M.Sc. Syllabus

In BOTANY

University of Calcutta

2018

The Regulations for two years M.Sc. course in Botany, Calcutta University

1. The University of Calcutta shall provide instructions leading towards course for M.Sc degree in Botany. A candidate who has passed the three year B.Sc. examination with Honours (Major) in Botany will be eligible for admission to this course on the basis of merit.

2. The duration of the course shall be two academic years and the examination for the M.Sc degree in Botany shall be held in four semesters over a total of 1000 marks. The duration of the semester shall be as follows:

1st Semester July - December

2nd Semester January -May

3rd Semester July - December

4th Semester January -May

3. The course shall comprise a total credit of 72 (seventy two), evenly distributed over the four semesters. The courses shall be grouped as core and optional and will carry credits according to the number of theoretical classes required, study hours and laboratory hours.

Semester wise distribution of courses:

	Courses	**No.of courses**	**Marks**	**Credits**
1st Semester	Core courses	4	260	20
2nd Semester	Core Courses	4	260	20
3rd Semester	Core Courses Optional Course Choice based Courses	2 1 2	130 20 100	10 1.5 08
4th Semester	Core Courses Optional Course Supportive Dissertation	2 1 1 1	130 20 20 60	10 1.5 1.5 7.5
TOTAL			**1000**	**80**

6. Grading of students' performance (as per CU rules)

Marks	**Numerical grade points**	**Grades**
75-100	5.00-6.00	Outstanding (O)
65-74	4.50-4.99	Excellent (A+)
60-64	4.00-4.49	Very Good (A)
55-59	3.75-3.99	Good (B+)
50-54	3.50-3.74	Fair (B)
40-49	3.00- 3.49	Satisfactory (C)
00-39	****	Fail (F)

Orientation of Courses in Four Semesters for M.Sc. in Botany

1st Semester

Core courses		*Marks(Theo.+Prac.)*	*Credits*
Bot C11	Microbiology	40+ 25	2.5+2.5
Bot C12	Phycology	40 + 25	2.5+2.5
Bot C13	Bryophytes,Pteridophytes and Gymnosperms	40 + 25	2.5+2.5
Bot C14	Cell Biology	40 + 25	2.5+2.5
	Total	**160 (Theoretical) + 100 (Practical)**	**10 (Theoretical) + 10 (Practical)**
		= 260	**= 20**

2nd Semester

Core courses		*Marks(Theo.+Prac.)*	*Credits*
Bot C21	Palaeobotany and Palynology	40 + 25	2.5+2.5
Bot C22	Taxonomy of Angiosperms	40 + 25	2.5+2.5
Bot C23	Phytochemistry and Pharmacognosy	40 + 25	2.5+2.5
Bot C24	Genetics and Genomics	40 + 25	2.5+2.5
	Total	**160 (Theoretical) + 100 (Practical)**	**10 (Theoretical) + 10 (Practical)**
		= 260	**= 20**

3rd Semester

Core & other courses		*Marks(Theo.+Prac.)*	*Credits*
Bot C31	Mycology and Plant pathology	40+ 25	2.5+2.5
Bot C32	Plant Physiology and Biochemistry	40+25	2.5+2.5
Bot-O-I	Optional Paper I	20 + 0	1.5+0
OA	Choice based credit course (CBCS 1)	50+0	4+0
OB	Choice based credit course (CBCS 2)	50+0	4+0
	Total	**200 (Theoretical) + 50 (Practical)**	**14.5 (Theoretical) + 5 (Practical)**
		= 250	**=19.5**
	Dissertation (To be Contd in Sem IV)		

4th Semester

Optional& other courses		*Marks(Theo.+Prac.)*	*Credits*
Bot C41	Plant Anatomy and Developmental Biology	40 + 25	2.5+2.5
Bot C42	Plant Biotechnology	40 + 25	2.5+2.5
Bot C 43	Ecology	20 + 0	1.5+0
Bot-O-II	Optional Paper II	20+0	1.5+0
	Dissertation (Contd from Sem III)	60	7.5

	Total:	120 (Theoretical) + 50 (Practical)+60 (Dissertation)	8 (Theoretical) + 5 (Practical) +7.5(Dissertation)
		= 230	= 20.5
	Grand Total Marks/Credits	**640 (Theoretical) +300 (Practical)+60 (Dissertation)**	**42.5 (Theoretical) +30 (Practical) +7.5 (Dissertation)**
		= 1000	**=80**

C – Core course; O – Optional course; S – Supportive course

Optional PapersBot OP:

Optional Paper I (any 1 of the following)

1. **O-I-A:** **Applied Virology**
2. **O-I-B:** **Molecular and Applied Phycology**
3. **O-I-C:** **Molecular and Applied Mycology**
4. **O-I -D:** **Molecular Plant Physiology**
5. **O- I -E:** **Advanced Cell Biology**
6. **O- I -F:** **Advanced Paleobotany and Palynology**
7. **O- I -G:** **Advanced Phytochemistry and Pharmacognosy**
8. **O- I-H:** **Taxonomy and Biosystematics**

Optional Papers II (any 1 of the following)

1. **O-II-A:** **Microbial Biotechnology**
2. **O-II-B:** **Plant protection**
3. **O-II-C:** **Green Nanotechnology**
4. **O-II-D:** **Plant Molecular genetics**
5. **O-II-E:** **Plant Molecular Biology**
6. **O-II-F** **Molecular stress biology**
7. **O-II-G:** **Immunology**
8. **O-II-H:** **Instrumentation and Biostatistics**

Scottish Church College

M.Sc. BOTANY

Affiliated to

University of Calcutta

Semester IV

(Session: 2019 – 2021)

Dissertation

Title: **A review on Nucleolar Organiser Region and its significance in plant chromosome analysis**

C.U. Roll No.: 223 - BOT - 191074

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A review on Nucleolar Organiser Region and its significance in plant chromosome analysis

A review on Nucleolar Organiser Region and its significance in plant chromosome analysis

Abstract:

Nucleolar organiser region constitute an important characteristic of eukaryotic genome, loaded with vast array of the rDNA gene superfamily and various proteins important to ribosome assembly. The present review addresses composition of the NOR in brief and focuses on the cytogenetic methods enabling visualization of the NORs on metaphase chromosomes. The staining methods facilitate analysis of NORs in karyotypes and the significance of such approach has been discussed with evidences from plant species. Being the species specific marker, the possibilities of NOR based cytogenetic analysis to aid relationships among taxa has been outlined.

Keywords: NORs, *Allium*, *Lens*, CMA band, FISH, rDNA

1. Introduction:

The cell nucleus contains the nucleoli which is a characteristic feature of eukaryotes. The primary function of the nucleolus is the transcription and processing of Ribosomal RNA (rRNA) and assembly of the ribosomal subunit (Raska et. al. 2006*)*. RNA polymerase I (RNA pol I) fulfils the process of assembling a ribosome subunit by the initial transcription of ribosomal DNA. Proteins associates with the rRNA to form the ribosomes.In higher eukaryotes the sub nucleolar compartments can be well distinguished when it is placed under electron and light microscopy. The sub nucleolar compartments consist of - fibrillar centres (FC) surrounded by dense fibrillar components (DFC) and granular component (GC) in which the FC and DFC are embedded(Dubois and Boisvert, 2016).Though inactive, rDNA is still assembled into nucleoli while in many cell types, only a subset of r DNA genes are transcriptionally active. RNA pol I transcribes the initial 47S r RNA precursor transcript, which is cleaved into 28S, 18S and 5.8S r RNAs. It is post transcriptionally modified by the help of small nuclear ribonucleoproteins (sno RNPs) and additional protein factors. The processed and modified rRNA finally assembles with rDNA proteins for export (Henras et. al. 2015).

The arrays of rDNA arrangement from head-to-tail tandem repeats are termed as nucleolar organizer regions (NORs) (Caperta et. al. 2007). When visualized by electron tomography during the metaphase, some clusters contain r-chromatin (ribosomal genes complexed with proteins involved in r DNA transcription)as 60-80nm fibres (Heliot et. al. 1997). Throughout mitosis the RNA polymerase I transcription factors remain associated with NORs (Roussel et. al. 1996). Every 43kb repeats includes a 13-14kb segment coding for the rRNA sequence and are separated from the next transcription unit by 30kb intergenic spacers (IGS). These spacers contain regulatory elements like the gene promoter, repetitive enhancer elements and terminator sequences.

NORs are reliable cytogenetic markers for species. Chromosomal localization of NORs constitute dependable species-specific feature to aid taxonomic and evolutionary studies.

3. Appearance of NORs

3.1. NORs in cell nuclei

NORs are visible only at a particular stage of cell division within the chromosomes. However, appearance of NORs in the nucleus depends on the phase of division, transcriptional activity of the cell. In simple eukaryotes, during mitosis there is no breakdown of the nuclear membrane. The nucleoli remain intact during “closed” mitosis and transcription is only momentarily inhibited during anaphase, to ensure r-DNA condensation and nucleolar segregation. Whereas with the “open” mitosis in complex eukaryotes (including higher plants), pol 1 transcription is shut down and nucleoli disappear during prophase(Clemente-Blanco et. al. 2009).

Pol 1 transcription resumes during telophase. Individual NORs start forming multiple small nucleoli, and begin to reform (Savino et. al. 2001) resulting in the formation of larger NORs. The mechanism of nucleolar fission is unknown but must involve very significant reorganization of many chromosomal territories within the interphase nucleolus. During disassembly, the granular centre is lost,followed by the DFC components. Pol 1 subunits and upstream binding factors modulate DNA conformation, and hence remain with rDNA arrays. In plants the small nucleoli then often fuse together to form a single nucleolus as interphase

progression. At the end of mitosis, the nucleoli reforms. The small round bodies called pre-nucleolar bodies are formed.

These pre-nucleolar bodies disappear as new nucleoli are formed where more than one active NOR is present in the nucleus, separate nucleoli initially forms at each active NOR (Mc Stay, 2020).

3.2 NORs during the metaphase stage

The striking morphological appearance as achromatic gaps on stained chromosomes made the NOR to be first recognizable on metaphase chromosomes, and these were sometimes referred to as the secondary constrictions (centromeres being considered as primary constrictions). (McClintock 1934). The presence of upstream binding factor alone is sufficient to produce a secondary constriction in the mitotic chromosomes (Mc Stay 2020). The ability of being selectively stained with silver nitrate in many plants, is a characteristic feature of active NORs (Goodpasture and Bloom, 1975). During metaphase, silver staining proteins remain associated with NORs, which are responsible for the less condensed rDNA. NOR can be located physically on chromosome during metaphase when the chromosome condenses. The cytogenetic techniqueallows us to localize NOR on chromosome and characterize the diversity and function of NOR in plant species.

The study of NOR in the chromosomes thus improves the cytogenetic background of a species because of the rapid visualization of ribosomal gene activities. It helps in understanding the relationship between various species and can be used as a marker in taxonomic studies by improving the karyological characterization of species varieties and cultivars. NOR localization and analysis on chromosomes contribute to resolve taxonomic ambiguity and genomic attributes. NOR helps in exploring the karyo-evolutionary trends and also aids in identification of new genetic resources, beyond only chromosome number and karyotype data (Moscone et. al. 1996).

4. Objective

An overview of the methods for NOR visualization in chromosomes and significance of NORs in karyotype analysis of plants.

4.1. Methods to identify NOR using various staining techniques:

The identification of the nucleolar organizer region (NOR) can be done by staining through various methods. Ribosomal DNA is the only DNA present within the nucleolar volume. The various techniques to identify NORs are: chromosome banding, FISH, Ag NOR staining, etc.

4.1.1 Chromosome banding:

The chromosome banding methods are based on chromosome staining with a dye or assay for a particular region (Bickmore, 2001). The total number of the bands or its resolution in the karyotype determines the condensation state of the chromosome and the heterochromatic content (Bickmore, 2001) as these become visibly apparent on the chromosomes. These banding methods may differentiate NOR as well, using specific dyes for chromosome staining (Guerra et. al. 2000). For example, the chromosome banding patterns are obtained by using the combination of fluorescent dye which includes chromomycin A3 and DAPI (4',6'-diamidino-2- phenylindole) (Kim et. al. 2001). The mechanism of the dye binding to chromatin and the occurrence of the processes in chromosome determines the CMA/DAPI and CPD (PI/DAPI) banding patterns. The stains like mithramycin (MM) also stain GC rich regions, which are concentrated in NORs (Guerra 2000). Following is an account of some frequently used chromosome banding and other methods for detection of NORs in plant chromosomes.

Banding with Chromomycin A

Chromomycin A (CMA) staining has proven to be very useful for cytogenetic characterization revealing a well-differentiated pattern of CMA+/DAPI- bands, which may exhibit interspecific polymorphism or heterozygosity (Kang et. al. 2008). Chromomycin A is a GC specific fluorochrome forming dimers in aquatic solutions in the presence of magnesium ions. The DNA-Chromomycin interaction is promoted by divalent cations of magnesium, manganese or zinc (Schweizer 1976). The dimer of these cations primarily binds to the 5'-dGpC-3' sequence and lies in the minor DNA groove. After the chromosome is stained with CMA, the GC pairs enriched regions display the bright fluorescence while the AT regions become dull. Fluorochrome staining with chromomycin A was used for characterization and comparing the banding patterns of chromosome in *Citrus* species (all having 2n=18) (Yamamoto et. al. 2007). In spite of having same chromosome number, the approach aided clarification of karyotype relationships and characterization

of *Citrus* species because every species exhibited a characteristic CMA banding pattern. The high reproducibility of chromosome analysis was demonstrated using the Chromomycin A staining method (Yamamoto et. al. 2007). For instance, the nucleolar organizer regions in all the three plant species *Vicia faba, Scilla siberica,* and *Ornithogalum caudatum* displayed very bright fluorescence with Chromomycin A3 (Schweizer 1976).

Figure 1: ChromomycinA-stained chromosome of *Allium nigrum* (Adopted from Maragheh et. al. 2018).

4.1.2 Fluorescence in situ hybridization (FISH)

This technique has been used to reveal the number and localization of 45S and 5S ribosomal RNA genes. It is useful for the physical mapping of the 5S-5.8S-25S (45S) DNA sequences of the ribosomal RNA genes by providing valuable chromosomal landmarks which has its importance in the understanding of evolution and diversification of species (Scaldaferro et. al. 2016). The sequences have been widely studied to understand the 5S and 18S-25S ribosomal gene localization, evolution of the chromosome, localization of the transgene, genetic maps and their linkage groups, phylogeny etc in different plants (Scladaferro et. al. 2016). FISH has been used in many plants to identify chromosomes using species-specific repetitive DNA sequences, ribosomal genes, and even unique DNA sequences (Guetat et. al. 2015). For detection of 18S-5.8S-25S r DNA sites, a 9kb fragment including a full length 18S-5.8S-25S r DNA repeat unit of wheat was used as a probe in a study with *Aloe* (Jha and Yamamoto 2012). Reliable recognition of the NOR-HC has been possible by *in situ* hybridization in many plant taxa (Guerra 2000).

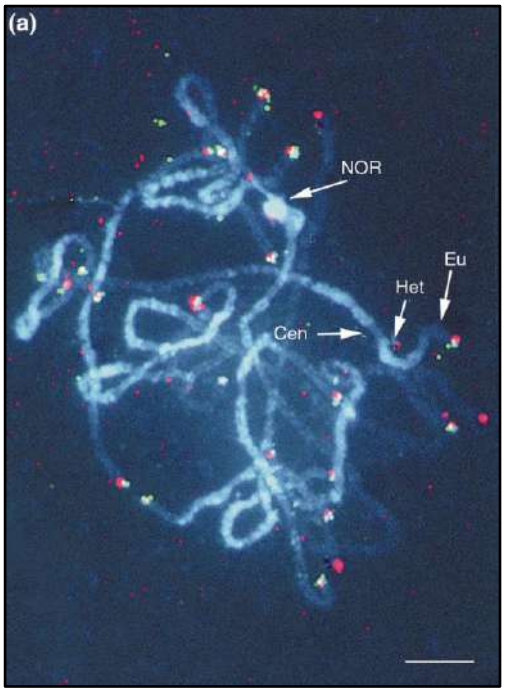

Figure 2: Fluorescent in situ hybridization showing the NOR region in tomato (*Solanum Lycopersicon*) during the pachytene stage of mitosis (Adopted from Jong and Zabel, 1999).

FISH method:

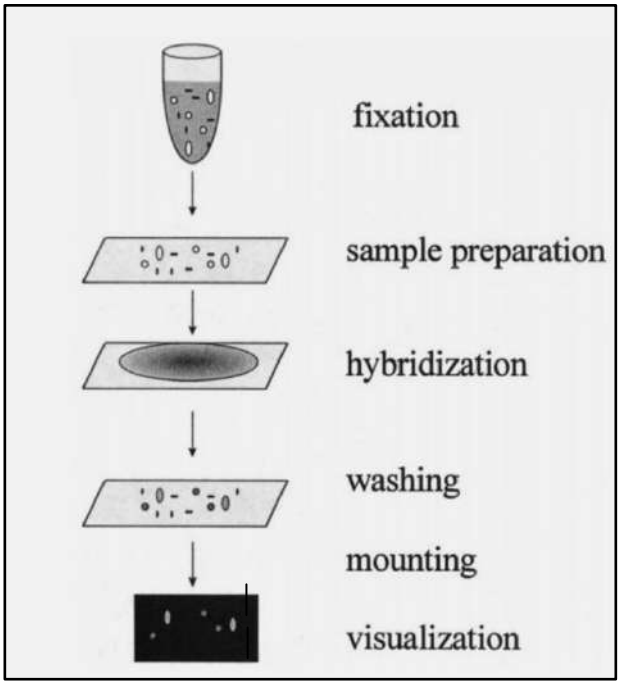

Figure 3: Key steps of FISH (Adopted from Moter and Gobel 2000)

4.1.3. Silver Nitrate (AgNO3) staining

NORs of many species can be selectively stained by silver solutions. Specific functioning of NORs in the nucleolus during interphase and early prophase is related to the mechanism of silver staijnig and thus silver staining can mark the active NORs and differentiate them from inactive NORs (Capoa et. al. 1982). Therefore, silver staining is appreciated in many finctional analyses and point out to variabilities, even beyond the scopes of FISH. The silver staining method depends on nucleolar proteins associated with actively transcribing ribosome cistrons (Howell, 1977). Probably the active NOR is detected by means of the protein accumulated in NOR at different stages from interphase to metaphase. The NOR proteins can be selectively stained in the nucleoli of resting nuclei for whole interphase or even in residual nucleolar material during prophase and metaphase (Pellicia et. al. 1978). There are several proteins in NOR of plants. There is a range of non-ribosomal and non-nucleolar proteins, splicing and translocation factors, exon function complex proteins, in addition to the expected ribosomal and nuclear proteins. In the nucleolar proteome, a small number of ribonucleoproteins are identified which maybe involved in production of the spliceosome U1snRNP in plants (Capoa et. al. 1982). It has also recently been shown that silver solution can selectively stain some specific chromosomal region other than actively transcribing NORs such as lampbrush chromosome loops, synaptonemal complex and chromosome core (Capoa et. al. 1982).

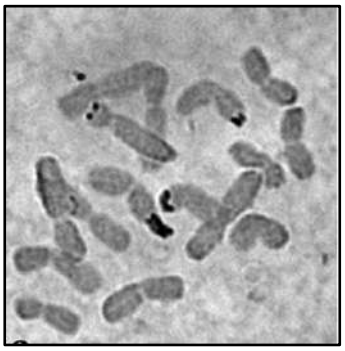

Figure 4: Nucleolar organizing regionin *Allium nigrum* that were detected by silver staining (Adopted from Maragheh et. al. 2018).

4.2. Signficance of NOR in karyotype analysis of plants

4.2.1 A case in Lentils (*Lens* spp.) show efficacy of CMA banding to detect NORs

Lens belongs to the family Fabaceae and it comprises of one cultivated and six wild species. In both domestic and international markets, the demand for the only cultivated species *L. culinaris* Medik is increasing due to its high protein content and various health benefits. Cultivated and wild lentils are

morphologically herbaceous annual plants and contain 2n = 14. Cultivars of Indian lentils were studied with fluorescence banding recently (Jha and Saha 2021). The analysisof NORs have been useful in phylogenetic inference on *Lens* species.

Table 2 variability of NOR sites in the *Lens* spp *

Species (2n)	Karyotype formula	NOR bearing chromosome	No. of CMA+ bands	Position of CMA band (NOR position)
L. culinaris	3m+1mSAT+2sm+1st	4th	2	intercalary
L. orientalis	3m+1mSAT+2sm+1st	4th	2	intercalary
L. nigericans	1M+4m+1stSAT+1st	6th	2	terminal
L. lamottei	5m+1stSAT+1st	5th	2	terminal
L. ervoides	5m+1stSAT+1st	6th	2	terminal
L. odemensis	3m+1mSAT+2sm+1st	3rd	2	intercalary

*(adopted from Jha and Saha 2021)

The lens species have same chromosome numbers but species delineation is achieved at karyotypes and then in the NORs, primarily addressed by CMA banding in this case. Interestingly, localization of NOR regions is found to be quite different based on the visible GC rich CMA+ bands. For example, it was located in the centromeric region in some species while in some others it was located in the terminal/ distal region (*L. nigricans*) and even in the interstitial regions (*L. culinaris*). This is how the variability of the position of the NOR was an important marker to differentiate the species of *Lens*. This was possible with the CMA3 banding since CMA banding is a preliminary step to identify GC rich NOR (Guerra 2000).

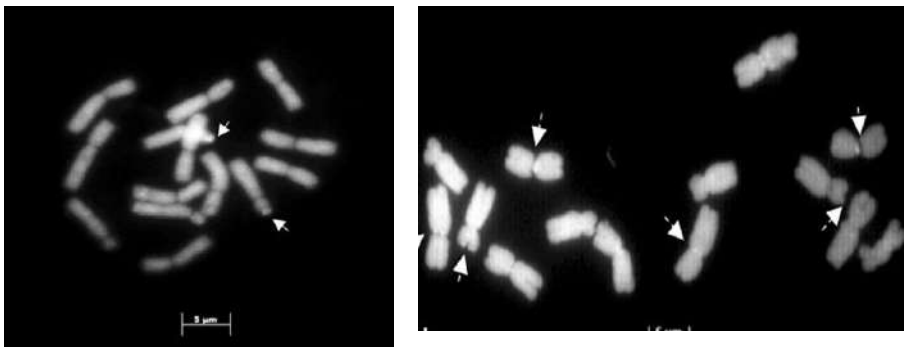

Figure 6: a) *L. nigricans* (2n=14), b) *L. orientalis* (2n=14) showing the terminal and interstitial position of NORs, respectively. The chromosomes are stained with the fluorescent dye CMA3. (adopted from Jha and Saha 2021).

4.2.2 A case in *Allium* reveals importance of FISH and CMA banding to detect NORs

The genus *Allium* is one of the largest world flora. The genus *Allium* L. comprises more than 800 species (Fritsch et al. 2010), which makes it one of the largest monocotyledonous genus. Many *Allium* species are economically important plants, including, e.g., the common onion (*A. cepa L.*), the bunching onion (*A. fistulosum*), leek (*A. porrum*), garlic (*A. sativum*), and many ornamental species such as A. moly L. or A. (Fritsch et al. 2010). A great diversity of various morphological characters (bulbs or rhizomes) and ecological habitat (Stearn 1992) is known in this genus. Among the great wealth of *Allium* species, the best known are the garden garlic and garden onion, both cultivated for thousands of years as edible plants worldwide. These plants have been used for centuries for their pungency, flavouring value, and medicinal properties, and in some parts of the world their use also has religious connotations (Kamenetsky and Rabinowitch 2006). The taxonomic limitations are disputed in *Allium* spp over the years.

Recently, karyotype analysis of the eight cultivated species of *Allium* has been conducted to help in overcoming ambiguities in species delimitations. FISH was used with 5S and 35S r DNA probes and banding methods (Maragheh et. al. 2018). *Allium* showed high level of interspecies polymorphism in the number and localization in the ribosomal DNA sites. The limitations of conventional staining and fluorochrome banding are overcome with FISH, to elucidate and confirm karyotype diversity based on NORs.

Previously, double staining with Chromomycin A and DAPI is used to identify the spatial relationship between the r DNA sites and positive CMA3 bands (Kolano et. al. 2013). *Allium nigrum* (2n=16) had only one chromosome with NOR in its diploid chromosome complement. The second site of 35S r DNA appeared to be transcriptionally inactive. *Allium sphaerocephalon* (2n=24) had four groups of chromosomes with 35S rDNA sites and NOR is present in only one chromosome in each group (Maragheh et. al. 2018).

Table 1 Variability of NOR sites in *Allium* spp *

Species and somatic chromosome number	Karyotype formula	5S rDNA	35S rDNA	NOR	CMA3
A. moly 2n=14	12m + 2sm	2	4	4	4
A. fistulosum 2n=16	14m + 2st	2	2	2	16
A. nigrum 2n=16	12m + 3sm + 1st	2	2	1	4
A. sphaerocephalon 2n=24	21m + 3sm	6	12	5	8
A. porrum 2n=32	24m + 8sm	13	8	8	8
A. oreophilum 2n=16	12m + 2sm + 2st	4	8	4	6
A. karataviense 2n=18	14m + 2sm + 2st	4	6	2	10

* (adopted from Maragheh et. al. 2018)

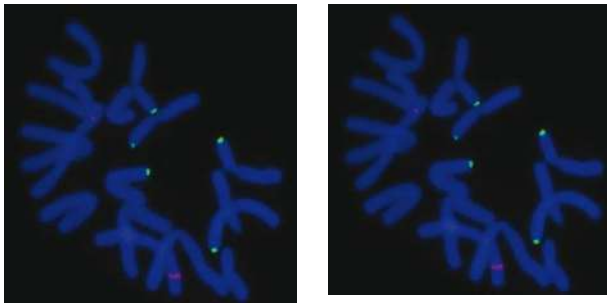

Figure 5. Chromosomes showing rDNA sites after FISH in *Allium roseum* (Adopted from Guetat et. al. 2015).

The study shows rDNA sites are colocalized with the CMA bands but the CMA bands might not be always co-localized to rDNA sites. So a confirmation of the GC rich NORs must be established with CMA banding and *in situ* hybridization. The case study in *Allium* reveals species specific diversity in karyotypes and NORs which enables resolving taxonomic ambiguities.

6. Conclusion

Taking the references of *Allium* and *Lens* elucidates how rDNA genes and their distribution follow a specific pattern within the group of angiosperm lineages. Therefore, the chromosomal detection of the rDNA sites is

the very important foundation of the genome based on which phylogenetic as well as taxonomic controversies can be solved. The localization and number of ribosomal DNA sites and their activities can be targeted with cytogenetic approach. Hence the preliminary data can be obtained to complement DNA sequencing experiments. Starting from the analysis of chromosome number and karyotype, further validation of differences can be possible by the methods used for staining the NORs.

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Agaricus bisporus: TYPES OF CULTIVATION, ITS NUTRITIONAL AND MEDICINAL VALUE

ABSTRACT: The White Button Mushroom (Agaricus bisporus) is exceptionally well known through the world and is the most significant mushroom of business importance in India. It tends to be effectively developed in where the natural conditions are positive; however it is developed in North-India in winter seasons because of the good conditions. The ideal temperature is 22 Degree Celsius – 25 Degree Celsius and that for organic product body arrangement 14 Degree Celsius – 18 Degree Celsius and a high level of relative dampness. The substrate for development is uncommonly pre-arranged fertilizer. The mushroom development rooms ought to have offices for temperature control and purification measures. Inside the house, rack or plate framework is generally received foe increment the space of development. Structures are developed of wood or bamboos or empty concrete blocks or two fold dividers. Great cleanliness is the key of business yet numerous contender molds and nuisance can emerged an enormous issue during the development and lead to a low item. This survey article is engaged upon the various methods of cultivation and its nutritional and medicinal value.

INTRODUCTION:

Development of white button mushrooms (Agaricus bisporus) began in the sixteenth century. Nonetheless, on a business scale, the development was started in Europe around seventeenth Century. Numerous homesteads for creation of catch mushrooms were set up and this assortment actually overwhelms the world creation and utilization. India, with its different agro climate conditions and plenitude of horticultural squanders, has been creating mushrooms, predominantly for the homegrown market, for over forty years [1]. Business creation got in the nineties and a few howdy tech trade arranged homesteads were set up with unfamiliar innovation coordinated efforts. However, significant portion of mushroom creation is as yet on little homesteads. [7] Agaricus bisporus, the Button mushroom, has a long development history and is these days developed for a huge scope in modern offices. Notwithstanding, ongoing examination

on the impacts of biodiversity on hereditary qualities and on the innovation of substrate making and development has opened additional opportunities for the creation of this mushroom under different conditions, with the goal of fulfilling the three elements of reasonable turn of events: human culture, economy, and the climate [2]. Progress is being made in the accompanying regions: high return of change of crude fixings to food varieties, restricted utilization of pesticides for controlling nuisances and microbes by utilizing biocontrol and safe strains, diminishing the utilization of energy by developing strains adjusted to different climatic conditions, and more noteworthy admittance to mechanical advancements and to hereditary advancement. [8]

Figure 1 The traditional source of compost is a pre- watered pile on the right and cut stems or rows on the left (Pennstate, 2017)

TYPES OF CULTIVATION METHODS: Mushroom cultivating normally includes 19 unique advances. The 19 different methods associated with mushroom development are:

Phase 1 treating the soil: We can produce our own spawn or can be procured from reliable laboratories situated in the region (chang and Miles 2004). Almost all agricultural Universities (Most commonly ICAR) supply the spawn. Spawn is the seed required for growing mushroom or we can also say that "Spawn is the grains covered with mushroom mycelium ". Technically, spawn is the vegetative mycelium from a selective mushroom grown on in any convenient medium like millet, wheat, sorghums etc [3]. After the production of the spawn, the next step involved in the mushroom cultivation is compost production which can be prepared by two methods; long, short method (Royse and Beeline, 2007).

COMPOST PREPARATION: The substrate on which the fungus grows is mainly made of a mixture of plant waste (grain straw / sugarcane, pulp etc), salt (urea, superphosphate / gypsum etc) , additives (rice bran / wheat bran) and water yields 1kg . Mushroom requires 220 grams of dry substrate. It is recommended that each ton of compost contains 6.6 kg, nitrogen is 2.0 kg

Phosphate and 5.0 kg potassium (N:P:K - 33: 10: 25) on a dry basis are 1.98% nitrogen, 0.62 % phosphate and 1.5 % Potassium respectively. The C: N ratio is a good matrix should be 25:30:1 during solidification and 16: 17: 1 in the final compost. [9]

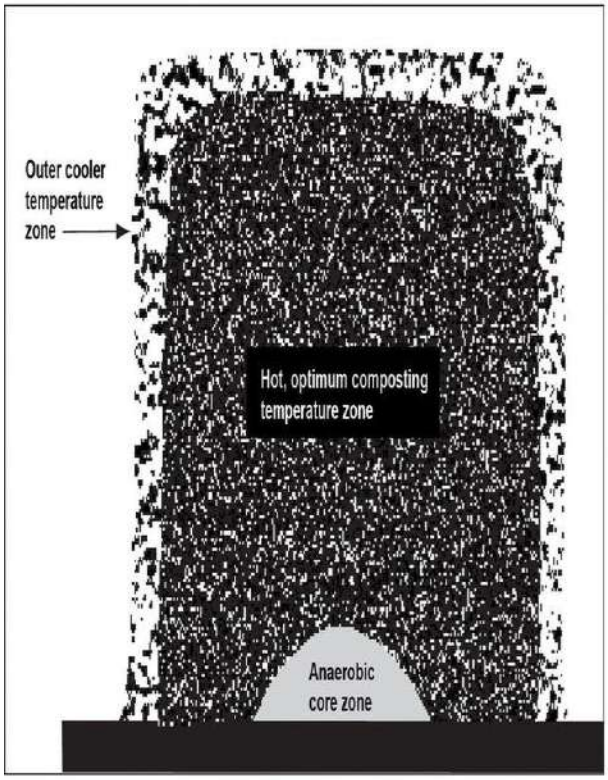

Figure 2 Cross phase of a compost pile displaying the exceptional temperature zones and air movement due to the chimmy effect.

(a) LONG METHOD OF COMPOSTING: Long term composting methods are usually carried out in areas where steam pasteurization equipment is not available. In this process, the first stage occurs about six days after the compost substrate is made. The thirteenth day of applying the patch. The fourth, fifth and sixth rounds will be held on the 16, 19 and 22 respectively (Fordyce, 1970). On the 25th day, add 10% BHC 125 grams in the seventh row and add the first on the Eighty-eighty day. Eight lines and then check whether the compost smells ammonia [4]. Only when the compost does not smell like ammonia is it ready to lay eggs. If not, please rotate a few more times within three days until the ammonia smell disappears. [10]

(b) SHORT METHOD OF COMPOSTING：In the first step of composting, the straw is layered and enough water is added to the pile, as well as fertilizer, wheat bran, molasses, etc (5 feet high wide, any length can be made of planks) . (According to Derikx, 1990) Turn it over the next day and water it again. On the fourth day, the pile was flipped a second time, plaster of Paris was added and watered, the third and final turning point was the twelfth, when the color of the compost changed to dark brown with a strong smell of ammonia. [11] The second stage is the pasteurization stage. The compost produced by the fermentation process mediated by micro organisms must be pasteurized to kill harmful competing microorganisms and convert ammonia into microbial protein (Alkaisi et. al, 2016). The air temperature is 600°c for 4 hours. In the end, the resulting compost should have a granular structure with a humidity of 70%, a Ph of 7, dark brown, harmless sweetness and be free of ammonia, insects and nematodes. At the end of the process, the substrate was cooled to 250°c. [11]

Figure 3 A self - propelled compost piles that moves on top of the compost pile (Royse and Beeline, 2007)

Phase 2: Stage II fertilizing the soil is the second phase of mushroom development. It is at this stage that the manure development is done or finished. Stage II fertilizing the soil is basic for the whole mushroom development measure. Sanitization happens at this stage, and it assists with slaughtering nematodes, bother growths, creepy crawlies and different irritations that might be available in the fertilizer (Loehr M. Stephanie, 2010). The smelling salts framed in stage I treating the soil are additionally eliminated at this stage since alkali is toxic and inhibitory to the development of mushrooms [5]. Purification arrangement of the fertilizer and the expulsion of smelling salts are the two principle objective of stage II treating the soil. Stage II fertilizing the soil happens in a few different ways including the bed (rack) framework, drafted framework, or the mass framework wherein the manure is either positioned in bed, plate or mass individually. The smelling salts and carbon dioxide is normally supplanted with outside air at this stage. [12]

Figure 4 This aerated substrate training device has a piped concrete ground beneath the substrate that forces air through the substrate to hold cardio situations at some point of the composting process (Loehr M.Stephania, 2010)

Stage II treating the soil is typically described by a controlled, temperature-reliant, environmental interaction that utilize air to keep up the manure in a temperature range most appropriate for the de-ammonifying microorganisms to develop and recreate. The development of these thermophilic (heat-cherishing) microorganisms relies upon the accessibility of usable

starches and nitrogen added as enhancements in Phase I treating the soil. Stage II treating the soil is generally kept up at a high temperature level or at a low temperature level because of the trouble experienced in the ideal administration of this phase of mushroom development (Loehr M. Stephanie). Toward the finish of Phase II treating the soil, the fertilizer temperature should be brought down to around 75° to 80°F prior to bringing forth (planting) can start [6]. The nitrogen substance of the fertilizer ought to be 2.0 to 2.4 percent, and the dampness content somewhere in the range of 68 and 72 percent. After the stage II treating the soil, the following stage in the mushroom development measure is generating – which can likewise be called planting. Bringing forth (planting) is the third stage in the mushroom development measure. [1]

Figure 5 Tunnel for the second and / or third stage of spawn grown system (Pennstate, 2017)

SPAWNING: A bring forth is the white, (Chang and Miles, 2004) stringy matter that frames the lattice from which mushroom develops. Generating or planting is the third phase of mushroom development. Mushrooms repeat physically during underground development, and angelically through spore arrangement. Mushroom propagation either physically or abiogenetically is typically influenced by microorganisms in the air, and this meddles with the ideal development and improvement of mushrooms. In this way, the effectively developing mushroom culture (mycelium) is set on mushroom development culture or manure – where the mushroom is permitted to develop a lot into a grown-up full scale organic entity or mushroom. This cycle of mushroom development is known as producing. Producing assists with giving mushroom a firm start being developed while forestalling or decreasing the odds of any microbial pollution. A mushroom produces numerous spores on the mushroom gills covering the underside of the mushroom cap as the mushroom develops over the mushroom fertilizer. Mushroom spores are not generally used to seed mushroom fertilizer for mushroom engendering in light of the fact that mushroom spores are not solid for mushroom spread. [13]

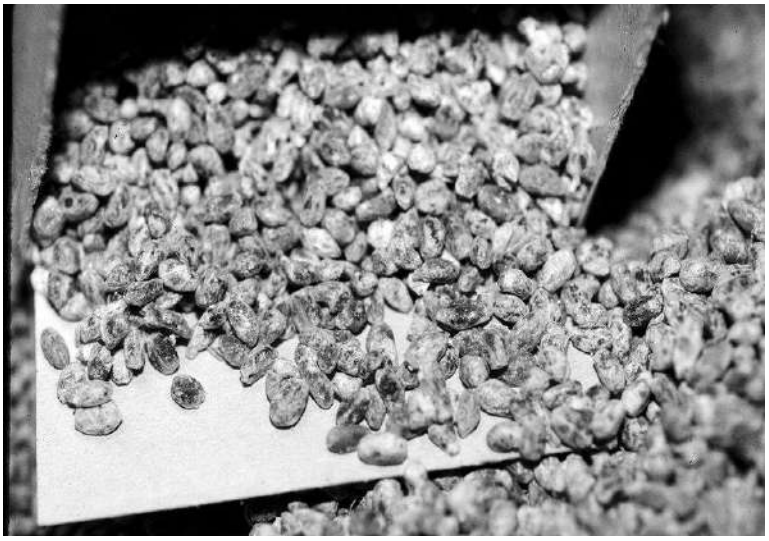

Figure 6 The seeds are used to grow compost with mushroom mycelium. The spawn is cooked, sterilized and the grains are cooled and inoculated with mycelium (Fordyce, 1970)

CASING: Casing is the following stage in the wake of producing. It is the fourth stage in mushroom development. (Reddy et. al 2011) Casing is completed once the mushroom manure is completely developed with generate. Casing is the top-dressing applied to the generate run mushroom manure on which the mushroom at last develop. It goes about as a water repository and where the rhizomorphs structure. The rhizomorphs structure when mycelium combines. Primordial (which are the initials of mushrooms) structure on the rhizomorphs. Rhizomorphs are the primary fundamental help of mushrooms on the grounds that without the rhizomorphs there will be no mushrooms. According to Reddy et. al, 2011, Casing assists with providing water to

the mycelium for development and advancement since dampness is significant for the improvement of a strong mushroom. It likewise forestalls and shields the mushroom manure from drying. Casing offers help for the creating mushrooms and it forestalls any underlying breakdown of the youthful mushroom following continued watering. The materials utilized for casing incorporate ground limestone, and peat greenery sphagnum (Reddy et. al 2011). It is critical to keep up irregular stock of water all through the period subsequent to casing to raise the dampness level limit of the mushroom manure before the mushroom pins or primordial starts to frame. Subsequent to casing, the following stage in mushroom development measure is pinning. Pinning is the fifth stage in mushroom development. [14]

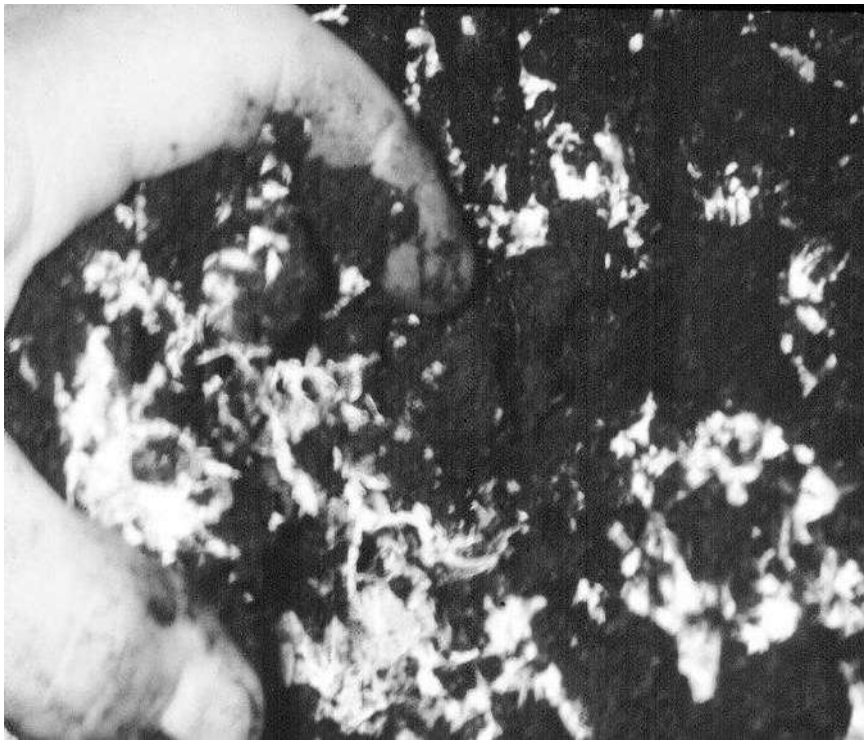

Figure 7 Spawn growth and thicker root like growth in the intestine (Loehr M. Stephanie, 2010)

RUFFLING: Ruffling of fertilizer on finish of produce run is done not long prior to casing. This training is especially valuable for round the year editing when 5-6 yields are taken each year and trimming period is diminished to around a month, as this training helps in weariness of manure sooner than ordinary. Unsettling of packaging following 3-4 days or so subsequent to casing is finished by certain producers to get uniform pinning. [15]

PINNING: Pinning is the stage at which primordial or mushroom pins start to shape. It is the fifth stage in the mushroom development measure. Pinning is the stage that follows casing. The mushroom pins, mushroom initials or primordial start to shape once the rhizomorphs have framed in the casing stage. As per Andrade et. al opinion, In spite of the fact that little, the mushroom initials or primordial are normally seen as outgrowths on the rhizomorphs [16] . The

mushrooms pins proceed to extend and develop bigger through the casing stage. The casing phase of the youthful mushroom ultimately develops into a develop and harvestable mushroom – which generally shows up in around 17 – 21 days after an effective packaging measure. The following stage in the mushroom development measure is harvesting. Harvesting is the 6th and last phase of the mushroom development measure (Andrade et. al 2003).

WATERING: According to Bahl, 1983, Mushroom contains almost 90% water and that gives us a thought how water is significant for the yield. Mycelium gets water from fertilizer during generates run and manure + casing during case run and from casing during organic product body development. Water level in casing is kept up 2ly. One path is by its ordinary splash when pinheads are pea estimated and afterward by keeping up RH at 80-85% during editing. In the event that one of the elements, (water showering and RH) during trimming is upset, it will influence crop efficiency [3]. Low RH during editing will bring about drying of beds, lightweight mushrooms, staining of mushrooms and harvest misfortunes. Drying of casing will seal the packaging medium bringing about mat arrangement, which gets impenetrable to water, and results in enormous yield misfortunes. (Royse and Beelinan, 2007) Water must be recharged in casing to oblige the water misfortunes from casing because of mushroom development and vanishing [17]. Lesser the water misfortune to room air, better it is. Bed dampness and RH are albeit two distinct variables yet are associated. Water showering on mushroom beds at pin breaks ought to be stayed away from [5]. The casing ought to be wet enough when natural air is acquired and room temperature brought down. As per Royse and Beeline, 2007, The wetness ought to be supported till pin heads become pea measured, and that is the stage when bed will require extra watering to permit pea-sized pins

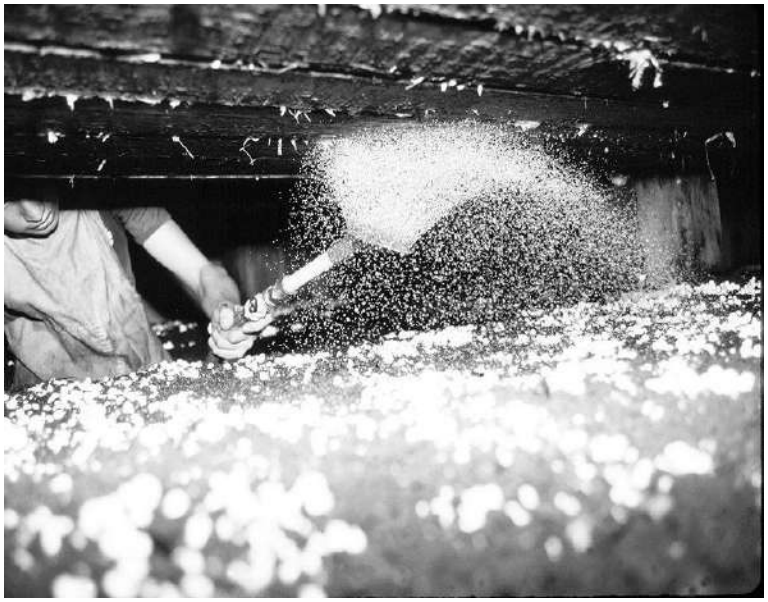

Figure 8 Most watering is performed with the aid of using hand, despite the fact that more recent farms use hand-propelled watering trees (De Gruyter, 2017)

to form into button estimated mushrooms. Watering to beds requires observing at each stage. RH in the trimming room is observed by utilizing dry and wet bulb thermometers. Two common stem thermometers are placed in the trimming room, setting one in the casing/manure bed and one lingering palpably close by (barely any cm separated). Bed temperature is 1-2°C higher than air temperature. PC control of AHU guarantees use of trimming boundaries with exactness during produce run, case run and harvesting. (Fordyce, 1970) The water utilized for water system (showering) on mushroom beds ought to be perfect, unbiased in pH and liberated from salts, weighty metals and different pollutions [10]. Water adequate for drinking/watering for vegetables/field crops is additionally useful for mushroom development. It is attractive to test the nature of water before the mushroom developing is begun at a specific site. (Bahl 1983)

HARVESTING: After the casing process, the First mushroom heads begin to grown in 12-14 days this is called the 1st flush. 1st flush is then picked from the mushroom harvest. The grower then waters the entire mushroom harvest. After another 5-6 days, the 2nd flush appears. This is also harvested in the same process and this process gets repeated again during the 3rd flush. Generally, the average harvest of mushroom has three independent buds, and each successive bud reduces the yield (Loehr M. Stephanie).

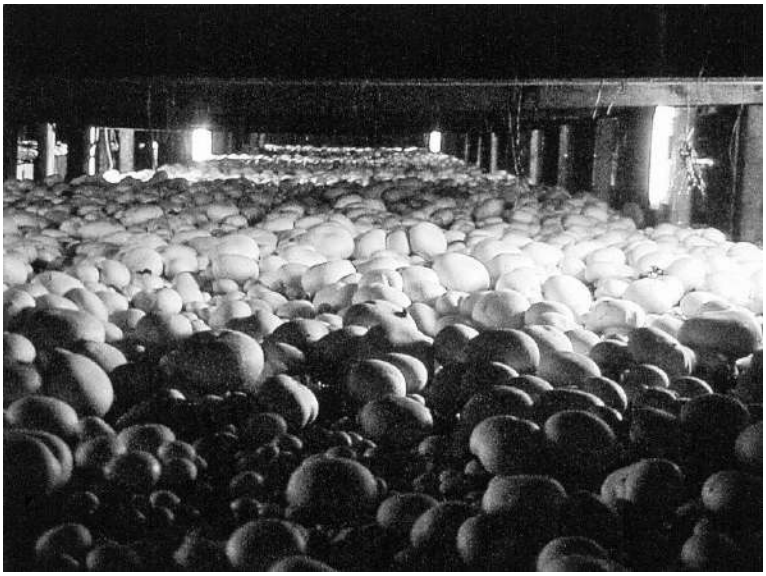

Figure 9 The mature mushrooms are ready to be harvested (De Gruyter, 2017)

Figure 11 Agaricus bisporus is successfully grown (Pennstate, 2017)

Figure 10 Fruiting bodies of white button mushroom

FRUITING: Under positive natural condition viz. Temperature (at first 23 +/- 20°c for about a week and afterwards 16 +/- 20°c), dampness (2-3) light splash each day for dampening the packaging layer, moistness (above 85%), appropriate ventilation and CO2 focus (0.08- 0.15 %) the natural product body initials which show up as pin heads begin developing and slowly form into button stage. (Derikx et. al 1990)

Use of sugar Mill waste water for Agaricus bisporus cultivation: Prediction models for trace metal uptake and health risk assessment (Vinod Kumar et. Al 2021) - This study investigated the risk of accumulation of trace metals in the fruiting bodies of white button (Agaricus bisporus JE Lange) mushrooms using treated sugar plant wastewater (SMW) in a sustainable way. To increase the content of moisture and nutrients in the straw substrate, regular watering and SMW of various capacities were loaded. The effect of SMW modification on A. bisporus yield biological efficiency and spawning run time was evaluated. Also, a model for predicting congestion of trace metals by A based on substrate properties (pH, organic matter, total nitrogen, total phosphorus, etc.). Mushroom fruiting bodies were developed using multiple linear regression (MLR) and artificial neural network (ANN) methods. [18] The results showed that maximum A. bisporus yield (158.42 ± 8.74 g/kg fresh substrate) biological efficiency (105.61 ± 3.97%) and minimum time of spawning run (15 days) were observed for SMW enrichment of 75%. To predict the incorporation of trace metals in Cd, Cu, Cr, Fe, Mn, and Zn, the ANN model performed better for R2 (>0.995), root mean square error (RMSE <0>0.99) and normalization of the model. Showed Bias (MNB <0>0.96) and MNB (<0.034). On the other hand, the Target Hazard Index (THQ) did not indicate a serious health risk for consumption of A contaminated with trace metals. Agaricus bisporus in both adults and groups of children. Therefore, the results of this study present new safe and sustainable methods of treated agricultural infrastructure wastewater management and A. bisporus cultivation. [20]

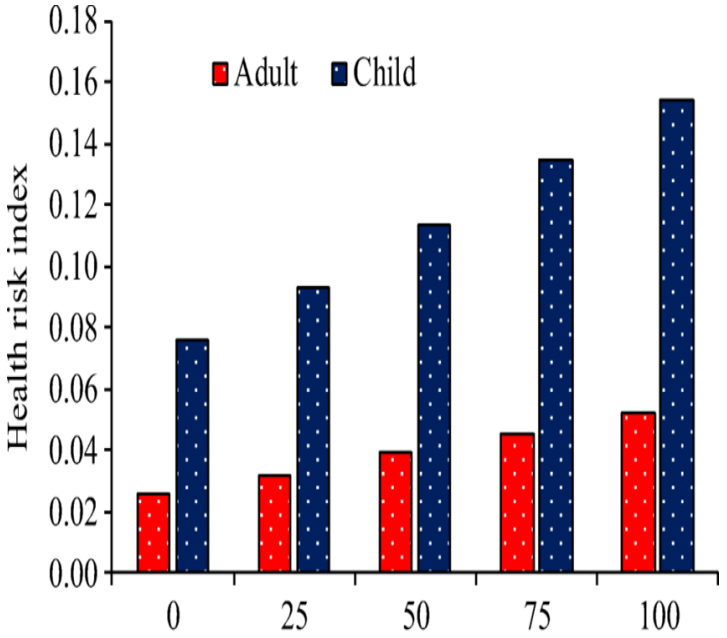

Figure 12 The general health risk index (HR) of adults and children related to the intake of trace element metals containing Agaricus Bisporus bacteria (Vinod Kumar, 2021)

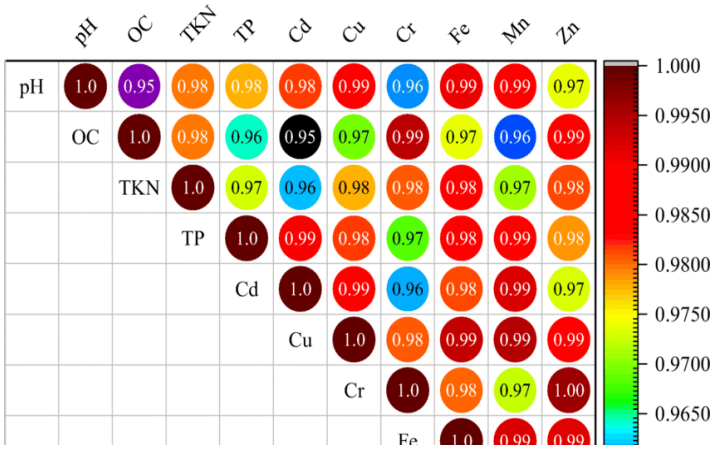

Figure 13 Pearson correlation matrix of the relationship between the physicochemical properties of mushroom substrate and micro metal properties (Vinod Kumar, 2021)

A new laboratory medium for the cultivation of A. bisporus - p. B Rainey, 1989 - Compost Malt Medium (CMM) is a novel experimental medium that can be easily prepared for the culture of Agaricus bisporus. CMM provides a growth medium similar to its commercial substrate A.bisporus derived from the infusion of mushroom compost. The compatibility of CMM as an artificial substrate of A.bisporus was proved. Laboratory studies of cultivated mushrooms, Agaricus bisporus (Lange) Imbach Biology, have been hampered by the lack of growth of in routinely used media. Artificial substrates commonly used for the growth of A. bisporus include: Malt extract agar (MEA; 2-4% malt extract) (Hume & Hayes 1972, Wood 1976, San Antonio & Thomas) 1972, Mathew 1961); po [19]. Tato dextrose agar (PDA) (Peerally 1979); complete yeast medium (CYM) (Elliott & Wood 1978); Commercially available malt extract (peptone) agar (MPA) (Masaphy et al. 1987), however, mycelia growth in these media late sectorization and strain denaturation are commonly seen (see Wood (1976 edition)). Also, the phenomena observed in these media do not necessarily reflect the situation in vivo (Rainey & Cole1987). A medium using powdered compost or compost extract can be used by spawning manufacturers and has also been reported to promote growth (Fritsche1978). However, these substrates are rarely used in laboratory research. [21]

Using tea waste as a new casing material in mushroom A. bisporus cultivation (Aysun Peksen et. al 2003) - This study investigated the possibility of using tea production and waste as a new casing material for mushroom (Agaricus bisporus) cultivation. We compared the physical and chemical properties of secondary waste, fermented tea waste, and a mixture of tea waste and peat with the effect on yield compared to peat casings. Obtained the highest yield in peat casing. The use of only tea production waste in the casing is not permitted for reliable harvesting when compared to peat. However, mixing tea production waste and peat at a ratio of 1:

1 (v: v) increased yields. There was no significant difference between car production, waste + peat and mushroom harvesting of peat casing materials after 30 days and 40 days. Yield was reduced due to the high salt content of the casing material, organic and inorganic compounds. However, the high iron content of the casing material showed a significant positive correlation with total 40-day production. [22]

Cultivation of Agaricus bisporus on some formulas compost and locally available casing I: Wheat straw based compost formulas and locally available casing materials (E Baysal et. al 2007) - 3 compost Formulas; The cultivation of Agaricus bisporus used various active substances based on straw hats: wheat brain, chicken manure and pigeon droppings. Locally available case materials from were used, including bor turf, agabash turf, kaikara turf, and a mixture of their perlites (80:20; v: v). Temperature diagrams for all compost formulations were measured at during composting at various depths to determine compost ability levels. The results showed that the internal compost temperature increased by the 8th and 9th days of composting, respectively, for the composts of Formula I, Formula II and Formula III. In the second round of composting, the maximum internal compost temperature was also measured for all compost formulations. The highest mushroom yield (1707.2 g) was recorded by straw blended with a mixture of keikara peat and perlite with pigeon manure as the casing material. [23]

Formula	Ingredients	Fresh weight (kg)	Moisture content (%)	Dry weight (kg)	Nitrogen (%)	Nitrogen (kg)
I	Wheat straw	460.0	15.0	400.0	0.5	2.00
	Wheat bran	137.0	17.0	113.0	2.4	2.71
	Ammonium nitrate	17.1	0.0	17.10	26.0	4.94
	Urea	10.1	0.0	10.10	44.0	4.84

Figure 14 White straw composting formulas (E.Baysal et. al 2007)

Compost Formulas	Casing materials	Mixture ratio (%, in volume)	Yield** Mean ± Sd***
Formula I*	Peat of Bolu	100	1253.2 ± 139.8 e
	Peat of Agacbasi	100	1351.2 ± 140.4 e
	Peat of Çaykara	100	1373.7 ± 86.1 de
	Peat of Bolu + perlite	(80:20)	1346.5 ± 89.5 cde
	Peat of Agacbasi +perlite	(80:20)	1468.2 ± 108.2 cde
	Peat of Caykara + perlite	(80:20)	1699.2 ± 106.5 cde
Formula II	Peat of Bolu	100	1427.2 ± 85.6 cde
	Peat of Agacbasi	100	1262.7 ± 138.7 bcde
	Peat of Çaykara	100	1467.2 ± 78.2 bcd
	Peat of Bolu + perlite	(80:20)	1162.0 ± 112.8 abc
	Peat of Agacbasi + perlite	(80:20)	1659.7 ± 186.4 abc
	Peat of Caykara + perlite	(80:20)	1403.2 ± 121.9 abc
Formula III	Peat of Bolu	100	1100.0 ± 131.6 abc
	Peat of Agacbasi	100	1363.2 ± 183.7 abc
	Peat of Çaykara	100	1501.7 ± 184.4 abc
	Peat of Bolu + perlite	(80:20)	1473.0 ± 118.7 abc
	Peat of Agacbasi + perlite	(80:20)	1337.7 ± 70.8 ab
	Peat of Caykara + perlite	(80:20)	1707.2 ± 355.8 a

Small letters given as superscript over yield values represent homogenity groups obtained by statistical analysis with similar letters within a column reflecting statistical insignificance at the 95% confidence level.
*Composts were filled into plastic bags as 7 kg weights basis.
**Results reflect observations of four plastic bags.
***Standard deviation

Figure 15 Agaricus bisporus productivity in various composting a casing material (Ferah Yilmaz, 2007)

Cultivation of Agaricus bisporus enriched with selenium, zinc and copper (Piotr Rzewski 24 May 2016) - Agaricus bisporus (white button mushroom) is a globally important culinary and medicinal species. Current studies have shown whether it can be grown with Se alone supplemented substrates or Cu and / or Zn (0.1-0.8 mol L-1) to produce nutritious fruiting bodies. I investigated first. As discovered in, substrate supplements did not affect the biomass produced up to 0.6 mol L-1 elemental concentrations, regardless of culture model. The 0.8 mol L-1 Se + Cu and Se + Zn supplemented biomass is similar to the control group still developed. Accumulation of trace elements in fruiting bodies generally reaches its maximum at 0.6 mol L-1 (for Se + Zn and Se + Cu + Zn) and 0.8 mol L-1 (for Se and Se + Cu). Increased over the concentration gradient. .. Organic Se accounted for the largest share of the total allocation of Se. Calculating, 10 g of A. bisporus desiccated substance obtained from an L-1 supply of 0.6 or 0.8 mill molar is 342 to 469% of the recommended daily intake (RDA) for Se, 43.4 to 48.5% for Cu, and 5.2 to Zn in the case of Zn. 5.8%. [24]

Agaricus bisporus production on substrates pasteurized by self-heating (Stephanie et. Al 2010) the purpose of this work was to determine whether the self-heating sterilization step is technically applicable to the cultivation of Agaricus bisporus. First we tested only the substrate (konkobu, pangoragurasu and a mixture of both ingredients and wood shavings). Next, two supplementary trials were conducted using soybeans, wheat bran, sheep dung, sesame, black

beans, and fry. The best production values (BE = 176.3% and Y = 26.6 kg/m2) were obtained using a formulation consisting of 25 ch of soybeans, black beans, wheat bran and fry at 9% supplementation added to spawning and casing. These results were comparable to those obtained with the phase II compost traditionally used for A. Cultivation of bisporus. [25]

Growing Agaricus bisporus on compost mixtures based on Chicken Manures and Banana Residues (December 2018 Zeina el sebaaly) - The present study evaluated the potential use of two widely available agricultural wastes at the local level: chicken manure (chicken manure) and banana waste (banned) to assess their potential to replace traditionally imported substrates. Horse dung). The trials therefore consisted of growing mushrooms on a substrate formed by some mixtures of two wastes mixed in various proportions (0%, 30%, 50%, 70% and 100% by volume) that were composted. (completely based on horse manure). Substrate Sikkuban: 100-0 Fruit formation was 1 to 3 days earlier compared to the rest of the substrate. Average yields were, on the contrary, highest (350.9 g/box) on substrate, and best on substrate following completely manure or banana waste compost. Individual mushroom weights ranged from 0-100 to a minimum of 36.8 g on a shikkuban and a maximum of 58.5 g on the control board. The yields of temperament refined-van: 70-30 and refined-van: 50-50 was comparable to controls (283.9 g/box and 294.2 g/box, respectively). Fruit counts did not differ significantly between all substrates. The cap diameter was the highest in Shikkuban, the 50-50 (6 cm) stem diameter was the highest in the control (2.25 cm), and the mushroom length was superior in both substrates compared to the other substrates. Stylish Room: All temperaments except 0-100 produced marketable fruit. As a result, it was possible to completely replace traditional compost with a mixture of Sikkuban: 70-30 and Sikkuban: 50-50, and more inexpensively at the level of this type of cultivation Lebanon. [26]

Amounts of components added to 400 Kg of each waste type	Straw-bedded horse manure	Deep litter chicken manure	Banana wastes (leaves and pseudo-stems)
Wheat straw (Kg)	200	200	200
Urea (Kg)	1.2	1.7	2.3
Ammonium nitrate (Kg)	1.2	1.5	2.5
Gypsum (Kg)	2	2	5
Protein supplements (Kg)	-	-	5

Figure 16 Composition of compost formula based on agricultural residues (Zeina el. Sebaaly, 2018)

Alternative substrates for higher mushrooms mycelia cultivation (December 2015 tetitetiana- Cultivation of 29 species of higher mushroom mycelium was investigated as an

alternative substrate, which is a waste of the Ukrainian local industry. The amount of mushroom mycelium obtained from the 12 substrates investigated changed significantly from 1.0 g / L to 22.9 g / L on the 14th day of cultivation. The surface cultivation adopted in this study makes it easy to select the medium (temperament) suitable for hyphal production. We have selected alternative substrates (glucose-peptone-compared to yeast medium) for all study species, from 24 hyphal growth-optimal soybean cakes to walnut cakes suitable for only 2 species. Utilization of temperament was evaluated biologically efficiently. The best indicator of biological efficiency varied from 19.0% to 41.6, depending on the type of mushroom. High biological efficiency of mycelium culture on substrates has been established: wheat seed cakes - Pleurotus djamor, Lyophyllum shimeji, Crinipellis schevczenkovi, Phellinus igniarius, Spongipellis litschaueri; Oat Seed Cake - Ganodermaapplanatum and G.lucidum- Hohenbuehelia myxotricha, Trametes versicolor, Morchella esculenta, Cordyceps sinensis, C. militaris and Agrocybe aegerita; Rape Cake - Auriporia aurea; Turtle Lina Seed Cake - Fomesfomentarius. This kind of cultivation is projected as a bio-process in which agricultural waste is converted into mycelium, and can be used in various forms of products with therapeutic effects (nutritional supplements in powders or tablets or as ingredients in functional foods). [27]

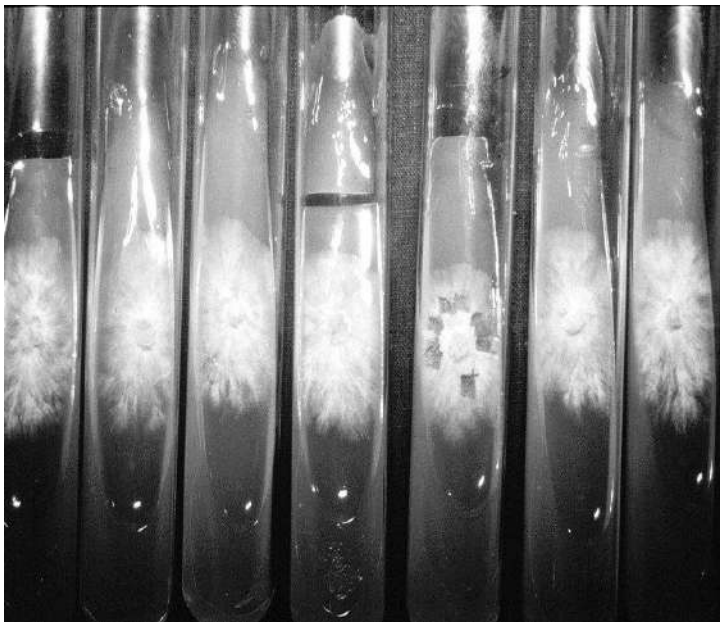

Figure 17 Mushroom mycelia culture (tetitetiana, 2015)

New cultivation method of button mushroom (Agaricus bisporus) utilizing mixture of sawdust and chicken manure (January 2016 Byung-Eui Lee) - Button mushrooms

(Agaricus bisporus) are grown on straw and rice straw-based compost. In this study, other composting machines, the carbon atom sawdust and nitrogen-causing chicken manure, were used to grow button mushrooms. Two steps required a sufficient composting process for successful cultivation. 1) In step 1, the substrates must be mixed regularly under aerobic conditions. 2) Should the temperature be maintained between 55 and 65? 3 or more days in a phase II course. The results showed that it is possible to successfully grow button mushrooms on sawdust-based compost. However, the production of mushrooms was lower than that of conventional composting machines (rice straw or straw). The new composting machine developed in this study may be useful, but further research may be needed to improve button mushroom cultivation more efficiently. [28]

Use of culture medium for Agaricus bisporus production (2004) - The present invention relates to a technique for culturing bisporus, in particular to a medium for producing bisporus and its application. Culture medium the present invention relates to a culture technique for a double whole mushroom, in particular, a type of culture medium for a double whole mushroom and its application. Glass growing medium consists of raw materials in the following proportions by weight: feed 45-55% feces 35-45%, cake manure 1-8%, urea 0.5-1.0%, 0.5-1.5 gypsum powder 0.8-1.5 %, aggregate 45-55wt%, dung material 35-45wt%, cake manure 1-8wt%, urine 0.5-1.0wt%, gypsum powder 0.5-1.5wt%, calcium superphosphate 0.8-1.5wt%, lime powder 0.2- 1.5 wt%, calcium carbonate 0~2f over calcium phosphate 0.2~1.5% lime powder, 0~2.0% calcium carbonate, 0~0.5% pana plate feed and feces component 90% over .0wt%, dichloroboth 0~0.5 wt%. Among the total weight of raw materials, grass material including rice straw, wheat straw, cottonseed hull, corncob, and corn stalk, and dung material including cow dung, family dung and poultry manure. Feed is straw straw, wheat grass, cotton coat, corn and/or corn stalks. The dung material may be cow dung horse dung and/or chick dung. The cake makes up at least 90 wt %. Cake manure, soybean cake, soy flour, cottonseed gourd and/or cornmeal are also good. The growing medium is used to grow tall stem manure double-hole mushrooms, which may be soybean cake, soybean meal, cottonseed cake and/or corn meal. The present invention has the advantages of easy availability of raw materials, low production cost, good applied crop fields, bioconversion efficiency of 60% or more, and a yield of more than 25 kg/m of double whole mushroom. Ionic effect, etc. The medium of the present invention is used for

cultivating bisporus in the field of high stem crop cultivation. The bioconversion rate of both spores is more than 60%, and the yield reaches 25 kg/m3. [29]

Method for cultivating Agaricus bisporus through needle mushroom cultivation waste (2013) - The present invention relates to a method for cultivating Agaricus bisporus through saliva from mushroom cultivation waste, and belongs to the field of edible mushroom cultivation technology. The formula for compost is 120-130 parts straw by weight, 48-53 parts needle mushroom waste, 28-33 parts cow dung, 6.5-7.7 parts cake fertilizer, 2.8-3.2 parts gypsum, and 2.8-3.23 parts superphosphate. And 1.8-2.2 parts of lime and Agaricus Bisporus are crushed, pre-wet, dummy construction, dummy rotation, secondary chamber fertilizer, frame rotation, sowing, scattering execution, grounding, as well as fruit-bearing management. According to this method, needle mushroom waste is used to replace some of the fresh ingredients for planting Agaricus bisporus, thus improving the yield and quality of Agaricus bisporus and reducing production costs. And the method will be bigger. It is meaningful for the rapid development of the edible mushroom recycling industry, protection of the ecological environment and the improvement of people's quality of life them. [30]

Microbial ecology of the Agaricus bisporus mushroom cropping process (2018-02-01 McGee, Connor F) - Agaricus bisporus is the most widely cultivated type of mushroom in the world. Incubation is initiated by inoculating Agaricus bisporus pure spawning on the bottom of semi-pasteurized compostable organic substrates. Thereafter, the A. bisporus mycelium breaks down organic matter, releasing nutrients to form colonies on the composted substrate. Often, a layer of peat, called "case soil", is placed on the surface of the composted substrate, which drives the development of mushroom crops and maintains the environmental conditions of the compost. Extensive research is being conducted studying the biochemistry and genetics of A. bisporus throughout the culture process. However, little is currently known about the wider microbial ecology coexisting in composted substrates and casing layers. The micro biome of compost and casings is known to play an important role in the production process of mushrooms. Microbial species present in composts and casings are known to be important nitrogen sources for (1) A. Mushroom mycelium (2) sugar residue release by decomposition of straw in composted substrates (3) plays an important role in inducing growth of mushroom mycelium and (4) acts as a pathogen by parasitizing mushroom mycelium/crop. Despite a long history of research on the

growing process of mushrooms, an extensive review of the microbial community present in composts and casings has not yet been completed. The purpose of this review is to provide a comprehensive overview of the literature investigating the compost and casing micro biome throughout the cultivation of A. bisporus mushroom crops. [31]

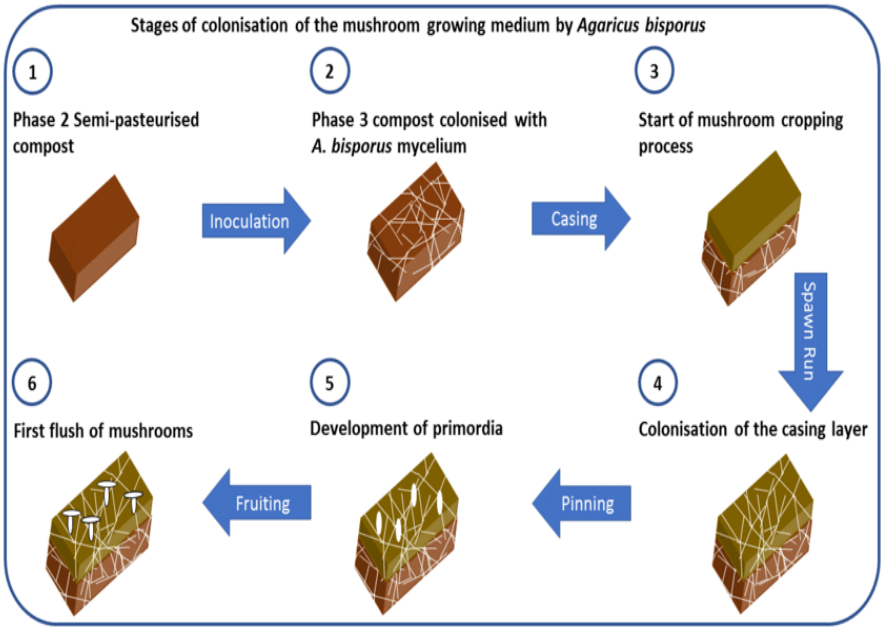

Figure 18 Colonization stage of Agaricus bisporus mushroom in culture medium (Conor Francis McGee, 2018)

Figure 19 A small amount of mushroom substrate fully colonized spawn development (Dr.Parveen Garg)

Use of Mustard Meal (De-Oiled Cake) During Composting To Bring Down the Cost of Mushroom Production (Dr.

Parveen Garg Department of Botany, Swami Shradhanand College, University of Delhi, Alipur) - Considering the constraint that uniform quality chicken manure is not available in the local market, the three combinations of wheat mustard nights (desulfurized cake) are combined with the production of this button mushroom (Agaricus bisporus). Tested for ability to influence production costs. .. Compost was prepared by mixing 18, 22, and 25% mustard nights and straw in a short composting method. 80% chicken manure, including straw, was the control group. All components were composted during the 18 days of two-stage composting using a tunnel for peak heating of as supported by Shandilya et al, 1976. Fresh grain scatter was acquired at Bharat Mushrooms in Delhi, India. Formaldehyde-treated casing soil (a 2: 1 mixture of two-year farm yard fertilizer and garden soil) used for the bed casing. The results proved that all combinations affected the harvest and promising factors for low cost production of mushrooms. When combined with 22% mustard flour, the maximum production of mushrooms was (226 kg per ton of compost). [32]

Non- Composted grain based substrates for Agaricus bisporus Production:

According to Bechara M.A et. al 2003, Research on mushroom growth of Agaricus Bisporus on an unconnected grain - based substrate to eliminate the need for a lengthy and often unpleasant odor composting process. They used grains, mixed grains with soybeans and commercial rye seeds. The treatment process includes the use of perlite as inert filler in various proportions (100%, 75%, 50% and 25%) of grains or grain seeds. For a mixture of millet and soybeans, the biomass (millet and soybeans) is fixed at 75% of the volume, and the ratio of millet to soybeans is variable [8]. To induce firmness, all substrates are covered with a sterile mixture of peat and calcium carbonate. The tray contains 25% activated carbon (U/V), which is found to be as

Figure 20 Non-Composted grain based for Agaricus bisporus (Bechara, 2003)

effective as non - sterile commercial trays. Among the various treatments, the three grain treatments had the highest yield of fungi, which were 100% millet 0% pearlite treatment (8.7kg/m2). In contrast, when soybeans are added to the substrates, the millet / soybean mixture does not produce fungi. The highest mushroom yield from commercial grains is 5 individuals. With 100% spawing 10% pearlite treatment, the yield is 3 kg/m3 .Compared with millet and composted grain substrate, the yield is lower, but the bio- efficiency is 117% . 25% spawing treatment 75% pearlite, while composting and processing of 75% millet. 25% pearlite are 98% and 55% respectively. The results indicate that mushroom can be grown on non- compostable substrates. However, further economic analysis is needed to determine the economic feasibility of alternative substrates. [19]

Benefits of Agaricus bisporus Mushroom: The six significant constituents of mushrooms are water, proteins, carbs, fiber, fat, and debris alongside minerals and fundamental amino acids (Heleno et al. 2010; Aliphatic et al. 2015). Mushrooms are a low-cost food and significant wellspring of protein in the battle against hunger [23]. The cell reinforcement and antibacterial have the capacity to forestall harm which is ascribed to free extremists Rhodes, phenolic compounds, and so on (Aida et al. 2009; Barros et al. 2007). They are additionally viewed as ready to decrease cholesterol and relieve stress and certain sicknesses (Bahl 1983).

Nutritional Value of Agaricus bisporus: As per Mustafa Nadhim Owaid, 2017, mushrooms have long been well known to mankind. They are called “Forest Vegetables " And " Forest Meat " . Mushrooms are not only delicious, but also aromatic and nutritious, but due to the deterioration of ecological conditions and pollution, under current conditions. Even for experienced mushroom pickers, this is not safe because they are known how to distinguish edible mushrooms from poisonous mushrooms. The fact is that mushrooms are osmotic bacteria that can absorb harmful substances in the soil and air [29]. In many industrialized countries, mushrooms are no longer picked. Forest mushrooms have been successfully replaced by cultivated mushrooms. They contain no harmful substances and can be used in food without any health risks. Mushrooms are the source of many nutrients necessary for human health. It has more protein i.e.; (3 grams per 100 grams) . They have a higher proportion of healthy amino acids than other vegetables and 70-90% of vegetables, protein is easy to digest. Mushrooms are low-calorie foods (less than 30 calories per 100 grams) . Almost no sugar, no cholesterol, no

fats i.e.; (10.2 grams per 100 grams). Mushrooms are rich in water soluble vitamins than any other vegetables [23].

According to Alexander Tsaren (2003-2021), Approximate content of vitamins per 100 grams :-

• B1 (Thiamine)	: 0-1 mg
• B2 (Riboflavin)	: 0-4 mg
• Nicotinic acid	: 0-5 mg
• Pantothenic acid	: 2-6 mg
• Folic acid	: 0.016 mg
• Vitamin B12	: 0.26 mg
• Vitamin C	: 2 mg

Agaricus bisporus is a great source of minerals (Alexander Tsaren 2003-2021) :

• Phosphorus	: 75 mg
• Potassium	: 620 mg
• Iron	: 1 mg
• Zinc	: 0-2 mg
• Sodium	: 7 mg

Medicinal Value of Agaricus bisporus: According to Maria Elena Valverde, 2015, Mushrooms have been burned-through since soonest history; antiquated Greeks accepted that mushrooms gave solidarity to fighters in fight, and the Romans saw them as the "Food of the Gods." For hundreds of years, the Chinese culture has loved mushrooms as a wellbeing food, an "mixture of life." They have been essential for the human culture for millennia and have significant interest in the main developments in history due to their tangible qualities; they have been perceived for their alluring culinary properties. These days, mushrooms are well known important food sources since they are low in calories, carbs, fat, and sodium: additionally, they are sans cholesterol [3]. Also, mushrooms give significant supplements, including selenium, potassium, riboflavin, niacin, nutrient D, proteins, and fiber. All along with a long history as

food source, mushrooms are significant for their mending limits and properties in conventional medication. (Talía Hernández-Pérez , 2015) It has revealed useful impacts for wellbeing and treatment of certain illnesses. Numerous nutraceutical properties are portrayed in mushrooms, like avoidance or treatment of Parkinson, Alzheimer, hypertension, and high danger of stroke [29]. They are additionally used to decrease the probability of disease intrusion and metastasis due to antitumoral ascribes. Mushrooms go about as antibacterial, safe framework enhancer and cholesterol bringing down specialists; moreover, they are significant wellsprings of bioactive mixtures. Because of these properties, some mushroom removes are utilized to advance human wellbeing and are found as dietary enhancements. [13]

- Anti-Oxidant and Immunodulating Activity: According to Apoorva Bhushan, 2018, the polysaccharides registered in Agaricus bisporus have a large amounts of 'a' and 'b' glucans, which are confirmed by Fourier Transform Infrared Spectroscopy (FTIR) and have Immunostimulatory effects. [2]
- Metabolic Effects: As per Royse D et. al 2003 , A total of 240 , 7 days old mixed quail chicks were randomly assigned to four treatment groups treated with Agaricus bisporus, with an internal of 21 days between adult male and female chicks. 35 days of the growing season. It was found that compared with the control, total cholesterol (TC) , triglycerides (TG) and low-level density lipoprotein (LDL) were significantly reduced. Compared with the control group, 2% of HDL cholesterol in mushrooms increased significantly. In summary, it can be said that 2% of mushrooms have a positive effect on cholesterol, TG, high- density lipoprotein (HDL) and (LDL) in the quail diet. [14]
- Anti-Cancer Effect: According to Winner EP et. al 2002, Malignancy is perhaps the deadliest sickness on the planet. As of late, cleaned some regular dynamic part from mushrooms, for example; polysaccharides showed the huge enemy of malignant growth action toward different disease cell lines. Basidiomycota is known to introduce therapeutic qualities, which are being credited to its glucan and other polysaccharides. The polysaccharides for the most part have a place with the beta-glucan group of mixtures and seem to apply their enemy of tumorigenic impacts by means of improvement of cell insusceptibility. Agaricus bisporus contains bioactive mixtures that have been appeared to display immunomodulating and anticancer properties. The

Canadian Cancer Society suggests utilization of Agaricus bisporus mushrooms due to its viability against human infections. As per Zhang et. al 2014 detailed that early colored Agaricus bisporus polysaccharides had solid Immunostimulatory and Antitumor bioactivity in vivo and in vitro. Agaricus Bisporus contains three principle polysaccharides alpha- glucan, beta-glucan and galactomannan (Smiderie et. al 2011) and galactomannan is fundamental Polysaccharides by 55.8% (Smiderie et. al 2011) , (Ren et. al 2012) revealed that the most widely recognized glucans removed from Agaricus bisporus are (1-3), (1-6) -D-glucan. Utilization of organic products juice advanced with alpha-glucan from Agaricus bisporus (5 g glucans/day) lip polysaccharides actuated tumor putrefaction factor (TNF) creation by 69%. No consequences for interleukin (IL-1b), (IL-6) and diminished creation of IL-12 and IL-10 were noticed (Volkmann et. al 2010). Then again Agaricus bisporus doesn't present exceptionally high B-glucan content (8-12/100 g dm) (MC Cleary and Draga 2016). [15]

- Anti-Inflammatory Activity: According to G. Dhamodharan et. al 2010, the calming action of Meth- anolic concentrates of Agaricus bisporus was examined on actuated macrophages and tracked down that some palatable mushrooms species have a potential enemy of inflammatory limit in vitro. [16]
- Anti-Microbial Activity: As per Sadler M 2003 , the investigation included disconnecting Erwinia spp. Ralstonia Solanacearum from contaminated plants followed by oppressing the confines and financially gained staphylococcus aureus (ATCC25923) , Enterococcus Faecalis (ATCC29212) , Escherichia coli (ATCC25922) , Pseudomonas Aeruginosa (ATCC27853) , Streptococcus Pneumonia (ATCC49617) , Protein Vulgaris (ATCC49990) , Candida Albicans , Aspergillus Niger (ATCC1015), Fusarium Oxysporum (ATCC16608) , Ustilago Maydis (ATCC14826), Microsporum Gypseum (ATCC15621) and Malassezia Furfur (ATCC14423) and were effectively offended by mushroom. [17]
- Skin Disorders: As per Loganathan et. al 1994 , this examination depended on an impact of cleaned tyrosinase from Agaricus bisporus on B16F10 Melanocytes for the melanin creation through impeding shade cell apparatus. Utilizing B16F10 Melanocytes showed that the incitement of Melanogenesis by decontaminated tyrosinase is expected to expanded tyrosinase ingestion. Cell tyrosinase action and melanin content in B16F10

Melanocytes were expanded by sanitized tyrosinase in a portion subordinate way. The outcomes showed that cleansed tyrosinase can be treated as a hopeful for the treatment of antilogous skin conditions. [18]

CONCLUSION: Agaricus bisporus mushroom is a valuable bio-factor for agro waste reusing and can be developed on different manures for example; manures of wheat straw, reed plants, paper, oat straw and some water plants. Agaricus bisporus has numerous dietary utilizations because of its substance of proteins, carbs, low calories, minor components, also nutrients. In the drug field, it was utilized for combination of nanoparticles with antimicrobial and anticancer exercises. The objective of this survey was to show late information about the kind of manures used to deliver and some new methods to cultivate Agaricus bisporus mushroom. Agaricus bisporus may address a significant development for their portrayal as a wellspring of medications and more clinical information are required for the assurance of therapeutic advantages of Agaricus bisporus.

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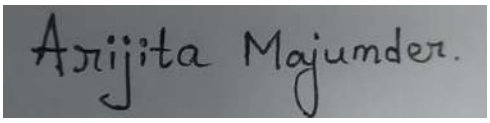

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Asian Soybean Rust: An upcoming threat to soybean cultivation

Abstract:

Asian soybean rust caused by the fungal pathogen *Phakopsora pachyrhizi*, is a great threat for soybean cultivation in all over the world. As it is the major disease of soybean field the appearance of this rust fungi completely destroys the grain production and yield causing a huge economic loss every year in every major soybean producing field. Soybean has an enormous application in different industries including one of the main sources of nutrition and seed oil. Currently Asian soybean rust is associated with every major soybean producing countries including India. The consequence of this disease is so devastating and explosive that is was previously considered as bioterrorism. *Phakopsora pachyrhizi* is an obligate biotrophic pathogen which completes its life cycle asexually through the production of uredospores. This pathogen shares a broad range of host that it can infect 31 plant species of legumes. The disease is mainly expands through the wind and when the uredospores lands on the leaf surface it initiates new infection. The main symptoms of soybean rust are the formation of small brownish to dark brown lesions, which contains one or more uredosori on the ventral side of the leaflets. At present application of fungicides like DMI, SDHI, QoI is the most effective means of control of soybean rust. At present six R genes (*Rpp 1-6*) were identified from different cultivars of soybean. Beside this several evidences support that application of non-host plants, transgenic soybean plants and genetic engineering also provides promising resistance against *P. pachyrhizi*. Biological control using beneficial microbes and RNAi techniques is another means of sustainable protection strategies of Asian soybean rust. In the present review the disease development, infection events and in depth effective control measures of Asian soybean rust has been enlightened.

Key words: Asian soybean rust, DeMethylation inhibitor, phytoalexins, R gene, *Rpp*, uredospores

Introduction

Rapid increase in the world population is a severe threat for various food producing industries including agricultural sectors. Providing sufficient food to all the individuals is an

important challenge which has been faced for a long time. Though advancement in the modern agricultural system able to provide considerable food security but still various biotic and abiotic stresses in the crop field is always been a severe problem that leads to huge economic loss. Rapid climate change introduces various abiotic stresses which causes significant yield loss. Beside this, attack of different plant pathogens and pests is also an important issue. One of such recent instances is the occurrence of Asian soybean rust caused by the fungal pathogen *Phakopsora pachyrhizi*. It is a major disease of soybean crop field that results in huge economic loss every year worldwide. Globally soybean (*Glycine max*) is recognized as an economically important crop, since it is a rich source of both protein (about 40%) and vegetable oil (about 20%).Thus soybean is also called as "two in one crop" (Chander et al. 2019). The dietary fiber and isoflavones present in soybean help to protect several chronic diseases including diabetes, obesity and heart diseases. It is suggested that in breast cancer treatment soybean can be used as it contains high amount of vitamins, minerals and polyunsaturated fats (Asif et al. 2013). Since its wide utilization and one of the important sources of foods, soybean cultivation is increasing consistently in all over the world. Soybean has a great adaptability to different latitudes, climatic and soil conditions which enabled them to become fourth most widely grown crop across the globe after wheat, maize and rice. But one of the major problems is the presence of biotic stresses in the cultivation field, leading to low productivity of soybean. There are more than 300 species of pathogens have been encountered worldwide which causes severe damage to soybean. Among which Asian Soybean rust by *P. pachyrhizi* is the most common and major disease in soybean field causing more than 90% yields loss (Hartman et al. 2005). The severity of Asian soybean rust is so explosive for the soybean crop fields that this disease was previously considered as the bioterrorism (Balardin et al. 2006). This pathogen mainly attacks the leaf portion which affects the photosynthetic ability of the plant leading reduction in weight and poor grain quality. This pathogen cannot survive the freezing low temperature but the disease can spread through the wind over a long distance and the consequences are so dangerous that it is now one of the most feared diseases of soybean field (Rosa et al. 2015).

Asian soybean rust is present in almost all the soybean growing field across the globe. It is known to occur in Asia and many other countries of Europe, Africa, Australia and America. However, this disease was first reported from Japan in 1902 when Hennings isolate this rust pathogen from the leguminous crop *Pachyrhizus ahipa* (Hennings 1903). Over the next 90 years,

the fungus was reported in soybean and related species throughout the Eastern Hemisphere, including Australia, China, Indonesia, India, Japan, Taiwan and other countries in tropical and sub-tropical regions (Bromfield 1984). In India the disease was first reported in 1970 and earlier considered as of minor importance and was known to occur in low hills of Uttar Pradesh, West Bengal and North East regions. After 1993 the disease was known to occur in all the soybean growing field in India including Madhya Pradesh, Maharashtra, Karnataka, Tamil Nadu, Kerala, Rajasthan and Himachal Pradesh, and becoming serious threat to soybean cultivation (Sharma and Gupta 2006).

Previously it was reported that there are two species of *Phakospora* exists namely *P. pachyrhizi* and *P. meibomiae* which causes soybean rust. *Phakopsora meibomiae* is less harmful and less aggressive compared to *P. pachyrhizi* and occurred mainly in the Central America. However, recent studies have reported that there are 80% sequence homology in the DNA content of both the species (Chander et al. 2015). One of the major characteristics of this rust fungi is the extremely broad host range. In this regards it was reported that *P. pachyrhizi* can complete their life cycle in 31 different species of leguminous plant including their main host *G. max* (Goellner et al. 2010). Generally rust fungi are mostly biotrophic and macrocyclic. Commonly five different spore stages are associated with their life cycle. But in case of *P. pachyrhizi* the sexual cycle is somewhat not prominent and unclear. This pathogen belongs to the class Basidiomycetes and known to have only asexual uredosorus stage. However in laboratory condition teliomorphic and basidial stage were distinguished but aecial stage is not yet reported (Green 1984). Symptoms of Asian soybean rust are mainly appeared on the aerial parts of the plants. Small, tan colored lesions are formed on the abaxial surface of the soybean leaflets (Ogot et al. 2017).It may also occur in petioles, pods and stems. The Pathogen has the ability to defoliate the soybean fields within a few days and may lead to complete crop failure.

Soybean cultivation in many areas invaded by *P. pachyrhizi* is endangered because soybean varieties with resistance to all isolates of pathogen have not yet been discovered. Moreover, most of the conventional methods used to eradicate the disease are found to be less effective. It is very important to control the pathogen to get rid of this serious disease of the soybean field. At present, three strategies have been followed widely to check the disease progression which incorporates use of different chemical fungicide, production of genetic

engineered resistant soybean plant and specific cultivation techniques (Kendrick et al. 2011). Chemical control using chemical fungicide belongs from the class Demethylation inhibitors (DMI) and Quinone outside inhibitors (QoI) is found to be most effective against *P. pachyrhizi* (Guicherit et al. 2014). Beside this biological control using beneficial microbes and other biological organisms like fungi and essential oils from different plants have been also tested *in vitro* and exhibited promising results (Langenbach et al. 2016). Genome analysis of soybean plant revealed the presence of six resistance gene which referred as *Rpp 1-6*. Further studies have shown that there are three genes present namely *GmEDS1*, *GmPAD4*, and *GmNPR1*which directly involved in the mechanism of *Rpp 2* mediated resistance in soybean plant (Pandey et al. 2011). Forward genetic screening can be used to identify different resistance genes and loci which provide quantitative resistance against Asian soybean rust. Different transcription factors also have been analyzed and found to be over expressed in plants with *Rpp 1* and *Rpp 2* gene product (Schneider et al. 2011). In order to draw resistance scientists have proposed another approach that is the application of recessive *R* genes. Three recessive *R* genes to *P. pachyrhizi* have been isolated and help to draw resistance in soybean plants (Langenbach et al. 2016). Elimination of susceptibility gene *(S)* is another approach to draw resistance against *P. pachyrhizi*. But it is very difficult to identify the *S* gene, till now scientists are able to identify only one soybean rust *S* gene (Uppalapati et al. 2012). Another effective molecular control is the application of RNAi mediated gene silencing. Different pathogenesis related genes in the fungi can be targeted and then editing can be done by using dsRNA (Koch and Kogel 2014). Beside this, different biotechnological methods are also investigated like *R* gene pyramiding, introduction of engineered *R* gene, use of non-host plant, application of antimicrobial peptides and production of secondary metabolites which found to be effective against the Asian soybean rust (Tremblay et al. 2009; Langenbach et al. 2016). The overall control measure strategies of Asian soybean rust are presented in the following Figure1.

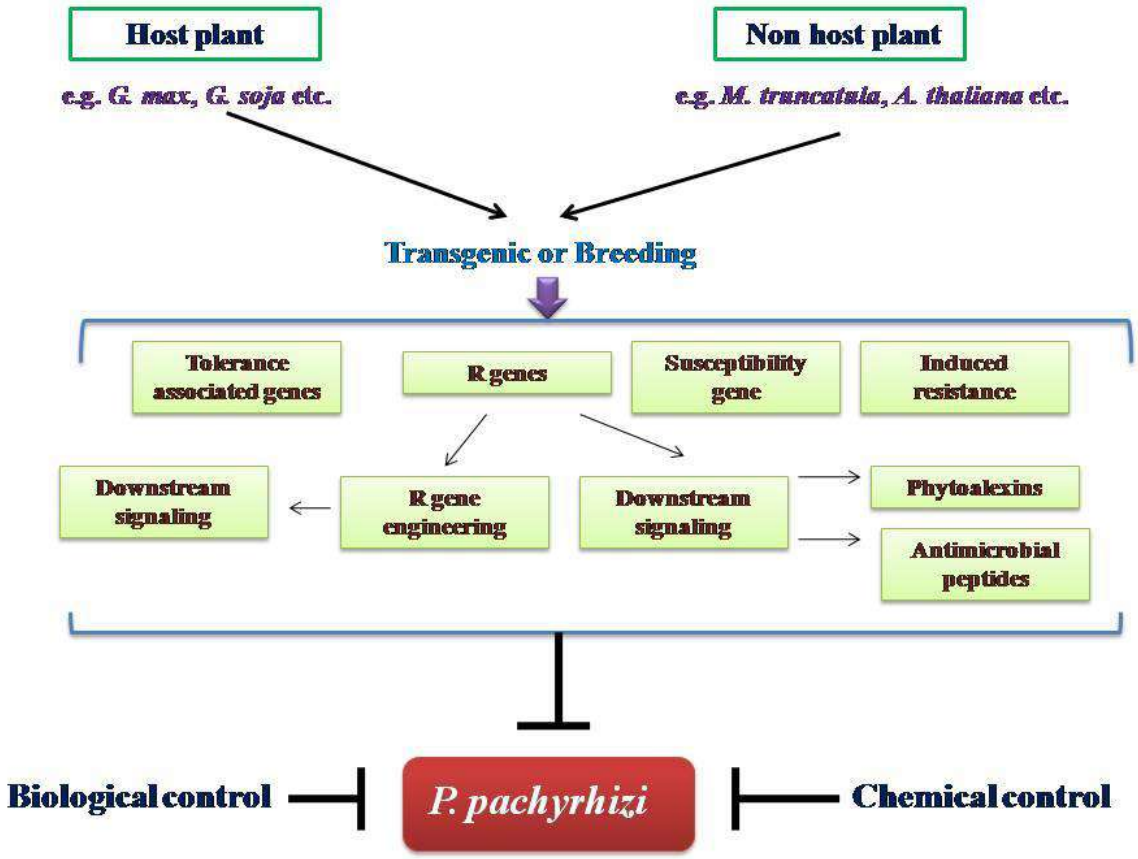

Figure1: Different disease management strategies taken to control Asian soybean rust.

To overcome the threat of the rust and to minimize the accruing yield loss it is become very important to know more about the disease and its eco-friendly integrated management. In the present review an attempt has been made to compile and synthesize the important information on geographical distribution, yield losses, casual organism, disease cycle, host range, disease management, rust resistant gene and biological control, chemical control and to reveal the gaps in knowledge on various aspects of this serious disease of soybean by the pathogen *P. pachyrhizi.*

The host: Soybean an important staple

Soybean holds an important place among all the agricultural crops since it is an excellent source of oil, foods and medicines. Soybean belongs to the family Leguminosae or Fabaceae (subfamily Papilionaceae). The correct scientific name of the field grown soybean is *Glycine*

max (L.) Merrill. Two subgenera of the genus *Glycine* are known to occur one is annual and other is perennial. *G. max* and *G. soja* are the two annual species. The cultivated soybean consists of both tap root and adventitious root system with bushy appearance generally devoid of secondary branches (Chaturvedi et al. 2011). Basic chromosome number of the field grown soybean is 2X= 40 and it is a paleopolyploids (Hymowitz 2004).

Soybean cultivation has a long history and it is now cultivated throughout the world over a vast area. Various scientists supported the fact that soybean cultivation was introduced first during the Shang dynasty in northern China during 800-100 BC. It is one of the oldest legume food and people are cultivating soybean for over 5000 years (Hymowitz 1970; Bromfield 1984). As time passes soybean cultivation gains its popularity and currently it is being cultivated in Korea, Japan, China, Europe, USA and other parts of the globe. As soybean is a legume crop it can fixes biological nitrogen which provides a great adaptability to the plant over a wide range of environmental conditions (Rosa et al. 2015).With the advancement of agricultural biotechnology soybean productions rises steadily over last few decades. Soybean shares the maximum global seed oil production which is 53% of all the other sources of grain oils (Asif et al. 2013). Besides this, soybean has vast utilities in health, food and other industrial sectors. Due to high nutritional value soybean based supplements and medicines are used to treat different diseases including breast cancer. Soybean contains a wide range of nutritional components (Table1) including vitamin-A,B,C,K, high amount of proteins, essential amino acids carbohydrates, omega-3 fatty acids, dietary fibers, minerals like iron, potassium, calcium and estrogenic substances (Friedman and Brandon 2001). Due to this ample amount of application and high nutritive property it is very necessary to prevent soybean from different biotic and abiotic stresses which will eventually secure future soybean cultivation.

Table1: Nutritional profile (per 100g dry matter) and amino acid composition of soybean seed (mg/g) (Lokuruka 2010; Asif et al. 2013).

Nutritional components	Amount	Amino acid composition	Amount mg/g protein
Proteins	36g	Aspartic acid	68.86
Simple carbohydrates	9g	Arginine	77.16

Complex carbohydrates	21g	Glycine	36.72
Raffinose	1.6g	Cystine	25.00
Stachyose	3.3g	Histidine	34.38
Saturated fat	2.8g	Lysine	68.37
Monounsaturated fat	4.4g	Glutamic acid	190.16
Polyunsaturated fat	11.2g	4-Hydroxyproline	1.40
Total fat	19g	Isoleucine	51.58
Soluble fiber	7g	Valine	41.55
Insoluble fiber	10g	Serine	54.05
Magenesium	280mg	Tryptophan	12.73
Calcium	276mg	Methionine	10.70
Iron	16mg	Tyrosine	41.55
Potassium	1797mg	Proline	52.91
Zinc	4.8mg	Phenylalanine	56.29

Different abiotic and biotic stresses on Soybean cultivation

Like other legumes soybean is also involved in biological nitrogen fixation which facilitate to their growth and productivity. Beneficial microbes and mycorrhizal fungi are colonized in the root nodules of soybean which enhances the nutrients uptake ability that contributed to their high grain productivity. Instead of this attributes cultivated soybean may be subjected to different environmental stresses. Different biotic and abiotic factors regulate the grain production capability of the plant. Among the abiotic stress flooding stress, acidity stress and tillage stress are mostly affects the plant which causes huge economic losses (Hasanuzzaman et al. 2016). Flooding causes several anatomical and physical injuries and might initiates anaerobic stresses in the plant. Flooding stress also hampers the microbial colonization as a result nodulation gets affected which causes significant yield losses. Moreover, it was found that flooding stresses down regulate the tolerance genes in soybean (Tewari and Arora 2016). Soybean plants which are cultivated to tropical area may also cope with acidity stress. During the acidity stress high concentrations of different ions may initiates osmotic stress. The symbiotic

association between the nitrogen fixing bacteria is totally disrupt during acidity stress and consequently yield loss occurs (Miransari 2016).

Like abiotic stress, biotic stresses including attacks of different pathogens, pests, aphides, parasites are some major problem in soybean cultivation. Beside Asian soybean rust several other fungal, bacterial, viral and parasitic diseases (Table2) affects soybean yield greatly. But the severity of Asian soybean rust is totally unmatched thus it is considered as the most severe soybean disease.

Table2: Different biotic stresses of soybean caused by different group of pathogens.

Causal agent	**Disease name**	**Pathogen**	**Symptoms/ affected portion**	**Reference**
Bacteria	Bacterial Blight	*Pseudomonas savastanoi* pv. *glycinea*	Symptoms appears on leaf, stems and pods, Small, angular translucent yellow spots are developed with characteristic yellow halo in the center.	Faske et al. 2015
	Bacterial Pustule	*Xanthomonas axonopodis* pv. *glycines*	Small light green color lesions are appeared on the infected leaf, lesions turns brown at maturity.	Sweets et al. 2008
Fungi	*Fusarium* root rot	*Fusarium solani*, *F. oxysporum*, *F .tricinctum*.	Reddish to Brown discoloration of the taproot, reduced nodule formation, seedling dumping-off.	Roth et al. 2020
	Rhizoctonia root rot	*Rhizoctonia solani*	Reddish lesions on the hypocotyls of seedlings near the soil line, plants	Tsror 2010

			stunted, yellow and wilting.	
	Sudden death syndrome	*F. virguliforme*	At early stage of infection discoloration and rotting observed in the roots, as disease progresses interveinal chlorosis appears as foliar symptoms that develop into necrosis.	Hartman et al. 2015
	Charcoal rot	*Macrophomina phaseolina*	Taproot and the lower stem turns gray or silver. Under the epidermis numerous black fungal specks (micro sclerotia) gives a "charcoal" like appearance.	Khan 2007
	Sclerotinia Stem Rot (White mold)	*Sclerotinia sclerotiorum*	Water-soaked lesions appeared near nodes in the stem, soon the lesions enlarged and fluffy white fungal growth develops in moist stem.	Fall et al. 2018
	Frogeye leaf spot	*Cercospora sojina*	Brown colored lesions develop on leaf surface surrounded by darker reddish brown or purple ring.	Mian et al. 2008

	Downy mildew	*Peronospora manshurica*	Pale green to light yellow spots develops on the upper surface of the young leaf as an early symptom, at maturity the spots turn into dark brown with yellow margin.	Sweets et al. 2008
	Stem Canker	*Diaporthe caulivora* (northern stem canker) *and D. aspalathi* (southern stem canker)	On the lower portion of the stem starting at nodes reddish brown lesions are formed, they expand with the time and become sunken cankers.	Faske et al. 2014
	Anthracnose	*Colletotrichum truncatum*	During maturity dark brown lesions formed on stems, pods, and petioles. Small black fungal patches appear in patterns on stems, pods and petiole.	Thasis et al. 2021
	Brown stem rot	*Cadophora gregata*	In lower stem development of brown pith occurs, symptoms also manifested as brown to yellow discoloration in between leaf veins.	Cummings and Bergstrom 2015
	Pod and stem blight/ Phomopsis seed decay	*Diaporthe sojae* and *Diaporthe longicolla*	On stem, pods and petioles small, raised black dots (pycnidia) are arranged in distinct rows. The upper portion of the	Rupe 1990

			plants may discolor and die, leading to plant death.	
	Powdery mildew	*Microsphaera diffusa*	On the upper leaf surface small, circular areas of white, powdery mold grows.	Sweets et al. 2008
	Cercospora leaf blight	*Cercospora kikuchi*	On the upper leaf surface purple to bronze discoloration found, on both sides of the leaves develop red and brown spots.	Paul 2013
	Septoria brown spot	*Septoria glycines*	Irregular brown and yellow patches develops on one side of the leaf, brown spots coalesce into large brown areas.	Lin et al. 2020
	Soybean rust	*Phakopsora pachyrhizi*	On the upper surface of the leaf very small tan or reddish brown lesions are formed, very small pustules develops on the underside of the leaf.	Kelly et al. 2015
Virus	Bean pod mottle	Bean pod mottle virus (BPMV)	The young leaves become mottled, green to yellow mottling is found. Leaves become wrinkled, and puckered.	Bradshaw 2007
	Soybean mosaic	Soybean mosaic virus	Symptoms are most severe on youngest leaves. Light	Hajimorad et al. 2018

			and dark green mottling of leaves occurs. The leaf curl downwardly, seeds are become small in size.	
Nematodes	Soybean cyst nematode	*Heterodera glycines*	On the roots small lemon shaped female worms found. Cysts color ranges from cream to dark brown. Soybeans can become yellow and stunted.	Tylka 1997
	Root knot nematode	*Meloidogyne incognita*	The infected plant may show yellowing, wilting and stunting. The presence of gall or swelling in the root system is a characteristic symptom of root knot nematode.	Sweets et al. 2008

Taxonomy and morphological overview of the pathogen

Taxonomy of fungi is traditionally based on the differences in phenotypical or morphological characteristics such as size and shape of the spores, reproductive organ, germination etc. It is very difficult to accurately classify any rust fungi based on their morphological features since they possess a complex life cycle and shares a wide range of host. In modern taxonomy DNA sequences are used to identify different group of fungi. So to classify any rust fungi it is very necessary to analyze the gene sequences for obtaining accuracy. The casual organism of the Asian soybean rust, *P. pachyrhizi* belongs to the phylum Basidiomycota (Alexopoulos and Mims 1979), Calss Uredinomycetes, Order Uredinales, family Phakopsoraceae Family and the Genus *Phakopsora* (Hennings 1903; Agrios 1997). The family Phakopsoraceae contains 10 anamorphic genera and 13 teleomorphic genera and it is considered as a diverse group. Classification of the genus is not so clear and unsatisfactory. The genus is thought to be

monophyletic origin and consist of more than 90 species (Ono et al. 1992). Historically there are two isolate of the fungi present known as Asian-Australian isolate *P. pachyrhizi* and the American isolate *P. meibomiae*. Previously taxonomist made no distinction between these two species but later genetic analysis showed that these two species shares 80% sequence homology within their ribosomal internal transcribed spacer region (Frederick et al. 2002).

*P. pachyrhizi*is an obligate biotrophic pathogen and thus can only grow on living host. Generally rust fungi exhibited five different spore stages in their life cycle which includes spermatia, aeciospores, uredospores, teliospores and basidiospores (Aime et al. 2017). These five spore types are highly specialized for causing any infection in a particular host (Goellner et al., 2010). The rust fungi which exhibit these five types of spore in their life cycle are called as macrocyclic fungi (Chander et al. 2019). But in case of *P. pachyrhizi* till now the uredial, teleomorphic and basidial stages are reported(Rosa et al. 2015). The aecial stages have not been reported yet (Green 1984). Beside this most of the rust fungi exhibit two separate haploid nuclei in the cells during their life cycle known as dikaryotic mycelium.

Uredospores are globose to sub-globose, ellipsoidal or ovate in shape and are essentially light-yellow brown to hyaline in color and open through a central pore to form a germ tube (Figure2). They form abundantly on the abaxial leaf surface, where they range from 100 to 200 µm in diameter (Sinclair and Backman 1989). The size of the spore is highly variable, in the range

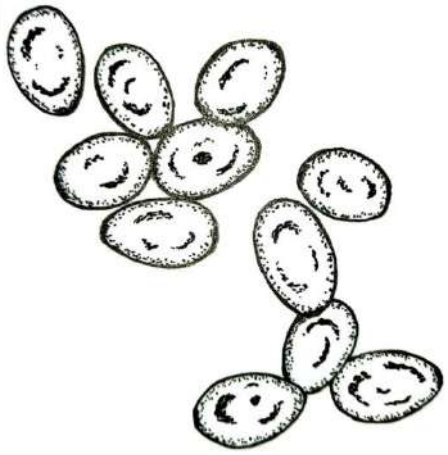

Figure2: Uredospores of *P. pachyrhizi.*

of 18-45×13-28 µm, depending on the environmental conditions and the host. A dome-like outer covering is present at the sporophores known as paraphyses, which are found surrounding the inner wall of the uredosorus, and unite at the base. The paraphyses are inward curving, hyaline to sub-hyaline, prominently capitate at the apex, with a narrow lumen, and a measure about 7-15 µm toward the apex (Sinclair and Backman 1989).

Geographical footprints of the soybean rust pathogen

The climatic condition which needed for the soybean cultivation, unfortunately also favors the development of soybean rust disease. Presently it is well established that soybean rust caused by two obligate biotrophic fungal species*: P. pachyrhizi* and *P. meibomiae*. The rust disease was first recorded in Japan in the year 1902 by Nakanishiki who identified the fungus as *Uredo sojae* (Yang 1977). Later, Hennings (1903) confirmed the fungus as *Uredo sojae* on leaves of wild grown soybean *G. soja* when it was collected from Tosca Province of Japan by Yoshinaga (Sharma and Gupta 2006). By 1934 the pathogen had been found in several Asian countries and as far south as Australia (Bromfield and Hartwig 1980). The soybean rust caused by these Asian-Australian isolates of *P. pachyrhizi* is widely referred as Asian soybean rust in literature. In the second half of the 20th century, the footprints of *P. pachyrhizi* appeared to be moving into several soybean growing countries in Africa. In 1996 the soybean rust spread in Uganda and Kenya. From these countries, the disease possibly expanded southwards to Rwanda, Zambia, and Zimbabwe in 1998, Mozambique in 2000 and South Africa in 2001 (Pretorius et al. 2001; Levy et al. 2002).The westward movement of the pathogen on the African Continent was reported from Nigeria in 1999 (Akinsanmi et al. 2001).

In South America the first report of *P. pachyrhizi* was came from Paraguay in March 2001 (Morel et al. 2004). It was subsequently reported in the state of Parana, Brazil in 2001 (Yorinori 2004). The disease was found in Hawaii in 1994 on cultivated soybean field in the islands of Hilo, Kakaha, Kauai and Oahu. (Kilgore and Heu 1994).

By 2002, soybean rust was widespread throughout Paraguay and in limited areas of Brazil, bordering Paraguay (Morel and Yorinori 2002). The pathogen also found in some limited area in northern Argentina (Rossi 2003)

In August 2004, the USDA and the Animal Plant Health Inspection Service (APHIS) confirmed a report of soybean rust in Colombia (Simmet 2004). On the 10th November 2004, the USDA issued a press release on the first report of soybean rust on the USA mainland (Rogers and Redding 2004).

In India, the appearance of soybean rust was first reported on in 1951 (Sharma and Mehta 1996).The first authentic report of soybean rust in India was available from Pantnagar in 1970 (Sarbhoy et al. 1972). Later, rust was also found in low hills of UP and Kalyani in West Bengal. Till 1974 the rust remained restricted in and around Pantnagar and subsequently disappeared from India. However, in 1980, after a lapse of almost 5 years the rust resurrected in high altitude areas of Meghalaya and later reports came from the plains of Assam in North east regions of India (Maiti et al. 1981; Sharma 1990). Since then rust is occurring in almost all Northeastern hill region in epiphytotic form. Till 1993, the rust remained confined to this region and afterwards it spread to other soybean production areas of India (Sharma and Gupta 2006). It is now established that soybean rust occurs in all major soybean production areas around the world. In the following Figure3 the worldwide occurrence of Asian soybean rust has been presented.

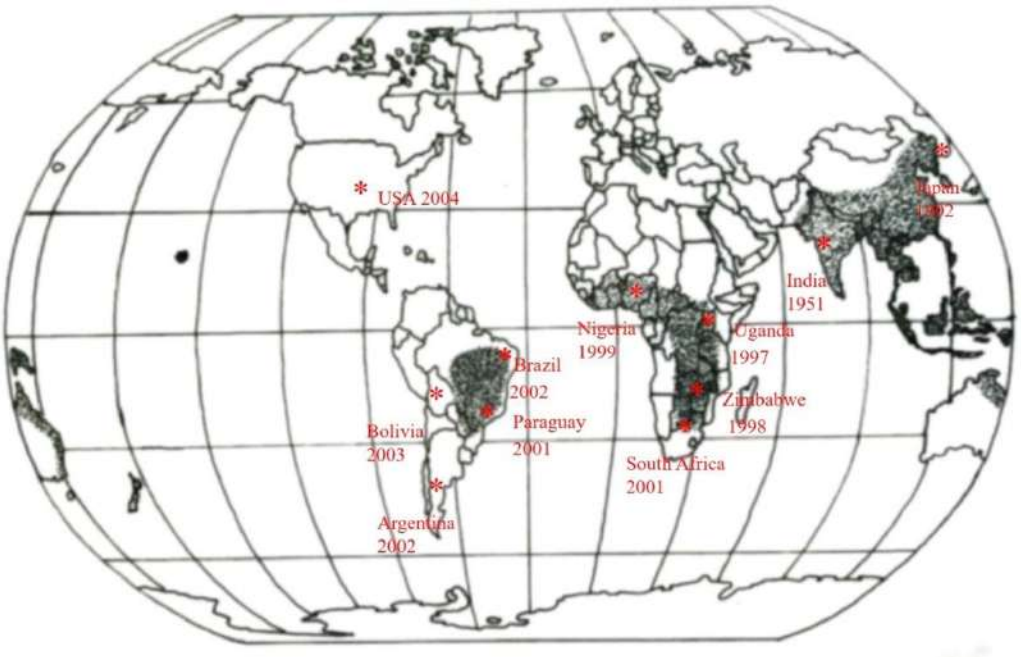

Figure3: Distribution of Asian soybean rust and the year it was first reported for each location.

Host range of the pathogen

P. pachyrhizi is an obligate parasite so this pathogen cannot complete its life cycle without exploiting a suitable host. If an obligate parasite cannot obtain a host it will fail to reproduce. Therefore *P. pachyrhizi* cannot survive independently of its host or on debris. So it must have to find another host on which it can survive under host free conditions.

This pathogen shares unusually wide range of host. Different reports suggested that *P. pachyrhizi* can cause natural infections on 31 plant species in 17 genera of legumes. Moreover it can also infect 60 other species of plants belonging from 26 additional genera when inoculated (Chu and Chuang 1961).

This fungus can infect a large number of dicotyledonous plants such as, common bean (*Phaseolus vulgaris*), wild soybean (*G. ussuriensis*), yam bean (*Pachyrhizus erosus*), cowpea (*Vigana ungiculata*), pigeon pea (*Cajanus cajan*) and kudzu (*Pueraria lobata*), both in field and in laboratory (Yang 1977; Hartman et al. 2011).

Soybean and kudzu is the most important host epidemiologically. Soybean is economically affected by the disease and kudzu is a common overwintering host (Silva 2008). The pathogen can also found on other important crop belonging from the family Fabaceae such as cowpea and pigeon pea plant. The disease does not usually cause severe epidemics in these crops, and their importance for spore production in natural condition is unclear.

Different researchers proposed different alternative host for *P. pachyrhizi*, but more studies are needed to reach a convenient interpretation. Because the researchers do not state the criteria clearly used to determine a 'host'. The basic criteria of determining a host can be sporulation. If the fungus can produce spores on the crop then it should be considered as an alternative host (Miles et al. 2003).

Indicator of the disease: the symptoms of soybean rust

The symptoms of Soybean rust caused by the fungus *P. pachyrhizi* are different from the other types of rust pathogens. The fruiting bodies are the uredosorus, which produce uredospores. Uredospores are asexual spores, and are borne on a short stalk within an uredosori. After 5-8 days of inoculation on colonized leaves, uredospores are germinated and released from uredosori through ostiole and dispersed by wind. Under suitable environmental conditions each uredospores gets germinated containing a single germ tube. After successful establishment of the pathogen symptoms are appeared on the infected plants. The main symptom of soybean rust is the formation of small brownish to dark brown lesions, which contains one or more uredosorus on the ventral side of the leaflets (Figure4).Generally symptoms are first appeared on the older and lower portion of the leaf when the flowering stage is over. Initially the lesions are small and appeared as water soaked, grey to brown in color but the size increases gradually as the disease progressed. Lesions are angular in shape with 2-5 mm in diameter. Uredospores can be visible prominently on the leaf surface which is present as rust spores cloud (Caldwell and Laing 2002). Symptoms are predominant on the leaves but in severe cases stems, pods and petioles can also be affected. The color of the lesions varies with the age and with the interaction between the host genotype and the pathogen isolates (Reis et al. 2012).When the symptoms present as reddish brown coloration it indicates semi-compatible interaction and when it is present as tan red coloration with low necrosis then it is considered as compatible interaction (Bromfield et al. 1980).

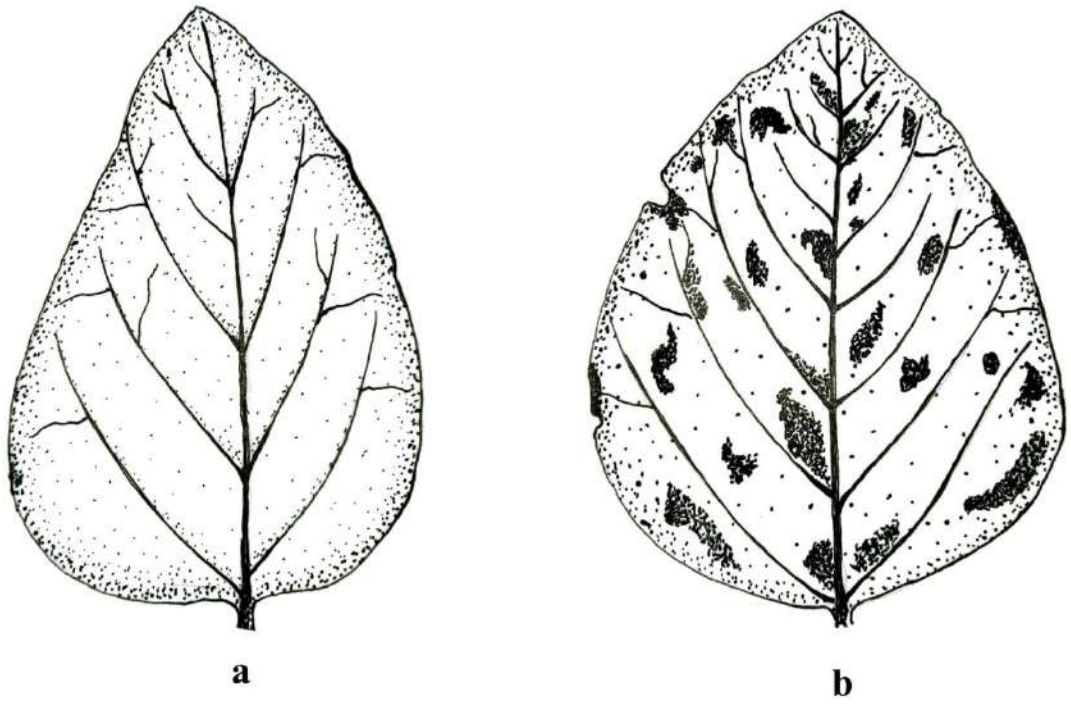

Figure4: A healthy soybean leaf before infection (a). Leaf after successful infection by *P. pachyrhizi* showing reddish brown lesions containing uredospores (b).

Once the symptom appears, chlorosis occurs rapidly which results in premature defoliation. Leaves turns into yellow or brown with patches of tan brown color uredospores. Number and weight of the seeds and pod gets reduced with early maturity of the grains. Sometimes the symptoms of soybean rust may be confused with bacterial pustule disease but in case of bacterial disease the water soaked lesions are associated with mucilaginous sticky substance containing full of bacteria (Guicherit et al. 2014).

The pathogen life wheel and infection process

Uredospores can be dispersed by wind and deposited on the host leaf surface which begins to germinate followed by the initiation of infection. Infection process takes place in the presence of water and an optimum temperature of 21°C to 25°C.Approximately eight days after the infection fungus begins reproduction that leads to hyphal aggregation forming the uredosorus primordium. Uredosorus have a light brown to red color. Uredospores start producing in about three to four days after the formation of uredosorus primordium (Rosa et al. 2015).

After germination of the uredospore on the leaf surface of the host plant the infection process begins. The growth of the germ tube is terminated by the formation of appressorium. Appressorium is a specialized, globose infection structure which is separated from the germ tube by the formation of a septum. The asexual cycle has been summarized in the Figure 5.

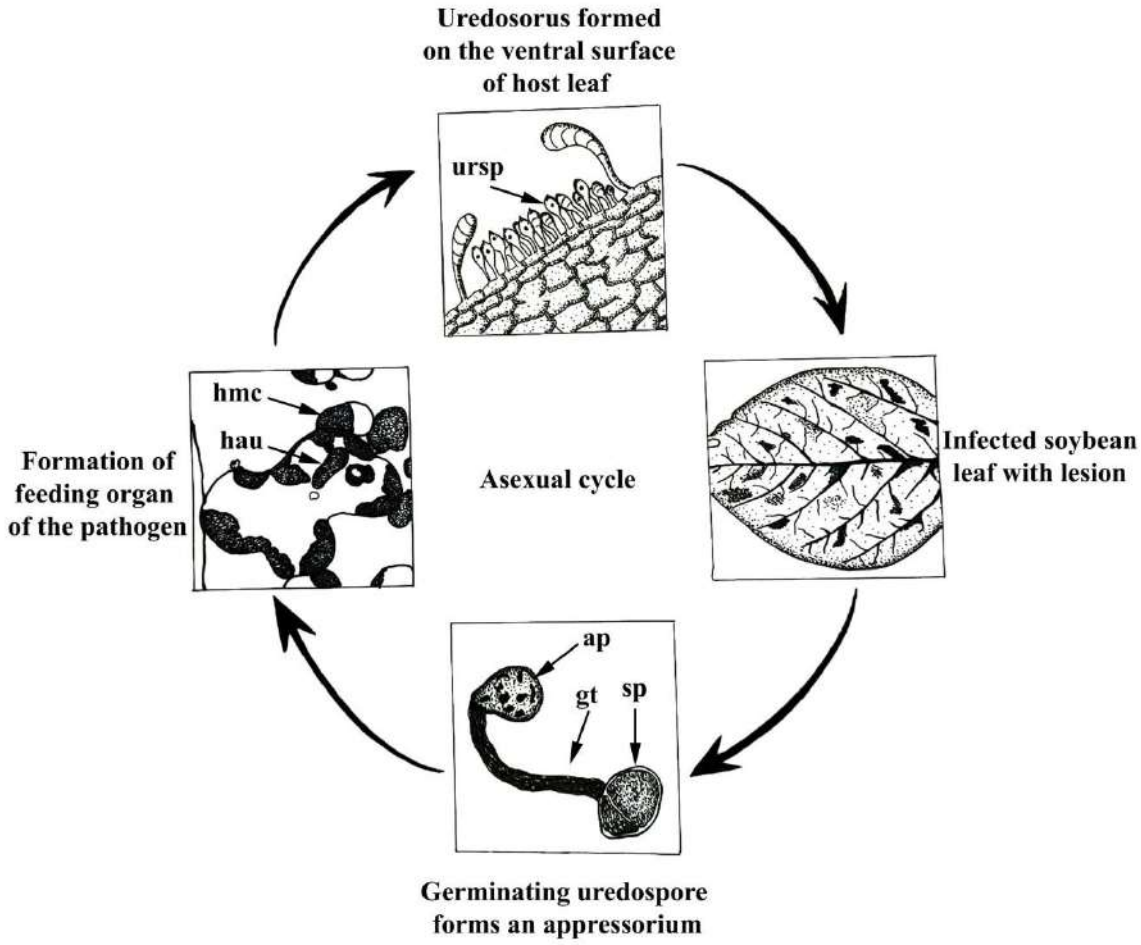

Figure5: Life cycle of *P. pachyrhizi*. app; appressorium, sp; spores, hmc; haustorial mother cell, hau; haustoria.

The fungus *P. pachyrhizi* penetrates into the host epidermal cells within the appressorium by building of an internal structure called the appressorial cone (Figure6) which then elongates into the penetration peg (Bromfield1984; Loehrer and Schaffrath 2011). The penetration of the parasite directly through the cuticle and epidermal cell wall of the host is a special characteristic feature of Asian soybean rust that helps to differentiates *P. pachyrhizi* from other rust fungi. In

contrast to *P. pachyrhizi*, majority of other rust fungi exhibited stomatal penetration (Loehrer andSchaffrath 2011; Rosa et al. 2015).

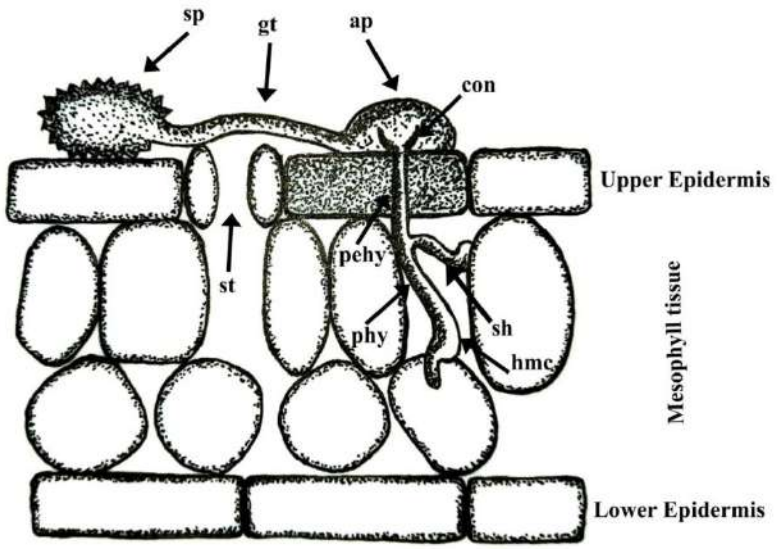

Figure6: Initial interaction of *P. pachyrhizi* with host plant. Sp; spore, gt; germ tube, app; appressorium, penh; penetration hypha, ph; primary hypha, sh; secondary hypha, hmc; haustorial mother cell.

The infection process of *P. pachyrhizi* includes a sequence of events (Figure7). The epidermal cells of the host plant undergo cell death after penetration by *P. pachyrhizi*, it is a very uncommon feature among biotrophic pathogens particularly in rust diseases (Keogh et al. 1980; Loehrer and Schaffrath2011). However, the cell death event does not affect the growth of the penetration hyphae, growth of the hyphae goes on and they branched after they reached the mesophyll tissue and differentiating into haustorial mother cells (Koch et al.1983; Loehrer and Schaffrath2011). The haustorial mother cell gives rise to formation of a specialized infection structure called haustoria which is an essential feeding organ of the pathogen. All obligate biotrophic fungal plant pathogens produce haustoria, it is a very common feature among them and failure of which is a knock-out criterion for infection (Loehrer and Schaffrath 2011). The fungal mycelium intensively colonizes the host tissue after the haustoria successfully established and then the production of new uredospores completes the pathogens life cycle (Koch et al. 1983).

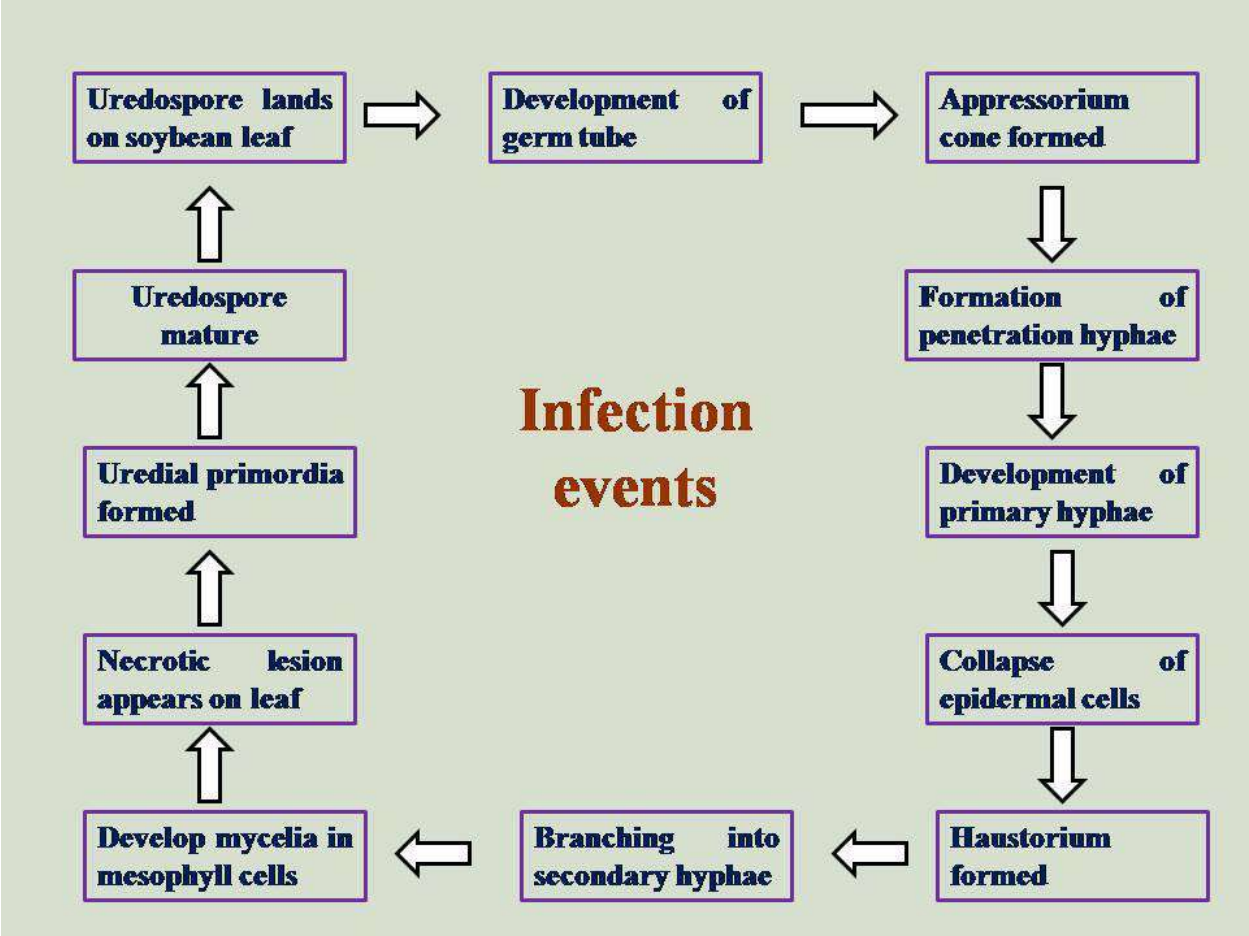

Figure7: Different series of events that takes place during the infection.

Disease epidemiology

Asian soybean rust favors the environments that are humid and warm. The spores of the pathogen can be able to germinate on the leaf of the host plant only after a continuous period of wetness, this situation will aid the growth of this disease. Temperature, rainfall and leaf wetness are some of the main factors which determines the severity of the disease (Tschanz et al. 1984).

Rust epidemics are most severe when the average daily temperature is less than 28°C and presence of an extended period of leaf wetness with relative humidity of 75-80% (Caldwell et al. 2002; Hartman et al. 1999). Dry conditions, excessive precipitation or daily mean temperatures greater than 30°C or less than 15°C inhibit rust development (Sinclair and Backman 1989). Moisture on plant surfaces is crucial for germination to occur (Caldwell et al. 2002). Hence areas where prolonged periods of leaf wetness due to dew, mist and light rain occurs provide optimum

conditions for germination (Kloppers 2002). Temperatures above 27°C for extended periods retard rust development even with adequate free moisture on the leaf surface (Casey 1979). Soybean rust develops more rapidly in the areas where rainfall occurs more evenly throughout the season, as compared to the areas where rainfall occurs in an uneven pattern. Hence rust development varies according to prevailing rainfall patterns.

The uredospores are the primary inoculum of soybean rust. These are asexual, small, lightweight spores, which are released from uredosorus when the infected leaf surface gets dry. They can be able to spread from one field to another through the air current. The uredospores deposited on the leaf surface, infects the host plant and starts germinating in the presence of 21°C to 25°C temperature (Reis et al. 2012). The parasite penetrates directly through the leaf cuticle and epidermal cell wall rather than through the stomata of the host plant. The direct penetration is a characteristic of the *P. pachyrhizi* fungus that differentiates it from other rust causing fungi (Rosa et al. 2015). The colonization begins shortly after penetration, the primary branching of hyphae gives rise to formation of dense mycelium filling the intercellular spaces and inserting haustoria in the mesophyll and epidermal cells. The fungus reproduction begins approximately at eight days after infection, and its first evidence is the formation of hyphal aggregation giving rise to the uredinia primordium. After about 3 to 4 days, uredospores are produced from the uredosorus (Rosa et al. 2015).

Phakopsora pachyrhizi is one of the five rust fungi that can interact without the formation of an appressorium. Telia are very rare but occasionally can form sub-epidermally, mostly on the ventral leaf surface, along with the uredosorus and at the edges of the lesions (Chander et al. 2019).

Control measures

To control soybean rust different strategies has been incorporated by different researchers. Chemical control is found to be very effective against the pathogen. Different groups of chemical fungicides are being applied and different countries have made different commercially available fungicides to draw protection. Biological control using beneficial microbes and different plant products are also a significant means of soybean rust. Genetic engineering, identification of resistance genes, use of non-host plants are some of the effective

strategies which found to be give protection against the rust pathogen. In the following context some of the potential disease management techniques have been discussed.

Chemical Controls

The importance and application of fungicides in crop the field has increased significantly as their efficacy of protection has evolved drastically. In the present time the application of fungicides is the most effective means of control of soybean rust. During 1960s the first report of chemical control of soybean rust was recorded. Based on the effectiveness of lime-sulphur, Bordeaux mixture, mercurials and zineb that are initially tested on soybean plant in Japan, scientists have showed their interest in development of fungicides to control soybean rust (Bromfield 1984). Currently application of chemical fungicides found to be an alternative for the producer of different location in controlling this aggressive pathogen. The most commonly used fungicides are belong from the group triazoles, carboxamide and strobulirins. Beside this several protectants and eradicant are also used with varying proportion of constituents and effectiveness (Yorinori et al. 2005). During the last few decades, the chemical control has changed according to the fungicide evolution and resistance of rust to the chemicals. There are certain factors like biological activity, translocation in plants, penetration, application strategies and time intervals that determines the efficacy of these fungicides.

Protective fungicides are used before the pathogen attack that ensures protective barriers against the fungal pathogens by inhibiting spore germination. Contact protective fungicides do not produce phytotoxicity as penetration is not necessary for their function. Mancozeb is widely used as a protectant spray. However, for high effective control four applications per season are necessary and the spray schedule has to be initiated before appearing of the symptoms. (Preez and Caldwell 2004). Recently due to the problem of the efficacy of two most commonly used fungicide DMI and QoI, copper based multisite fungicide like dithiocarbamate and chloronitriles with combination of systemic chemicals has been tested to obtain resistance (Miles et al. 2007).The group sterol biosynthesis inhibitor includes three groups of fungicides viz amines, DMI and hydroxyanilides. These compounds are so much versatile and used in various crops including soybean. Triazole is another group of fungicides with potential eradicative property and long residual effects. Triazoles such as frutriafol and tebuconazole are extensively used in the management of Asian soybean rust which gives significant resistance against *P. pachyrhizi*.

There are some triazole fungicides which are commercially registered for Asian soybean rust including cyproconazole, epoxyconazole, fluquinconazole, tetraconazole, metconazole and flutriafol (Silva et al. 2004). Strobilurins is also used in the rust disease management program. Strobilurins in combination with triazole exhibited significant resistance against the rust fungi in soybean. The main strobirulins registered to control soybean rust includes pyraclostrobin, trifloxystrobins and azoxystrobin (Sierotzki et al. 2013). In the following Table3 some of the commercially available fungicides are listed which are used to control *P. pachyrhizi*.

Table3: Some of the major fungicides with their trade name and active ingredients used to control Asian soybean rust (After Yorinori et al. 2005).

Sl. No.	Active constituents	Trade name	Dose of usage (ml/ha)
1.	Triforine	Funginex®	1500
2.	Cyproconazole	Alto®	300
3.	Flutriafol	Impact®	800
4.	Tebuconazole	Folicur®	1000
5.	Triademenol	Shavit®	500
6.	Carbendazim	Punch Xtra®	350
7.	Propiconazole	Tilt®	500
8.	Difenoconazole	Score®	300

Biological controls

Protection against Asian soybean rust using beneficial microbes and other biological organism has been obtained. The bio control of soybean rust not yet clearly found through studies, but some macro parasites such as *Trichothecium rosae* and *Simplicillium lanosoniveum* seem to be potential biocontrol agents. These organisms colonize in the uredosori of *P. pachyrhizi* on the infected leaves which eventually reduce both sporulation and disease severity (Kumar et al. 2002; Ward et al. 2012). It was also found that *T. rosae* mediate the lysis of the growing uredospores by causing shrinkage and hypertrophy. In addition to this some of the *Bacillus* species were also found to be effective against *P. pachyrhizi*. The bacterium *Bacillus* is used as an active constituent of the commercially available fungicides named as Ballad® which

is a potential protective agent in soybean rust disease management. Moreover, it was found that the reduction of rust severity as well as the germination of *P. pachyrhizi* uredospore completely inhibited by the application of *B.subtilis* and *B.pumilus* (Dorighello et al. 2015).A soilborne bacterium, *Xanthomonas parasitica* that are spread by rain splash, parasitizes uredia of various cereal rust fungi and causes uredospore lysis (Pon et al. 1954).

Beside these antagonistic microbes, it has been demonstrated that farnesyl acetate, a naturally occurring plant volatile emitted by rust infected plants, negatively affects haustorial development. Thus, this compound might be used to control soybean rust in an environmentally compatible manner (Mendgen et al. 2006).Other natural compounds such as Acibenzolar-S-methyl (Cruz et al. 2013), essential oils from *Hyptis marrubioides*, *Aloysia gratissima*, and *Cordia verbenacea*, were also reported to be effective in the suppression of soybean rust (Silva et al. 2014). These are the some evidences of biological control of soybean rust. However, more studies and trials are needed to obtain resistance through biological control.

Crop management

Introduction of new cultural techniques and modification of the present cultural methods helps to prevents diseases in the crop fields. Very few agronomic practices have been discovered so far for soybean rust management. Moreover, such practices are mainly dependent on disease avoidance mechanisms and are restricted to specific site and/or climatic zones. It was observed that sown during dry season in Nigeria control soybean rust, and if sowing done in wet season, early planting appeared as an important factor to avoid soybean rust (Twizeyimana et al.2011). Most agronomic practices such as row spacing, sowing date, cultivar maturity and cropping and tillage system does not directly affect soybean rust incidence or severity. However, such practices may influence disease pressure due to extended time that the crop is exposed in the field (Chander et al.2019).However, it was found that several crop management strategies like selection of planting sites, use of early maturing variety, weed management in the field and utilization of short pod filling stage give protection against *P. pachyrhizi* (Bromfield 1984). Physiological age of the soybean plant greatly affected the disease development. In a study it was observed that the early maturing varieties are more susceptible to *P. pachyrhizi* than the late maturing varieties (Tschanz and Tsai 1982).

Use of Coffee oil (Dorighello et al. 2015) seed treatment with shale water (Mehta et al. 2015) root drenching with saccharin (Srivastava et al. 2011) are also important crop management practices, but their actual efficacy for effective soybean rust control is still awaiting assessment under field conditions.

Silicon is a beneficial agent for yield and plant growth, it is also gaining recognition for its prophylactic role in alleviating diseases, insects attack, unfavorable climatic conditions, and in improvement of chemical and physical soil properties (Liang et al. 2005). Soybean rust disease severity has reduced up to 65% as well as delaying the disease onset by the use of soil- and/or foliar-applied silicon (Rodrigues etal. 2009; Lemes et al. 2011). There is genetic variability for silicon absorption within soybean germplasm and high absorption of silicon protects the soybean crops against soybean rust disease (Labrecque et al. 2012). How silicon boosts soybean rust disease resistance is still not clear. Additional information such as appropriate source of silicon and its mode of application are also needed for more precise and long-lasting control of *P. pachyrhizi*.

Secondary metabolites

Accumulation of secondary metabolites in response to invading pathogens attacks is a very common defense mechanism of plants. In most of the cases these metabolites are cell wall degrading agents and antimicrobial chemicals which draw a barrier against an infection. Secondary metabolite plays an important role in the interaction of *P. pachyrhizi* and soybean plant during the disease progression. It was reported that during the infection events isoflavonoids compounds like genistein, glyceollin and daidzein is accumulated which affects the spore germination and reduces disease severity in both resistant and susceptible soybean plants (Lygin et al. 2009). The role of phytoalexins is also observed in providing resistance against soybean rust. In *Glycine tomentella* an alternative host of *P. pachyrhizi*, accumulates an isoflavonoid upon infection which inhibits uredospore germination (Chung and singh 2008). Accumulation of medicarpin in *Medicago truncatula* a non-host plant of *P. pachyrhizi* supported the potential ability of phytoalexins in defeating the rust. it was reported that medicarpin negatively regulate the infection events by blocking the uredospore germination (Ishiga et al. 2015). The molecular investigation on this chemical pathway could identify the specific genes

which can be used to construct resistant transgenic soybean plants. Moreover these metabolites can be used as natural fungicides that could provide resistance in susceptible hosts. The importance of phenylpropanoid pathway in disease resistance has been studied by gene silencing. When the phenylalanine ammonia lyase enzyme gene gets silenced the *Rpp-2* mediated resistance in infected plants was compromised (Pandey et al. 2011). Beside this, antimicrobial peptides can also provide resistance against soybean rust. However the efficacy of these peptides is not well studied. Intragenic antimicrobial peptides from the other organisms can be used to construct transgenic plant. For instance penetratin and dermaseptin SI are two IAP which provides soybean rust resistance by inhibiting uredospore germination (Brand et al. 2012).

Non-host resistance

Incorporation of non-host plant is another promising approach of soybean rust resistance. Application of non-host plant is an effective technique to identify the resistance trait. Use of these plants enable the utilization of vast genetic resources. It is a complex type of resistance strategies which shares defense mechanism with the host plant. It is very difficult to distinguish host and non-host plant since there is many intermediate resistance (Bettgenhaeuser et al. 2014). Genetic analysis of these resistance traits in soybean plant may provide alternative resistance beside chemical fungicides and biological control. In terms of non-host resistance *Arabidopsis thaliana* and *M. truncatula* are best described non host plant. However the initial stage of development of *P. pachyrhizi* in both, *A. thaliana* and soybean are similar. Although there is no report of hyphal proliferation in leaf mesophyll cells of *A. thaliana*. To encounter the preinvasion resistance in *Arabidopsis* penetration mutants were used. Three mutant *pen1, pen2* and *pen3* were prepared and it was observed that hyphal proliferation occurred in these mutants. However despite of hyphal proliferation in mesophyll tissue fungus did not complete its life cycle and failed to colonize. This indicates the preinvasion resistance in this plant. The post invasion resistance was also encountered in this plant with triple mutant *pen2, pad4, sag101*. This study reveals that interspecies transfer of these genes can induce resistance against *P. pachyrhizi* (Langenbach et al. 2013).

Phakopsora pachyrhizi can infect various plants but its occurrence in non-host plant is very rare. *Medicago truncatula* is the only legume non host plant in which sporulation does not

occurs. The mutants in this plant were identified by forward genetic screening as the plant contains diploid genome. The mutant *M. truncatula* exhibited altered resistance to *P. pachyrhizi*. Genetic screening identified a irg 1 (inhibitor of rust germ tube differentiation) mutant which inhibits the formation of pre-infection structure in this plant (Uppalapati et al. 2012). Moreover, the interaction between *P. pachyrhizi* and *M. truncatula* induces several genes which are associated with isoflavonoids, phenylpropanoid and flavonoid pathway. These groups of chemicals block the uredospore formation which eventually slows down disease progression (Ishiga et al. 2015). In addition to this the role of phytoalexins in rust resistance is also explored. These genes provide resistance in soybean plants against *P. pachyrhizi*.

Resistance genes

It is very interesting that pathogens exhibit different levels of virulence and aggressiveness in different hosts. These host specific physiologic specializations are known as pathotypes or pathogenic races (Chander et al. 2019). Identification of these pathotypes in different population of pathogens is essential to trace resistance genes in host plant. With time and continuous evolution diversity has been increased in the virulence of pathogens. To overcome the virulence of these pathogens superior genetic resources are needed to achieve a long durable resistance. In plant use two types of defense against a pathogen, race specific resistance and race non-specific resistance (Rosa et al. 2015). During the infection process pathogens releases effectors to establish a successful infection. In response to effectors plant induce their resistance mechanisms by activating different downstream signaling cascades. At the early stages of infection pathogens releases pathogen associated molecular pattern (PAMPs) which is identified by pathogen recognition receptors (PRR) present in plant and initiate immune responses. This type of immune response is known as PAMP triggered immunity or PTI. This plant defense response is the first line of defense and not so durable. At the later stage plant exhibit effectors trigger immunity (ETI) which is a long lasting and durable resistance. Due to ETI plant initiate different signaling cascade which results in R gene expressions (Mackey and McFall 2006; Langenbach et al. 2016)

Bromfield (1984), first classifies resistance and susceptible soybean varieties based on the lesion color and number of uredosorus present during the infection. There are three types of infection reactions present in soybean plant. When infected soybean varieties developed tan

color lesions (TAN) contain no resistance gene considered as susceptible while those developed reddish-brown (RB) lesions contains resistance genes considered as partially resistant. In some cases where no symptoms occurs are considered as immune (Bromfield, 1984). Till now there are eight resistance genes were identified (*Rpp 1-8*) (Langenbach et al. 2016). The first resistance gene of soybean rust *Rpp 1* was found in the PI 200492 variety of soybean. In Taiwan PI 200492 was inoculated with nine isolates of the pathogen where six and seven isolates failed to develop uredinia and symptoms. In 1973 PI 200492 was found resistant to all the isolates tested in India (McLean and Byth 1980). In Uganda and Nigeria when PI 200492 treated with different isolates of the pathogen it showed less disease severity and also the pathogens were remains less virulent. Another version of *Rpp 1*resistance gene (*Rpp 1b*) was identified in different varieties of soybean including PI 587855 and PI 587886. This resistance gene is the allele of *Rpp 1*. When soybean variety PI 587880A containing *Rpp 1b*, infected with a number of isolates collected from kudzu it developed a moderate level of uredospores with combination of TAN and RB symptoms. It was reported that in South America *Rpp 1b* containing soybean varieties are resistant while in continental US susceptibility was observed (Kato and Yorinori 2008). Similarly different dominant R genes were also identified from different varieties of soybean plants which is presented in the following Table4.

Table4: List of resistance genes indentified in different soybean variety in different country.

Resistance gene	Variety/ Plant line	Original name	Origin	Chromosome no.	Reference
Rpp 1	PI 200492	Komata	Japan	18 (G)	McLean and Byth, 1980; Rosa et al. 2015
Rpp 1	PI 561356	Jin Yun Dou	China	18 (G)	Kim et al. 2012
Rpp 1	PI 594177	Himeshirazu	Japan	18 (G)	Yamanaka et al. 2015
Rpp 1	Xiao Jing Huang	Xiao Jing Huang	China	18 (G)	Yamanaka et al. 2015
Rpp 1b	PI 594538A	Min Hou Bai Sha Wan Dou	China	18 (G)	Chakraborty et al. 2009

Rpp 1b	PI 587886	Bai Dou	China	18 (G)	Ray et al. 2009
Rpp 2	PI 230970	No. 3	Japan	16 (J)	Bromfield and hartwig 1980; Rosa et al. 2015
Rpp 2	PI 197182	Raub 16.1422	Malayasia	16 (J)	Rosa et al. 2015
Rpp 3	PI 462312	Ankur	India	6 (C2)	Hartwig and Bromfield 1983
Rpp 3	PI 628932	FT-2	Brazil	6 (C2)	Chander et al. 2019
Rpp 3	D86-8286	D86-8286	USA	6 (C2)	Kato and Yorinori 2008
Rpp 4	PI 459025	Bing Nan	China	18 (G)	Hartwig 1986
Rpp 5	PI 200487	Kinoshita	Japan	3 (N)	Garcia et al. 2008
Rpp 5	PI 471904	Orba	Indonesia	3 (N)	Garcia et al. 2008
Rpp 6	PI 567120B	MARIF 2767	Indonesia	18 (G)	Li et al. 2012; Chander et al. 2019
Rpp 7	PI 605823	SAMPLE 87	Vietnam	19 (L)	Chander et al. 2019

Beside the dominant resistance genes, R gene pyramiding is another potential strategy of soybean rust resistance. R Gene pyramiding is the process of combine two different R gene into a single genetic background. Several evidences supported this process as a potential technique of conferring resistance. Hyuugaa cultivar variety of soybean in Japan exhibited natural R gene pyramiding. In this variety the combination of two R genes showed better resistance than the single R gene (Langenbach et al. 2016). Along with R gene mediated resistance identification of susceptible gene (S) and the knockout the S gene is also a important strategy of resistance. Different researchers applied this technique and got promising result towards the Asian soybean rust resistance.

Host induced gene silencing and RNAi technique

Gene silencing by using small double stranded interfering RNA has been a potential technique in various disease management. To control soybean rust RNAi can be used to silence genes of *P. pachyrhizi* which are critical for disease development. Host induced gene silencing is another version of RNAi technique which is very effective against different fungi, virus, oomycetes, nematodes, bacteria and sucking insects (Langenbach et al. 2016). Previously it was reported that gene silencing of nematodes by using siRNA in soybean plant provides protection against the pathogen. However there is no such report found about the soybean rust resistance by using this technique. Although different destructive rust fungi has been controlled in different crops by gene knockout technique which includes *Puccinia graminis*, *P. striiformis* and *P. triticina* (Yin et al. 2010; Panwar et al. 2013). These evidences support that host induced gene silencing could be used as an alternative strategy in case of soybean rust resistance. Several stage specific genes of *P. pachyrhizi* has already been identified including kinase family protein, cell wall degrading enzymes, metabolism linked genes etc. (Stone et al. 2012; Link et al. 2014; Langenbach et al. 2016). Targeting these genes might provide resistance in soybean.

Conclusion and future outlook

Asian soybean rust caused by *P. pachyrhizi* is a devastating disease of soybean field thus it is a great threat to soybean production worldwide. This virulent pathogen can spread a wide range of area since the uredospres are dispersed by wind and can initiate several infection cycle in a single growing season. It is very difficult to study the pathogen outside of its host since it is an obligate parasite. Till now majority of the researches on Asian soybean rust are mainly targeted on the disease development, epidemiology and control measures. Though these researches are centered on the diseased plants but it is still not clearly known whether all the soybean cultivars are resistant to *P. pachyrhizi* or not. The interaction between the host and pathogen during the infection events should be explored more thoroughly to identify the crucial elements of disease progression. More studies on the pathogen are required as there is not sufficient data present about *P. pachyrhizi*. Gene expression analysis during the infection could identify novel strategies for induced resistance against the fungus. Though researchers have already indentified some of the crucial genes in soybean but still more information is needed

about the up regulation of different genes during appressorium formation and epidermal penetration to achieve a clear idea on the asexual cycle of *P. pachyrhizi*.

Chemical control is one of the effective means of controlling rust. There are many novel compounds like DMI, QoI and SDHI which holds a promising control of soybean rust in near future. But it was also found that excess and long term use of these fungicides achieve insensitivity in *P. pachyrhizi*. Since R gene mediated resistance in the cultivars variety exhibited long lasting and potential resistance it is very essential to explore more transcriptional analyses on this disease. Engineered R gene and induction of phytoalexins biosynthesis pathway is also promising way of rust resistance. Beside this exploration of non-host plant mediated resistance would provide a vast range of germplasm resources in upcoming years. More application of new biotechnological techniques like gene knockout, gene silencing and genome editing tools like CRISPR/Cas9 has to be incorporated to achieve promising resistance in soybean. It can be concluded that genetic tools along with biological control would provide a sustainable soybean production in future.

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Lopamudra Bakshi

Biopesticidal activity of *Tagetes* spp.

I. Introduction:

Marigold belonging to the genus *Tagetes* is an annual herbaceous plant of Asteraceae family. *Tagetes* is native of Mexico and other warmer parts of America and are cultivated elsewhere in the tropics and subtropics. In India, these were introduced by the Portuguese (Gupta and Vasudeva,2012).The name marigold is however applied to several genera of Asteraceae with golden or yellow capitula inflorescence . There are about 33 species of the genus *Tagetes*, out of which, five species have been introduced into the Indian gardens viz. *Tagetes erecta* L. (Aztec or African Marigold), *Tagetes minuta* L. (*Tagetes glandulifera* Schrank), *Tagetes patula* L.(French Marigold), *Tagetes lucida* Cav. (sweet scented Marigold), *Tagetes tenuifolia* Cav. (Striped Marigold) (Rydberg, 1915).*Tagetes* is plant of various uses having ornamental, ritual, medicinal, anthelmintic, insecticidal, colorant, food, and forage applications.(Neher,1968, Vasudevan and Kashyap,1968).Healing properties of *Tagetes* species have been reported in folk medicine and alternative medicines through ages (Montellano, 1986). Despite being native to America, *Tagetes erecta* is often called as African marigold. This species is widely cultivated in India. Various medicinal uses of the different parts of this plant were explored in scientific literature (Shetty *et al*, 2015).

Biopesticides are pest management agents based on living micro-organisms or natural products. They have proven potential for pest management and they are being used across the world. (Chandler, 2011). Biopesticides may be of microbial or of plant product in origin. Many phytochemical pesticides show broad spectrum of activity against pests and other diseases. They have long been considered as potent alternative to synthetic chemical pesticides as they are biodegradable, target specific, and produce little or no toxic effect to the environment or to human health. Besides, cost of production of Biopesticides is significantly lower than the synthetic chemical pesticides (Hajek, 2004). Plants can produce a wide range of secondary metabolites such as phenol, flavonoids, terpenoids, quinones, tannins, alkaloids, saponins, coumarins and sterols which can play a very important role in plant defence and can protect large number of crops from pest and pathogens. The major components of essential oil of the aerial flowering plant of *Tagetes* mainly constitute monoterpene hydrocarbons and acyclic monoterpene ketones viz. dihydrotagetone, tagetones, tagetenones, piperitone, limonene, (E)-β-ocimene, linalyl acetate, linalol, terpinolene, n-nonyl

aldehyde, β -phellandrene, piperitone, and β – caryophyllene (Gupta and Vasudeva, 2012, Singh *et al,* 2015) . About 19 and 31 phytochemicals were extracted from the methanolic extracts of leaves and flowers of *T. erecta* (Devika and Justin, 2014). The major component of *T.erecta* flower are carotenoids (cis and trans isomers of zeaxanthine) and cis and trans isomers of lutein and lutein esters (Leigh *et al,* 1999).*T.minuta,* rich in many secondary metabolites compound including acyclic, monocyclic and bicyclic monoterpenes, sesquiterpenes, flavonoids, thiopenes, aromatics.(Brene *et al,* 2009;Lawrence,1996;Bansal *et al.*1999).The major constituents of *T.minuta* essential oils are Z-β-ocimene, limonene and acyclic unsaturated monoterpenes, ketones, dihydrotagetone, tagetones(E,Z) and ocimenones(E,Z).(Thappa *et al.*1993; Lawrence,1996; Bansal *et al.*1999; British Pharmacopia,1988).

The major components of *T. patula* essential oils are (Z)-β-ocimene, lionene, (E)-tagetone and (Z)-tagetone, methyl heptanol, β-caryophyllene, piperitone, piperitenone, α-terpinolene, (Z) and (E)-tagetenones, (Z,Z)-allocimene and (Z)-β-ocimene epoxide.(Gupta and Vasudeva,2012; Singh *et al.*2015).

T. lucida essential oil from aerial part mainly contain phenylpropenes and terpenes. (Gupta and Vasudeva, 2012). In fact, the essential oil is dominated by methyl chavicol (estragol) at levels up to 97.3%. (Marotti *et al.* 2004; Ciccio,2004). Other compound include linalool, (E)-β-ocimene, β-caryophyllene, germacreneD, methyllisoeugenol, bicyclogermacrene, spathulenol and caryophylleneoxide. (Caballero-Gallardo *et al.*2011; Vera *et al.*2014). Another compound found in *T. lucida* is α-terthienyl. (Ciccio *et al.*2004).

The essential oil of *T. tenuifolia* from aerial parts contain (Z)-ocimene, (E)-ocimene, dihydrotagetone, tagetones, limonene and β-ocimene. (Helthelyi *et al.*1987). According to recent reviews the essential oils are characterized by dihydrotagetone, tagetones, ocimenones and piperitone.(Gupta and Vasudeva,2012). The essential oil from leaves and flowers contain (E)-tagetenone, dihydrotagetone, (E)-tagetone and (Z)-β-ocimene. (Marotti *et al.*2004).

In the present review an extensive study has been carried out to elucidate the role of *Tagetes* spp as biopesticide especially as insecticides, mosquitocides, nematicides, fungicide and herbicide.

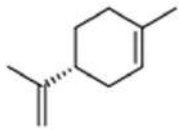
Tagetes erecta

Tagetes lucida

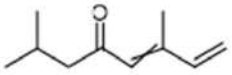
Tagetes patula

Tagetes minuta

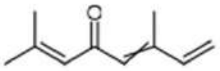
Fig 1. Some *Tagetes* spp. found in India



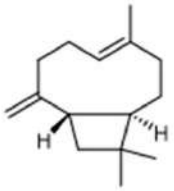
limonene



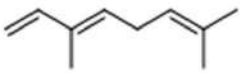
tagetone
(represented as a mixture of
(*E*)- and (*Z*)-isomers)



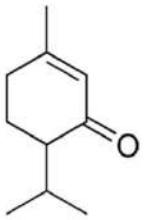
tagetenone
(represented as a mixture of
(*E*)- and (*Z*)-isomers)



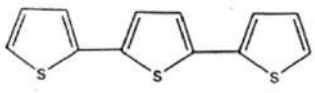
β- caryophyllene



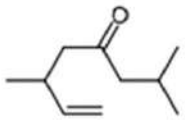
(*E*)- β-ocimene



piperitone



α-terthienyl



dihydrotagetone

Fig 2. Important chemical compound
present in essential oils (EOs) of *Tagetes* spp.

II. *Tagetes* spp. as insecticides:

Plant extracts are widely used as insecticides as they are ecofriendly and safe to the environment. Unlike chemical insecticides, plant extracts as alternative insecticides are non toxic to human and animals and do not cause environmental contaminations (Almeida *et al*, 1999).There are earlier reports of *Tagetes* species which have insecticidal (Sarin, 2004; Nikkon *et al.,* 2009, Weaver *et al.,* 1994,1997), larvidical (Marcia *et al.,* 2011) and mosquitocidal effects.(Nikkon *et al.,* 2011; Shetty, 2015). Several compounds obtained from essential oil from *T. erecta* plants are responsible for its insecticidal property. The major components in the essential oil of aerial parts, capitulum and leaves of *T. erecta* are limonene, (Z) –β-ocimene, eugenol, linalol, β-caryophyllene, linalyl acetate, piperitone (Salehi *et al*, 2018). Ravikumar (2010) evaluated the chemical composition of *T. erecta* and *T.patula* and elucidated its insecticidal property. The hexane extract of *T. erecta* flower showed better insecticidal property against *Acyrthosiphon gosypii*(1000ppm) and *Spodoptera frugiperda* (5000ppm). *T. erecta* showed insecticidal activity against *Tribolium* spp which are considered to be common pests of cereal silos, mills, and warehouses. The quantity and quality of stored food is highly affected by the presence of these insect species. (Nikkon *et al*, 2009). Prescence of insecticidal pyrethrin was found in the callus tissue of *T. erecta* maintained on revised Murashige and Skoog's medium (RT) as static cultures. The percentage of pyrethrin further increased by feeding the tissue with various concentrations of ascorbic acid. The maximum pyrethrin content (1.68%) was observed in 6 weeks old tissue when grown in the medium supplemented singly with 1000 mg/l of exogenous ascorbic acid. When pyrethrin mixture was screened against *Tribolium* spp immediate 'knock down' effect was observed. Knock down effect is the shock effect which is characterized by the inability of the insects to walk and finally progressing to death (Sarin 2004).

Santos *et al* (2016) reported that *T. erecta* and *T. patula* have phytotoxic compounds that can be utilized as a natural insecticide. The hydroethanolic and ethanolic extracts of both the plant species of *Tagetes* showed strong antioxidant property and insecticidal activity against maize weevil *Sitophilus zeamais*. This is a cosmopolitan insect that can attack many hosts like wheat, corn, rice, barley and oats. This insect can attack the seeds both in the field and also in the store house and can cause serious economic loss. In this investigation it was observed that after 60 hours from the beginning all the insects treated with *T. erecta* plant extract reached a maximum value for mortality (80% of dead insects) and the treatments with

T. patula reached its peak of mortality in 84 hours. TLC analysis revealed presence of polyphenols in the plant extract. Silveira (2009) studied the effect of marigold plants as a resource plant to natural enemies in onion fields. The experiment was set in a certified organic farm using marigold rows at a center of an onion field. Samples were taken from marigold and the onion plants 5 m (near) and 30 m (far) from the flowering strips. Higher numbers of arthropod pests were observed in onion plants 30 m from the marigold strip, while higher numbers of predators and parasitoids were found at 5 m distance Therefore, marigold rows next to onion fields resulted in higher number of entomophagous species, potentially enhancing the natural control of onion pests. The main phytophagous species collected *Thirps tabaci* (Thysanoptera: Thripidae) was 57% more abundant far from marigold than near it. A similar result was found for the second most abundant species, *Therioaphis trifolii* (Hemiptera: Aphidae), which was 15% more abundant far from the marigold strip. The opposite was observed with natural enemies predators, especially *Stomatothrips angustipennis*, *Stomatothrips rotundus* and *Franklinothrips vespiformis* (Thysanoptera: Aeolothripidae), which were twice as abundant in onion plants near the marigold strip In this field study, marigold strips were used as an alternative to insecticide sprays for control of onion pests.

Marques *et al* (2011) evaluated the activity of essential oil from *T. erecta* against 3rd instars of *Aedes aegypti* and determined the amounts of larvicidal thiophenes in all plant tissues. The oil obtained by steam distillation and analyzed by gas chromatography/mass spectrometry showed 14 compounds. The main compounds were piperitone (45.72%), D-limonene (9.67%), and piperitenone (5.89%). The essential oil was active against larvae of *Aedes aegypti*, with LC50 of 79.78 µg/ml and LC90 of 100.84 µg/ml. The larvicidal thiophene contents were higher in the roots and flowers as demonstrated by high-performance liquid chromatography analysis. Thus, *T. erecta* constitutes a good source of varied compounds showing larvicidal activity against *Aedes aegypti*.

The Mosquitocidal activity in Ethanolic, chloroform and petroleum ether extracts of *T. erecta* flower against different strains of *Culex quinquefasciatus* was studied by Rahman *et al*, 2009. Among the tested samples the chloroform soluble fraction showed the highest toxicity and constituently the LC50 values (14.14 µg/mL, 1.706 µg/mL, 36.88 µg/mL and 75 µg/mL) for all instars larvae of *C. quinquefasciatus*. The larvae showed comparative tolerance in the course of increasing age and time.

Essential oil of *T. minuta* showed both repellent and growth inhibitory properties against insect pests. (Jacobson,1983). Without any damage of seed germination and colour, the oil of *T. minuta* reduced grain damage due to insect infestation. For this reason it is used as alternative of synthetic insecticide in maize storage by farmers (Morgan,2015). Several insecticidal compounds have been isolated from *T.minuta* (Maradufu *et al*,1978; Perich *et al*,1995; Philogene *et al*,1985; Wells *et al*,1992). Simultaneous steam distillation and extraction of shoot tissue with methylene chloride contains primarily dihydrotagetone and also limonene, trans-tagetone and cis-tagetone. (Weaver *et al*,1994). By using supercritical carbon dioxide, extraction of *T.minuta* contain cis-ocimene and limonene as abundant compounds.(Daghero *et al*,1999). Primarily studies have been done with the group of insects such as Lepidoptera viz. fall army worm (Rao *et al*,2000); Anopheline (Basabose *et al*,1997); and other mosquitoes(Perich *et al*,1995;Philogene *et al*,1985) and Coleoptera (Weaver *et al*,1994,1997;Keita *et al*,2000). Essential oil of *T.minuta* was used asantitick agents especially for controlling *Hyalomma rufipes* tick. (Nchu *et al*,2012). Weaver *et al*,1994, studied that the floral, foliar and root extracts of *T. minuta* showed insecticidal activity against adult Mexican bean weevils but their flower and leaf extracts were more effective as insecticides. The essential oil of this plant is also used in the control of *Rhipicephalus microplus* in cattle. (Andreotti *et al*,2013). Methanolic extracts of *T. minuta* showed insecticidal activity against three different insects *Tribolium castaneum* (Red flour beetle), *Rhyzopertha dominica* (Lasser grain borer), and *Callosobruchus analis* (Pulse beetle). (Shahzadi *et al*,2010). This plant offered a multipurpose tool for managing plant pathogenic soil nematodes and soil dwelling pests. Delivery methods can be of different types such as direct soil incorporation of *T. minuta* biomass (green manure), alternative cropping with *T. minuta* or use of *T. minuta* essential oil as a botanical insecticides.

An important photoactive components (i.e.,α-terthienyl) of *T. minuta* were used for mosquito control.(Amason *et al.*1981). A compound of the essential oil of *T.minuta* was found to be E-ocimene, which showed the larvicidal activity against *Ades aegypti* (Maradufu *et al.*,1978). The essential oil of *T. minuta* also showed high effectiveness against mosquitoes of *Anopheles gambiae*, which is responsible for malaria. (Zoubiri and Baaliouamer, 2014).

Ciccio(2004) stated that *T. lucida* was used as botanical insecticide. Among the *Tagetes* species *T. minuta* and *T. lucida* are appeared to be the most effective for their use as biocidal crops. These are less harmful to human health and nature. (Marotti *et al*,2010). The essential oil of *T. lucida* was used against *Sitophilus zeamais* (Coleoptera:Curculionidae) using the

area performance method. Most oil components were oxygenated monoterpenoids or phenolic compounds. The oil was repellent at doses between 0.063 and 0.503 micro L/cm^2. (Nerio *et al.*,2009). The essential oil from *T. lucida* was used as alternative to the current commercial repellents to control *Tribolium casteneum.* The ethanolic extracts of *T.lucida* was used to control the aphid (*Aphis brassicae*) on cabbage plant. High reduction of aphid population was observed during the first six days after application. After nine days, however the pesticidal effect was lost and another application was needed after nine days from the first application to obtain long period of protection on Cabbage against the infestation of Aphids. (Hendawy,2015).

The western tarnished plant bug, *Lygus Hesperus* knight (Hemiptera;Miridae) and the whitefly , *Bemisia tabaci* Gennadius (Hemipters;Aletrodidae) are two major pests of cotton and other crops throughout the Western United States. The application of aqueous foliar extracts of *T. patula* showed a significant mortality rate in *Lygus hesperus*. Highest mortality was observed with the lowest concentration of the methanolic extract. Both aqueous and methanolic extracts of *T.patula,* exhibited dose dependent toxicity against *Bemisia tabaci*. Differences among doses appeared less marked for the methanolic extracts compared to aqueous extracts. (Jeffery *et al*.,2020).

III. *Tagetes* spp. as nematicides:

Nematodes are unsegmented roundworms that are usually microscopic in size. There are many different kinds of nematodes which live in terrestrial habitat (Krueger,2007). Nematodes can be free-living that feed on fungi, bacteria, nematodes, or other microscopic organisms. Nematodes that feed on plants are called plant-parasitic nematodes. Plant-parasitic nematodes can seriously damage or even kill crops, turf, and ornamental plants. They generally feed on plant roots causing swelling or galls within the roots obstructing the flow of water, mineral salts and nutrients. Plant-parasitic nematodes are difficult to control because they live underground or inside of plants. African (*T. erecta*) and French marigolds (*T. patula*) are the most commonly used species which are well known for possessing nematicidal property. Marigold can suppress about 14 plant parasitic nematodes such as lesion nematode (*Pratylenchus* sp) and root knot nematode (*Meloidogyne* sp) (Suatmadji, 1969). Nematode suppression by *Tagetes* spp is influenced by crop plants, nematode species, and soil temperature (Ploeg and Maris, 1999). There are a number of methods marigold species are found to adapt for nematode suppression such as by acting as trap crop

(Rangaswamy *et al.,* 1993, Pudasaini *et al.,* 2008), by exerting allelopathic effect (Gommers and Bakker ,1988) by enhancing the nematode antagonistic microorganisms (Ko and Schmitt, 1996, Wang *et al,* 2003) or by acting as host plant for nematode (Wang *et al,*2003, McSorley *et al,* 2009). The main method by which marigolds suppress plant-parasitic nematodes is through a biochemical interaction known allelopathy. Allelopathy is a phenomenon where a plant releases compounds in the microenvironment and are toxic to other organisms (Halbrendt, 1996). Marigold plants produce a number of potentially bioactive compounds, among which α-therthienyl is recognized as one of the most toxic substance. This sulfur-containing compound is abundant in marigold tissues, including roots. It has nematicidal, insecticidal, fungicidal, antiviral, and cytotoxic activities, and it is believed to be the main compound responsible for the nematicidal activity of marigold (Arnason *et al.,* 1989; Marles *et al.,* 1992). Thus nematodes may be killed either by entering the root system of a marigold plant or contacting soil containing marigold's bioactive compounds (Wang, 2007). The efficacy of *Tagetes* spp as biocontrol agent to control root knot nematode *Meloidogyne incognata* was evaluated both in the field and soil amendment experiment by Kalaiselvam and Devaraj *et al,* 2011. In this investigation when marigold species *T.erecta, T. patula* and *T. minuta* were preplanted with tomato crops reduction in the numbers of second stage juveniles (J2s) in subsequent tomato plantation was observed than control. Four different concentrations of water soluble extract of marigold cultivars were filtered and added to the Petridish infested with the eggs of *M.incognita*. Root exudates of *T.erecta* were lethal to J2 of *M.incognata* and were inhibitory to egg hatching at concentration of 75 % or higher. *T. erecta* was also reported in effective management of *M. incognita* when it was grown in infested soil (Prasad *et al*, 1992). The bioactive compounds of different marigold species and cultivars may differ in composition, quality, and quantity. Thus, certain species may be highly effective against one nematode species but have limited to no impact on or possibly increase populations of other plant-parasitic nematodes. Wang, 2007 studied the genera of nematodes commonly found in Hawaii and the marigold cultivars that are resistant to these nematodes. *T. patula* 'Single Gold', *T.* hybrid Polynema and *T. erecta* 'Cracker Jack' effectively suppressed four root-knot nematode species: *Meloidogyne arenaria, M. incognita, M. javanica,* and *M. hapla*. 'Polynema' and 'Cracker Jack' were good hosts for reniform nematodes but that marigold *T. patula* 'Boy-O-Boy' suppressed reniform nematode populations. Another important fact was revealed by Siddiqui and Alam, 1988, that aerial parts of *Tagetes* spp also produced α-terthienyl. They compared the nematicidal activity of different parts (leaf, flower, seed, and root) of *T. lucida* on reniform, lance (*Hoplolaimus*

indicus), and spiral (*Helicotylenchus indicus*) nematodes, and reported that the flower extracts had the strongest nematicidal activity, followed by seed, leaf, and root extracts. Similar result was obtained by Hassan *et al*,2003 who reported leaf extract of *T.patula* was toxic to juveniles of *M. javanica* in petridish assay. These findings suggest that aerial parts of marigold is more toxic than roots. Bakker *et al* (1979), Grommers (1972) and Nivsarkar *et al*,2001 reported that photoactivation is necessary for nematicidal activity of α-terthienyl. This may be a reason of lower toxicity of root than the aerial parts (Siddiqui and Alam, 1988). However Faizi *et al*, 2011 reported that nematicidal activity occurred even without photoactivation .Hamaguchi *et al* (2019) observed that, under dark conditions (without photoactivation), α-terthienyl was an oxidative stress-inducing chemical that effectively penetrated the nematode hypodermis and exerted nematicidal activity, suggesting high potential for its use as a practicable nematode control agent in agriculture. They investigated the nematicidal activity of α-terthienyl against the model organism *Caenorhabditis elegans* and the root-knot nematode, *Meloidogyne incognita*. It was observed that induction of two major enzymes, glutathione S-transferase (GST) and superoxide dismutase (SOD), was restricted in *C. elegans* hypodermis following treatment with α-terthienyl. The susceptibility of nematodes to α-terthienyl changed when the expression of GST and SOD was induced or suppressed.

Several cultural practices are practised in the field to control nematodes using marigold. Among different techniques crop rotation using marigold as cover crop is the most frequently used method to control nematodes. Marigold (*Tagetes* spp.), which is a popular bedding plant, can be used as such a cover crop. *T.erecta* produces more biomass than several cultivars of *T. patula* and thus establishes well in the field, making it ideal for use as a cover crop. However, *T.patula* 'Single Gold' can also generate a significant amount of biomass, similar to *T. erecta* 'Cracker Jack', and thus may be an ideal marigold cover crop (Wang 2007). Krueger (2007) suggested planting of marigold as cover crop should be done atleast two months ahead of planting of susceptible plants such as vegetables crops. Furthermore it should be planted at the same site where the susceptible crop is going to be planted next. Cover crop technique can reduce the nematode number in the soil. However if there is heavy infestation in the soil then the protection of next crop from nematode infection cannot be completely ensured. Thus it is important to determine the nematode population number before planting marigold. Reynolds *et al*. (2000) compared the effects of *T. patula* and *T. erecta* as rotation crops with the traditional practice of growing a rye (Secale cereale). Rotation crop and chemical fumigation were implemented before trans planting tobacco

(*Nicotiana tabacum*) in a field trial. Marigolds reduced *P. penetrans* population densities below the economic threshold for the cash crop for 3 years and increased tobacco yield by 197 kg ha−1 compared to rye and chemical fumigation. Similarly, Seigies and Pritts (2006) found that rotating *T.erecta* with strawberry lowered nematodes to below detection levels, Intercropping is another method of nematode management. Intercropping is the practice of cultivating two or more crops concurrently within the same field (Andrews and Kassam, 1976). Tsay *et al.* 2004 conducted a greenhouse experiment to evaluated the effects of intercropping water spinach (*Ipome areptans*) with some plants belonging to Asteraceae on root knot nematode *Meloidogyne incognita* numbers. They found that *T. erecta* was among those species that significantly reduced root galls on spinach was significantly reduced when intercropped with this species. Similarly, El-Hamawi *et al*. (2004) observed that *M. incognita* did not form galls on soybean (Glycine max) plants when intercropped with marigold species *T.erecta* and *T.patula*. Allelopathic plants may prove valuable under conditions where multiple nematode species are present since they have the ability to suppress multiple nematode pests. For example, banana plantings typically have mixed populations of nematode species with different feeding habits (Kashaija *et al.*, 2004; Wang and Hooks,2008). When *T. erecta* was intercropped with banana, populations , four important banana nematode pests, *Radopholus similis, Helicotylenchus multicintus, R. reniformis*, and *Hoplolaimus indicus* were suppressed (Alam *et al.*, 1979).Xie (2007) investigated the efficiency of crop rotation and intercropping systems with *T.erecta* for root knot nematodes (*Meloidogyne* sp) in angelica (*Angelica sinensis*).Plants of angelica were intercropped with *T.erecta* plants in row-intercropping (RI) and plant intercropping (PI) models. The crop rotation model showed higher nematode control efficiency than intercropping models. In addition, marigold can be added as a green manure in the field (Siddiqui and Alam, 1987b) and applied as a plant extract similar to nematicides (Mateeva and Ivanova, 2000, Natarajan, 2006).Lesion nematodes like *Pratylenchus penetrans* and *Pratylenchus pratensis*, parasites of ornamentals, coffee and other important crops were suppressed by *T. patula.*(Wang *et al.*,2007).

Fig 3. Intercropping with *Tagetes* spp. for nematode control

IV. *Tagetes* spp. as bactericide and fungicide:

Plant based antibacterials have huge therapeutic potentials. It can be used as alternative of sysnthetic antibacterials as they can serve the purpose with lesser side effects. (Lwu *et al.* 1999). Essential oil (EO) of *Tagetes* sp. showed biopesticidal nature. It was documented that EO components especially terpenoids such as dihydrotagetones, tagetones and ocimenones are most effective as antimicrobial agents. (Senatore *et al*.,2004). Various *Tagetes* oil analysed by GC/MS were shown to contain limonene, α-terpinolene, dihydrotagetene and oscimenene and seen to inhibit gram-positive bacteria and fungi (Helthelyi *et al*.,1988). *T.minuta* is one of the most promising species among marigold used as antibacterial and antifungal agents. Ethanolic extracts of this plant were tested against selected microorganisms (Anthony *et al.* 2015). Essential oils and plant extracts of *T.minuta* have been reported to have antifungal activity against pathogenic fungi. (Grange and Ahmed, 1988). The most commonly occurring components of *T.minuta* EOs is limonene, 1,8-cineole,α-pinene, β-pinene and camphor. *T.minuta* showed antifungal activity against some fungi, such as, *Rhizoctina solani, Fusarium oxysporum, Penicillin digitatum, Aspergillus niger, Verticillium fungicola* and *Trichoderma harzianum*. (Matasyoh *et al*. 2007; Saha *et al*. 2012). Various *Tagetes* oils appearded to inhibit gram-positive bacteria and fungi. (Hethelyi *et al*. 1988). Fungicidal activity of *Tagetes* was reported against *Helminthosporium oryzae*. (Lapis *et al.* 1978). Tomato is attacked by a number of insects and disease because of its fleshy nature. In field experiment marigold reduced canker disease of tomato by 62.82%andearly blight by 61.53% in comparison to control. (Nahak and Sahu, 2016). It was found that intercropping with *T.erecta* reduced early blight of Tomato caused by *Alternaria solani*.(Gomez-Rodriguez *et. al.*,2003). The flower extracts of *T.patula* exhibited toxicity against soil borne fungus *Fusarium oxysporum* f.sp. *lycopersici* causing wilt disease in tomato plant. (El-Khallal,2007). Fruit spot is a common disease of tomato plant caused by *Pseudomonas syringae.*(Reddy *et al*.2001). Foliar extracts of *T.minuta* showed antimicrobial activity against both gram-positive and gram-negative bacteria. The major component of the extract, quercetagetin-7-arabinosyl-galactoside showed significant antimicrobial activity. (Tereschuk *et al*., 1997). Hernandez *et al.*,2008 indicated that *T.lucida* extracts also showed high activity against gram positive bacteria and phytopathogenic fungi. Cespedes *et al*,2006 observed that EOs of *T.minuta* showed antibacterial activity against phytopathogenic bacteria *Pseudomonas savastanoi* pv. *phaseolicola, Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *manihotis*, which are responsible for several plant disease. Thembo *et al*.,2010 reported that

extract of aerial parts of *T.minuta* showed effective result against fungus, *Fusarium verticillioides* and *Fusarium proliferatum* in comparison to agricultural fungicide. Highest result was observed with hexane, dichloromethane and methanol. Aqueous extract had no activity on fungal strains. (Thembo *et al*.,2010). Methanolic extracts of *T.patula* showed dose dependent toxicity against *Botrytis cinerea*, *Fusarium moniliforme* and *Pythium ultimum.* For, *Botrytis cinereal* the dose dependent inhibition reached at 39.3% under sunlight, 57.4% with uv-A radiation and 24.8% in dark. In case of *Fusarium moniliforme* under sunlight the inhibition was 50.9% while it was 47.3% for uv-A and 33.8% in dark. *Pythium ultimum* showed highest inhibition under sunlight (72.6%) and 62.7% and 51.4% inhibition were shown in uv-A radiation and dark respectively (Mares and co-workers,2004). Initially tests of fungicidal activity were carried out by the method of Gottestein *et al.,* 1982. Fungitoxic effect of essential oil of *T.minuta* on phytopathogenic fungi *Cladosporium cucumerinum* by TLC method was studied by Cespedes *et al.*,2006 . Methanol-chloroform and ethyl-acetate extracts of *T. lucida* showed antifungal activity. (Damian-Badillo *et al*.,2008). Leaves of *T.minuta* and *T.filifolia* showed strong fungitoxicity against *Sclerotium cepivorum, Colletotrichum cocodes* and *Alternaria solani*.(Zygadlo *et al.,* 1994).*Tagetes* fungicide showed antifungal effects on *Fusarium oxysporumf.sp. Niveum, Fusarium oxysporumf.sp. Capsicum* and *Fusarium graminearum.* The tests were done by measuring the diameters of the fungistasis circles. A remarkable inhibition was observed when the concentration of *Tagetes* fungicide was 1μg/ml. A large number of osmophilic particles were observed in the cytoplasm of watermelon leaves after infected by *Fusarium oxysporum f sp. Niveum*. After treatment with *Tagetes* fungicide the number of osmophilic particle decreased compared to infected leaves. (Lili *et al.,* 2020). Coumarins have been reported in 10 to 30 % amount in the *T. lucida* (Caspedes *et al.,* 2006). Chen *et al.* 2016 evaluated the antimicrobial activity of coumarins against *Ralstonia solanacearum*, which causes severe damage to cell membrane.

V. *Tagetes* spp. as herbicides:

The herbs or unwanted weeds in the agricultural field causes significant economic loss since they are responsible for reducing the quality and quantity of crops. Besides development of herbicide resistance is a common problem nowadays for increasing uses of synthetic herbicides in the field for weed control. One of the solutions of this problem can be use of allelopathic plants or their products in the field to control weeds in a sustainable manner (Singh *et al*,2003). Batish *et al,* (2006) investigated the herbicidal activity of *T.minuta* leaf powder towards the control of two invasive weeds *Echinochloa crus-galli* and *Cyperus*

rotundus of rice fields. Results revealed that *T. minuta* leaf powder applied to rice field soil significantly reduced emergence and growth of both the weed species in the pots under green house and in rice field plots with simultaneous increase in the yield of rice. The effect was quite similar to that observed with the herbicidal application under field conditions. Inhibitory effect was more on *Cyperus rotundus* than *Echinochloa crus-galli*. Wichittrakam *et al* (2018) studied the inhibitory effects of crude extracts and its fractions of leaves of *T.erecta* on germination and seedlings growth of *Echinochloa crus-galli* (L.) Beauv Germination and seedling growth were drastically inhibited by hydrolyzed and acidic extracts. Flavonoid and phenolic concentrations were also found to be higher in these extracts. The hydrolyzed fraction was further selected and finally formulated into soluble concentrate product (SCT) and its inhibition potential and modes of action were investigated. The SCT drastically inhibited the seed germination of *E. crus-galli,* inhibiting both imbibition, α-amylase activity and also seedling growth. Results showed that a potential natural herbicide can be developed from the hydrolyzed fraction of a hydroethanolic extract of *T.erecta* leaves. Laosinwattana (2018) studied the chemical composition of essential oil extracted from *T.erecta* leaf and also evaluated its herbicidal activity against *Echinochloa cruss-galli* (L.) Beauv . Gas chromatography – mass spectrometry detected relatively high amounts of monoterpenes, consisting mainly of piperitone (17.12%), piperitenone (10.46), and ocimine (8.59%); identified sesquiterpenoids consisted mainly of neophytadiene (16.18%) and caryophyllene (11.10%). The essential oil was formulated as emulsifiable concentrate (EC-EO) for herbicidal applications. In Petridish bioassay EC-EO completely inhibited germination of seed of *E.cruss-galli* at concentration of 2mlL-1. Post emergence application was tested on 21-day old plants. Leaves of treated plants showed wilted and desiccated condition indicating EC-EO interfered with photosynthetic metabolism. The EC-EOs also caused loss of membrane integrity and increase of thiobarbituric acid reactive substances. Santos (2015) evaluated the phytotoxic potential and antioxidant activity of *T.patula* and *T.erecta* extracts. Both the extracts showed reduction in germination of *Lactuca sativa* and *Allium cepa* seeds. The development of seedling was also retarded. Presence of phenolic compounds and flavonoids were detected in the extract and the antioxidant activity was also determined. The above studies showed the strong potential of use of *Tagetes* extract as natural herbicide as an alternative to chemical herbicides in the agriculture field.

VI. Conclusion:

Marigold (*Tagetes* spp) which is considered to be a traditional medicinal plant native to Mexico and other warmer part of America, has significant therapeutic value and is used in treatment of a number of ailments. Five species of *Tagetes* viz. *T.erecta, T.minuta*, *T.lucida* and *T.tenuifolia* are introduced and cultivated widely in India. This review mainly emphasizes on the role of *Tagetes* as biopesticides especially as insecticide, mosquitocide, nematicide, bacteriocide, fungicide and herbicide. The essential oils obtained from the aerial part of *Tagetes* spp mainly contain monoterpene hydrocarbons (ocimenes, limonene, terpinene etc) and acyclic monoterpene ketones (tagetones, dihydrotagetone and tagetenone) in addition to lower amount of sesquiterpene and oxygenated compounds.These components are mainly responsible for insecticidal, larvicidal and mosquitocidal property. On the other hand *Tagetes* spp also showed nematicidal effect and thus they can suppress nematode species by acting trap crop or by extertingallelopahic effect. *Tagetes* spp produce a potentially bioactive component α-therthienyl that is mainly responsible for nematicidal action. This sulphur containing compound has nematicidal, insecticidal, fungicidal, antiviral and cytotoxic effect. Cover crop technique and intercropping are common practices for controlling nematodes using marigold. The essential oil of *Tagetes* spp. also shows Strong bacteriocidal, fungicidal and herbicidal effect.

Thus from the extensive study in this review, it is revealed that apart from therapeutic uses, *Tagetes* spp can be utilized as potential biopesticide as an alternative to chemical pesticides. It is not only ecofriendly and safe to environment but it is also cost effective. However before application of marigold as biopesticide in the field of agriculture, residual traces of its phytochemicals would need to be assessed to nullify their off target effects on beneficial arthropod community as well as on human health.

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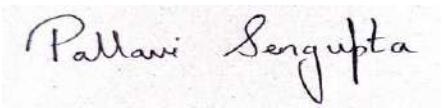

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A comprehensive study on an emerging disease of Barley

Abstract:

Barley, a predominant cereal crop that is used globally as malt for beer brewing, whisky production, fodder for animals, for human consumption, flavoring agents, packaged food items, in cosmetic industries as well as in pulp and paper industries etc. and are often subjected to a variety of diseases. Out of a list of devastating diseases *Ramularia* leaf spot (RLS) caused by *Ramularia collo-cygni* (Rcc) has turned into an epidemic resulting in huge amount of yield loss. The difficulty in recognition and isolation of the pathogen from the host led to the acceleration of spread of *R. collo-cygni* worldwide culminating in destruction of barley across wide areas. To curb the rapid outbreak, various molecular techniques such as PCR are being applied for rapid detection of the fungi. As controlling measures application of fungicides, seed treatment, use of resistant varieties, crop rotation, removal of diseased plants, awareness among mass is being widely used. The attribute features of the pathogen permit it to sporulate even under the snow. Weather parameters such as moisture, light intensity, rainfall, temperature, dew, humidity, precipitation is responsible for escalating RLS among barley. This review calls attention on the production of barley worldwide and in India, its economic importance, vulnerability to the number of pathogens with special emphasis on RLS, the symptoms occurring in barley by Rcc, infection strategy of the pathogen, disease cycle, molecular techniques for detection of the fungus, yield loss of barley and on the measures available to control the spread of RLS as well as highlights the future aspects of research.

Key words: Cereal crop, *Ramularia collo-cygni*, symptoms, disease cycle, molecular diagnosis, outbreak

1. Introduction

Sustainable crop production should be maintained in the context of huge climatic change and food security for coming future (Brown and Funk 2008; Turner et al. 2009). Due to the unexpected upraise in global population, increase in agricultural production can be helpful to fulfil the culinary requirement of the world. Apart from this, crop cultivation can reduce the unemployment issue of some developing countries. Out of the most important crops cultivated throughout the world, barley comes fourth following wheat, maize and rice (Akar et al. 2004). Barley (*Hordeum vulgare* L.) is primarily cultivated for food supply, but recently it is significantly used as animal feed, malt products and as raw materials for food processing, beverage and textile industries that can't be neglected (Kifle 2016). Due to the presence of higher soluble dietary fibre and comparatively low lipoprotein (LDL) content, food manufacturers prefer barley over wheat (Oakenfull 1996). Further attention was gained when the presence of β-glucans was evident in barley, which can reduce blood pressure level and glycemic index. This whole grain also contain few bio-chemicals like phenolic acids, flavonoids, lignans, tocols, phytosterols, and folate which exhibit antioxidant, anti-proliferative property (Idehen et al. 2017), and

appears to be associated with weight loss and increased satiety (Baik and Ullrich 2008) and can lower the risk of certain diseases including cancer, cardio-vascular disease and diabetes (Idehen et al. 2017). Apart from this, barley may provide a field for multiple research-works as they are the model crop for plant breeding, genetics, cytogenetic studies, pathological investigations, virology and biotechnological studies (Hockett and Nilan 1985; Hagberg 1987).

Hordeum vulgare is considered to be old world's cereal. Evidences showed the existence of barley in early Egyptian era along the riverside of Nile around 17,000 years ago (Badr et al. 2000). Barley is among world's earliest domesticated crop (Purugganan and Fuller 2009). Mutational change was initiated in the Middle East around 8000 BC and spread towards Northern Scotland (Helbaek et al. 1969; Pourkheirandish and Komatsuda 2007) and surprisingly archaeological studies revealed that the crop was domesticated at around 8000 BC (Nesbitt and Samuel 1996). Though, *H. spontaneum* C. Koch and *H. vulgare* L. are similar in their morphology (Zohary 1969) but *H. spontaneum* is reported to be the wild ancestor of modern barley, and recent detailed taxonomical analysis had documented *H. vulgare*, *H. spontaneum* and *H. agriocrithon* as subspecies (Bothmer and Jacobsen 1985). Early before domestication today's barley (*Hordeum vulgare* L.) was available as its old wild form (*H. spontaneum* C. Koch), which was used by hunter–gatherers as a food source (Sato 2020). Furthermore, genomic analysis revealed the genomic affinity between ancestral barley and modern domesticated barley (Bothmer et al. 2003). The ancestral species can colonize in vast geographical regions with fertile crescent, which includes its primary habitat like Israel, Turkey, Iraqi Kurdistan, Southwest Iran, Jordan and many more (Harlan and Zohary 1966; Nevo 1992), along with that *H. spontaneum* can also be found in some secondary habitats which incorporates areas like Mediterranean maquis, abandoned fields, and roadsides (Badr et al. 2000). Other than that, its presence has been reported from the Himalayas, Greece, Egypt, southwestern Asia, Morocco, Ethiopia and in their surrounding regions (Molina-Cano et al. 1987).

Immediately after domestication, barley plants were modified agronomically for better traits such as spring growth season (Yan et al. 2006), hull-less caryopsis (Taketa et al. 2008), six row spikes (Komatsuda et al. 2007). These mutated versions affect almost all cultivated varieties of barley within a few thousand years. Genomic diversity corresponds with multiple uses and diverse natural habitat made them suitable for worldwide cultivation except in tropics (Sato 2020).

Top five global barley producers are European Union, Russian Federation, Ukraine, Turkey and Canada and net global production of barley is estimated about 160 million tons per year (Akar et al. 2004), out of which 18% of the world-wide yield is produced by the developing countries only. An excellent adaptive capacity is shown by these plants, they can grow in cold, drought or non-fertile soils and much more tolerant than multiple other crops (Gürel et al. 2016). As a result, barley can be cultivated in adverse conditions; they can be cultivated on the mountain slopes of Tibet, Nepal, Ethiopia, and the Andes (Cook 2013). Barley was reported to be cultivated in most diverse regions like 330m below sea level near Dead Sea to 4200m high on Atipano. Interestingly, it can grow as a rain fed crop in the regions of North

Africa, the Middle East, Afghanistan, Pakistan, Eritrea, and the Yemen (Akar et al. 2004). Sixty-five percent of total cultivated barley is used for animal feeding, 33% is used for malting and only 2% of total yield is used as human food (Sullivan et al. 2013). The following graph (Fig 1) demonstrates top 10 barley producing countries in 2019, where the statistical data represents Russia as the highest barley yielding country followed by France, Germany, Canada, Ukraine, Australia and so on.

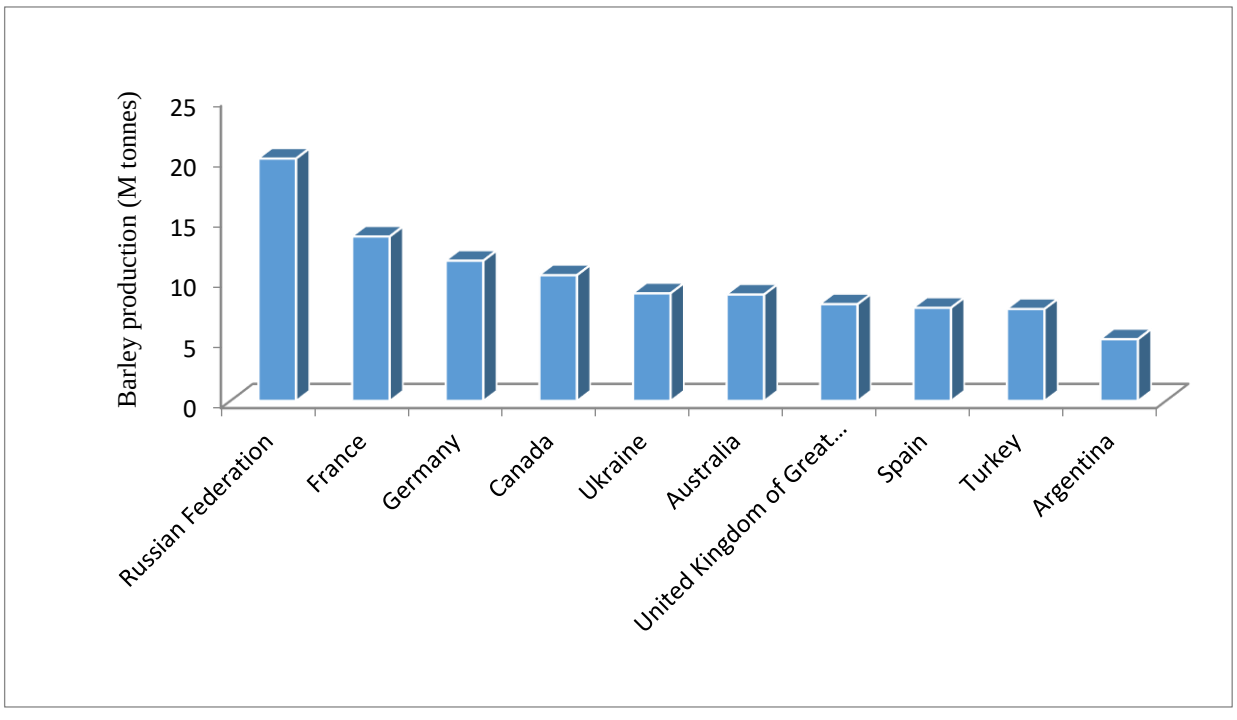

Fig 1. Production of Barley in different countries(source: FOASTAT, 2019).

Russia, no doubt serves highest percentage of global barley yield. Nevertheless, by analysing the available data it could be observed that barley procuring top 7 countries together produce 50% of total yield in the year of 2019 and rest 50% was produced by other countries. Out of them, contribution of Spain, Turkey and Argentina is remarkable. A graphical representation of respective percentages of barley production has been shown in Fig 2.

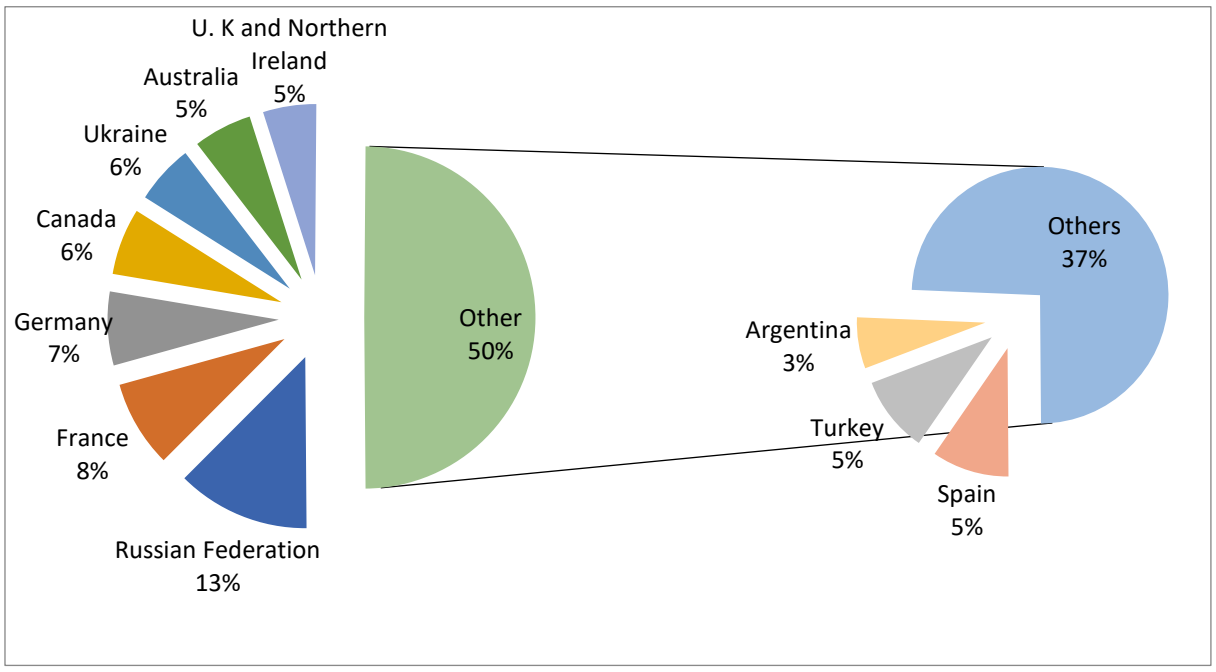

Fig 2. Percentage contribution by the countries in global barley production, (Data source: FOASTAT, 2019).

Considering total export and import of *H. vulgare*, it is observed that China occupies the 1st place in the matter of importing barley. As per report, this huge amount is imported mainly for malting. After China mainland, there is Saudi Arabia, Iran, Netherlands, Belgium and Germany. Whereas, France takes the 1st place in internationally exporting barley followed by Russia, Australia, Argentina, Ukraine, Canada, UK and so on. According to the previous graph it was evident that France contributes almost 8% of total world's production, which clearly spells out the reason behind its top position in barley export. Graphical depiction for both global export and import of barley (in the year of 2019) is provided in Fig 3 (a and b).

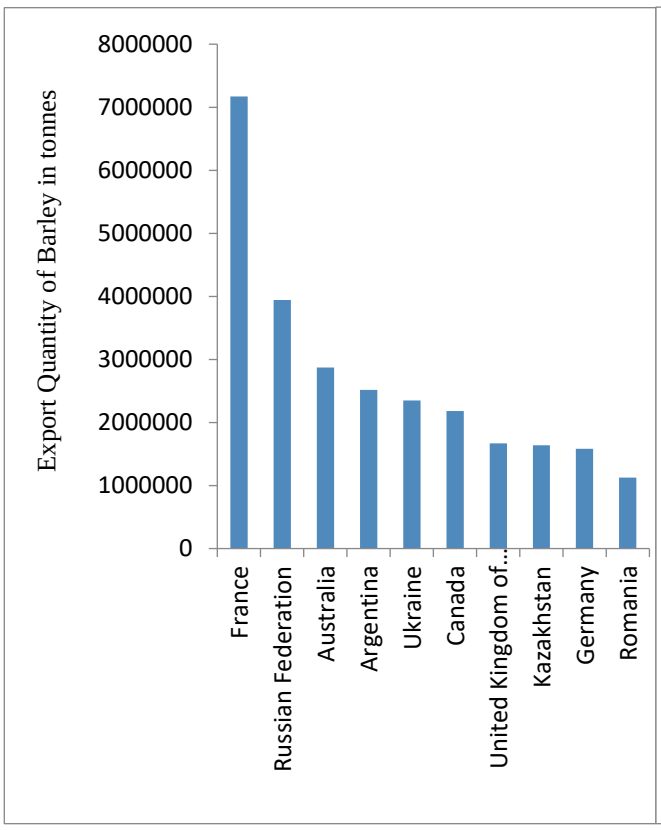

Fig 3a. Top 10 Countries, Export Quantity of Barley (source: FOASTAT, 2019).

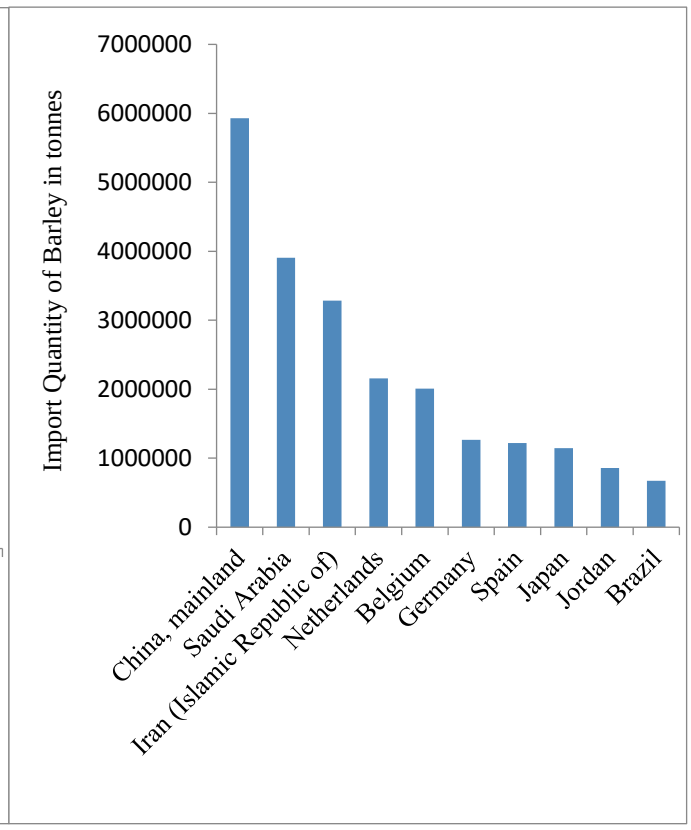

Fig 3b. Top 10 Countries, Import Quantity of Barley (source: FOASTAT, 2019).

Total barley production is very less in India as compared to other counties. Whereas, technological modifications and improved varieties played a vital role in increasing the yield of barley in India (Gupta et al. 2019). Nowadays India is independent in barley production. In India, barely is emerging as an important winter crop being grown in northern plain of the country which includes Rajasthan, Uttar Pradesh, Punjab, Bihar, Madhya Pradesh, Kashmir, Haryana and Jharkhand. Rajasthan has taken the first place in barley production by replacing UP in recent years (Verma et al. 2012), and Uttar Pradesh, Madhya Pradesh, Haryana and Punjab are next to it. India's annual production is almost 1.2-1.5 million tonnes on average (Khatkar et al. 2016), which contributes almost 1.02% of global barley yield in the year of 2019 (FOASTAT). Here is a graphical representation has been given in Fig 4, which shows the exponential growth of barley production in India (all the data are collected from official website of FOASTAT) in last consecutive 10 years (2010-2019). The pie chart (Fig 5) provided underneath depicts the state wise contribution in total barley yield of India (Khatkar et al. 2016).

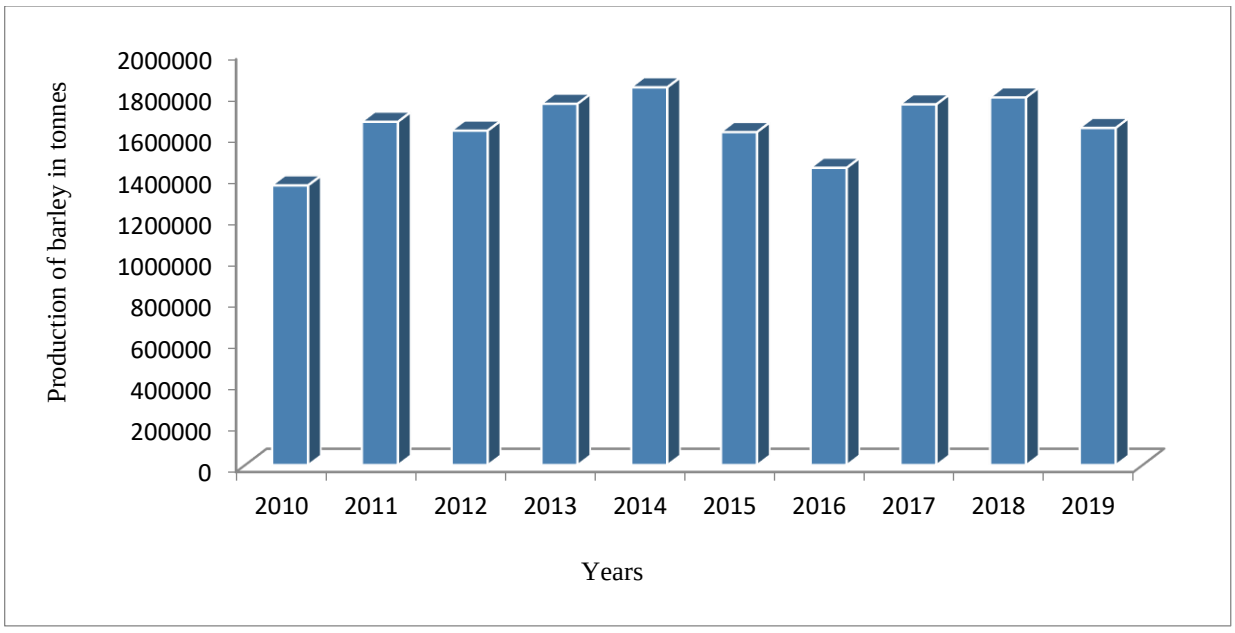

Fig 4. Production of barley (*Hordeum vulgare* L.) in India (2010-2019), Data source: FOASTAT.

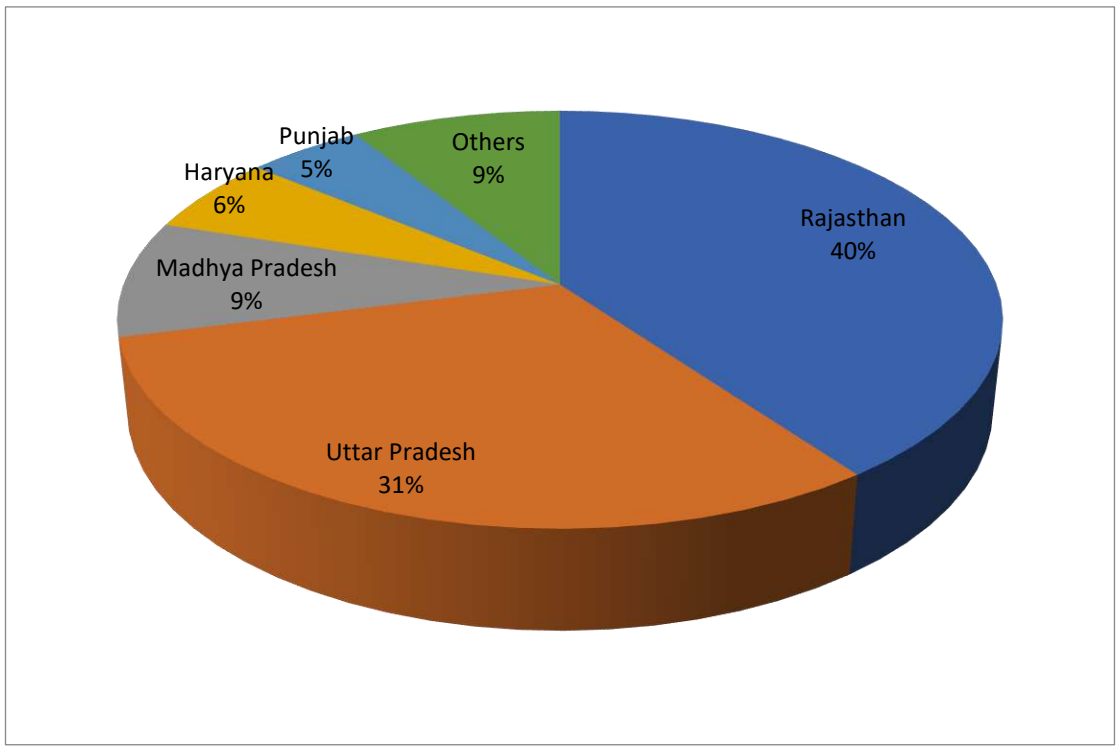

Fig 5. Regional production percentage of barley in India (Khatkar et al. 2016).

2. Barley: An emerging economically important crop

Barley is one of the most important cereal crops after maize, rice and wheat (Langridge 2018). Barley is known as poor man's crop as it is stress tolerant with low input requirement and is mostly grown in countries where maize is not available (Olson et al. 1987; Kumar et al. 2014).

2.1 Commercial Importance

Although the cultivation of barley started for human consumption but since 1980s only 5% of the total produce is used by humans as food (Langridge 2018). Barley straw can be used as an alternative raw material in pulp and paper

industry. It is a good source of vegetal collagen for the cosmetic industry (Tricase et al. 2016). About 20% of the total barley produce is used as malt for beer brewing and whiskey production (Langridge 2018). The food industry also uses barley malt as syrups for adding flavour, colour and sweetness to the commercially prepared food (Vasan et al. 2014). The global export of barley accounts is valued at nearly US$9 billion with Germany and France being the biggest exporters (Langridge 2018).

2.2 Nutritional and Medicinal Importance

Barley grain consists of 70% starch, 11-34% dietary fibre, 3-20% soluble fibre, 5-10% β-glucan, 10-20% protein, 2-3% free lipids and nearly 2.5% minerals. Moreover, barley is also known to contain low fat, excellent amount of vitamin A, antioxidants and complex carbohydrates all of which makes barley a nutritional crop (Tricase et al. 2016). The barley crop specifically *H. vulgare* proved to be an excellent source of dietary fibre which can significantly reduce the risk of cardiovascular disorders (Tricase et al. 2016, Boanta et al. 2019). Barley water is used to relief kidney pain and kidney inflammation (Hussain et al. 2020). There are records of the plant leaves being used as laxative or purgative which helps to empty the intestine (Hussain et al. 2020). It can also be used for the treatment of cough, flu, sterility, haemorrhoids, anaemia, diarrhoea, rheumatism and cystitis (Boanta et al. 2019). Cell wall of barley grains contain β-glucan which possess cholesterol lowering effects and efforts are been made to incorporate it into food items. The commercial product beta-fibre Barlív^{TM} which is natural source of soluble fibre contains β-glucan (Newton et al. 2011). Furthermore, phenolic acid found in barley is responsible for anti-oxidative boon that the plant possesses. Moreover, the bioactive compound also exhibits anti-proliferative and antiradical potential. The polyphenols due to their antiradical scavenging property play an eminent role in reducing the risk of cardiovascular disorders. Little amount of lignans found in barley has antibacterial, antiviral, antioxidant, antiestrogenic and antitumor activities. The bioactive flavonoids help in diminishing the occurrence of coronary heart diseases and cancer (Idehen et al. 2017).

Barley is a great source of tocopherols which are known for their cholesterol lowering and antioxidative activity (Tricase et al. 2016). Tocopherols and tocotrienols together can suppress cancer, promote apoptosis, clear antherosclerotic blockages in carotid artery, prevent lipid peroxidation of biological membranes, decrease the risk of cardiovacular diseases and induce the immune system. The phytosterols have cholesterol lowering effects and they can also prevent colon cancer (Idehen et al. 2017). Few other active compounds found in barley which are of biological significance include pentosans (cholesterol lowering), phytates (reduction of colon cancer), folate (reduction of cardiovascular risks and neural tube defects), policosanol (cholesterol lowering), alkylresorcinols (antioxidant) and arabinoxylans (cholesterol lowering) (Tricase et al. 2016).

2.3 Other Importance

Earlier in India it was considered the holiest of all crops (Kumar et al. 2014). During the ancient age barley was used as a source of food and beverages (Tricase et al. 2016). Currently, nearly 66-71% of the total barley produce is used as animal fodder (Langridge 2018). In addition to being used as green forage and silage, barley straw is good for animal bedding, as covering material for hut roofs and for inhibiting the growth of algae (Kumar et al. 2014; Tricase et al. 2016). Barley is a versatile crop that can be grown in arid places such as Middle East as well in the lower temperature of the arctic and subarctic zones (Newton et al. 2011). Although in India, the cultivation of barley as food crop is restricted to the hill areas where tribal lives but in the semi-arid regions of Africa, Middle East, Ethiopia, Tibet, highlands of Nepal and in some other Asian country's barley is still predominantly consumed as a food crop (Kumar et al. 2014). The effortlessness with which barley can be grown over a wide range of environmental parameters indicate the presence of rich genetic diversity (Newton et al. 2011). The wild barley serves as a good source of genes which can be readily crossed with other cultivars to produce quality crops. Moreover, the life-span of cultivated barley is 10 years or more (Horsley et al. 2009). Barley is used as a model organism for analysing the pathogen resistance, development, biochemical and physiological process by subjecting the plant to mutation and also for the production of best quality crop (Gubatz et al. 2007; Langridge 2018). Moreover, the large size of the barley caryopses makes it an excellent material for researches on seed development (Langridge 2018). The importance of isolated aleurone layer of barley includes studying the effects of abscisic acid and gibberellic acid and deciphering the control of gene expression related to hormonal signals (Chandler et al. 1984; Langridge 2018).

Barley is one of the well-studied crops in terms of genetics, genomics and breeding (Kumar et al. 2014). The progress made in barley research led the characterization of genomic regions which play a role in disease resistance (Kumar et al. 2014). The *Mlo* gene discovered in Ethiopian barley protects the plant from powdery mildew which affects nearly 10,000 plant species. There are scopes that the resistance mechanism of gene can be used as universal weapon to defeat powdery mildew in other plant species as well (Langridge 2018). Similarly, *Yd2* gene discovered in Ethiopian barley confers resistance against barley yellow dwarf luteovirus (BYDV) which infects all important cereal crops. The incorporation of the *Yd2* gene in 17 different cultivars of barley showed positive results indicating that the gene can be used in other breeding programmes (Paltridge et al. 1998). The International Barley Genome Sequencing Consortium upon developing a physical map of barley genome concluded that the barley gene space could be the epicentre for trait isolation, exploiting natural genetic diversity and for understanding the evolution of world very first domesticated crops (Stein and Mascher 2018).

3. Diseases occurring in barley

Barley being a highly important crop, its demand in market is continuously increasing mostly because of its use in various areas such as poultry, cattle feed, for human consumption, malt manufacture (Gangwar et al. 2018) and also for

production of beer (Langridge et al. 2018). Certain biotic and abiotic stresses showed adverse effects on the barley resulting in low production, degradation in barley quality and yield loss (Gangwar et al. 2018). Among the biotic stresses, diseases are the main cause of its yield loss. Table 1 is here to show a clear chart of diseases of barley along with respective pathogen and symptoms.

Table 1. Disease name, causal agent, pathogen type and symptoms of various barley diseases

Name of the disease	Causal organism	Type of pathogen	Symptoms of the disease	Reference
Crown rust	*Puccinia coronate* f. sp. *hordei*	Fungus	Uredinia are linear in shape and have a light orange coloration. These are then surrounded by chlorotic tissues. Uredinia are then converted into telia that appears to be blackish brown in color.	Paulitz et al. 2011
Common root rot and seedling blight	*Cochliobolus sativus*, *Bipolaris sorokiniana*	Fungus	Roots, internode of the infected plant develop lesions that are brown in colour.	Gangwar et al. 2018
Kernel blight (Black point)	*Alternaria* spp., *Cochliobolus sativus*, *Fusarium* spp.	Fungus	-	Gangwar et al. 2018
Loose smut	*Ustilago tritici* (Pers.) Rostrup (*Ustilago nuda* f. sp. *tritici*)	Fungus	The infected ears of barley appear to be black or brown in color due to accumulation of large amount of teliospores.	Gangwar et al. 2018
Covered smut	*Ustilago hordei* f. sp. *hordei*	Fungus	This disease can be recognized in barley by the appearance of blackened barley ears.	Gangwar et al. 2018
Leaf (brown) rust	*Puccinia hordei* Otth	Fungus	-	Gangwar et al. 2018
Dwarf bunt	*Tilletia controversa*	Fungus	-	Gangwar et

	Kühn			al. 2018
Ergot	*Claviceps purpurea* (Fr.) Tul.	Fungus	-	Gangwar et al. 2018
Powdery mildew	*Blumeria graminis* f. sp. *hordei*	Fungus	Greenish- yellow dots on the lower surface of the infected leaf followed by the appearance of reddish or yellowish brown rings of dead tissues.	Paulitz et al. 2011
Downy mildew (Crazy top)	*Sclerophthora rayssiae* var. *zeae*	Fungus	-	Gangwar et al. 2018
Rhizoctonia root rot	*Rhizoctonia solani, R. oryzae*	Fungus	Discoloration of leaves and stunted growth. The tips of crown roots appear tapered and the root tips appear to be brown in colour.	Paulitz et al. 2011
Net type net blotch (NTNB)	*Pyrenophora teres* f. *teres*	Fungus	-	Gangwar et al. 2018
Spot blotch	*Bipolaris sorokiniana* (*Drechslera sorokiniana*), *Cochliobolus* sp.	Fungus	-	Gangwar et al. 2018
Stem (black) rust	*P. graminis* f. sp. *tritici, Puccinia graminis* f. sp. *secalis*	Fungus	-	Gangwar et al. 2018
Fusarium head blight (Scab)	*Fusarium graminearum* Schwabe	Fungus	Kernels of barley appear to be discolored giving a tan or orangish tan to brown or somewhat dark brown coloration. As the infection spreads the peduncles appear to be dark brown in color.	Gangwar et al. 2018

Spot type net blotch (STNB)	*Pyrenophora teres* f. *maculata*	Fungus	-	Gangwar et al. 2018
Scald	*Rhynchosporium secalis* (Oud.) J.J. Davis	Fungus	Mostly barley leaf sheaths and blades are affected by the fungus. Symptoms include greenish to bluish grey-colored lesions, the central portion of which slowly changes from greenish grey coloration to steel grey which further changes and appears to be slightly tan or white in color.	Paulitz et al. 2011
Septoria speckled leaf blotch (SSLB)	*Septoria passerinii* Sacc.	Fungus	Elongated, chlorotic lesions that coalesce and appear straw or are greyish green in color.	Gangwar et al. 2018
Yellow (stripe) rust	*Puccinia striiformis* West. f. sp. *hordei*	Fungus	Chlorotic spots or patches at the infection site spreading on veins, necks, leaf sheaths and glumes.	Gangwar et al. 2018
Stripe disease	*Drechslera graminea* (Rabenh.) Shoemaker.	Fungus	The initial symptoms involve pale colored and tiny lesion on young leaves followed by yellow to straw coloration of stripes on diseased leaves which then turn brown in color. The brown coloration of leaves is again followed by or accompanied with the splitting up of diseased leaf blades. Greyish brown, erect, twisted, ears are observed and the plants are subjected to premature death.	Gangwar et al. 2018
Anthracnose	*Colletotrichum cereal* Manns.	Fungus	-	Gangwar et al. 2018
Tan spot	*Pyrenophora tritici-*	Fungus	-	Gangwar et

	repentis (Died.) Drechs.			al. 2018
Pythium root rot	*Pythium arrhenomanes*, *Pythium graminicola*, *Pythium tardicrescens*	Fungus	-	Gangwar et al. 2018
False loose smut	*Ustilago avenae* (Pers.) Rostr. (*U. nigra*)	Fungus	-	Gangwar et al. 2018
Take-all	*Gaeumannomyces graminis* var. *tritici*	Fungus	Stunted growth of barley, the roots become surrounded by a dark blackish layer.	Paulitz et al. 2011
Barley yellow dwarf	Barley yellow dwarf virus (BYDV)	Virus	Irregular patches along the margins and leaves of young leaves, the leaves appear to be bright yellow in colour. Other symptoms involve notched leaf margins, reduction of flowering and tillering, formation of small and fewer kernels.	Paulitz et al. 2011; Gangwar et al. 2018
Barley mosaic	Barley mosaic virus (BMV)	Virus	-	Gangwar et al. 2018
Barley yellow streak mosaic	Barley yellow streak mosaic virus (BYSMV)	Virus	-	Gangwar et al. 2018
Barley stripe mosaic	Barley stripe mosaic virus (BSMV)	Virus	-	Gangwar et al. 2018
Basal glume rot	*Pseudomonas syringae* pv. *atrofaciens*	Bacteria	-	Gangwar et al. 2018
Bacterial stripe	*Pseudomonas syringae* pv. *striafaciens*	Bacteria	-	Gangwar et al. 2018

Black chaff and bacterial streak	*Xanthomonas translucens* pv. *translucens*	Bacteria	-	Gangwar et al. 2018
Bacterial leaf blight	*Pseudomonas syringae* pv. *syringae*	Bacteria	-	Gangwar et al. 2018
Aster yellows	Aster yellows phytoplasma	Phytoplasmal diseases	-	Gangwar et al. 2018
Molya disease	*Heterodera avenae*, *Heterodera filipjevi*	Nematode	Barley when infected shows a somewhat bushy roots and the plant appears to be have achieved stunted growth, a deficiency of nutrients in the plant may also indicate that it has been infected.	Gangwar et al. 2018
Stunt nematode	*Merlinius brevidens*, *Tylenchorhynchus dubius*	Nematode	-	Gangwar et al. 2018
Cereal root knot nematode (Barley root Knot nematode)	*Meloidogyne naasi*, *Meloidogyne chitwoodi*	Nematode	-	Gangwar et al. 2018
Root lesion nematode	*Pratylenchus* spp.	Nematode	When infected leaves turn yellowish in color, shows stunted growth and reduced number of tillers.	Paulitz et al. 2011

Several diseases occurring in berley is being listed in the above table 1 and yield loss due to some diseases are represented in Table 2.

Table 2. Yield loss caused by pathogens affecting barley

Disease of Barley	Yield loss (%)	Reference
Powdery mildew	1-14	Gangwar et al. 2018
Stripe rust	20-70	Gangwar et al. 2018
Net blotch	10-44	Gangwar et al. 2018
Spot blotch	10-20	Gangwar et al. 2018
Barley yellow dwarf	8-38	Paulitz et al. 2011
Scald	Up to 40	Paulitz et al. 2011
Scab	Up to 40	Paulitz et al. 2011
Septoria speckled leaf blotch	23-38	Gangwar et al. 2018
Brown rust	60	Gangwar et al. 2018
Pythium root rot	13-36	Paulitz et al. 2011

At present a lot of diseases have been reported to occur in barley plants but in this review, we have given special emphasis on *Ramularia* Leaf Spot (RLS) in barley caused by *Ramularia collo-cygni* (Rcc) as the disease has been declared as an epidemic (Havis et al. 2015). The pathogen is creating havoc throughout the world thereby causing majority of yield loss and this is resulting in reduction in the availability of barley grains across the globe.

4. *Ramularia* Leaf Spot (RLS) disease of barley

Ramularia Leaf Spot (RLS) disease of barley (*H. vulgare*) is an emerging disease caused by *R. collo-cygni* (Rcc) (Salamati and Reitan 2006; Havis et al. 2015), a fungus belonging to the Mycosphaerellaceae clade of Dothideomycetes class (Stam et al. 2019). RLS was first reported over a century ago in Italy by Cavara in 1893 and at that time, the pathogen was known as *Ophiocladium hordei* (Sachs 2006) but later it was renamed and accepted worldwide as *R. collo-cygni* (Sutton and Waller 1988; Sachs 2006). This disease has spread out in Europe involving New Zealand (Harvey 2002), Scotland (Oxley et al. 2002), Denmark (Havis et al. 2015), Norway (Salamati and Reitan 2006), Germany, Austria, United States, Czech Republic (Walters et al. 2008), Argentina (Khier et al. 2002; Havis et al. 2015), Chile, Spain, Mexico, Russia, Columbia, Estonia, Slovak Republic, Iceland (Havis et al. 2015), and Uruguay (Pereyra 2014;

Havis et al. 2015). RLS caused serious damage in several areas of South America (Havis et al. 2015). Though till now, the disease has not been reported in India (Havis et al.2015).

Barley belongs to the family Poaceae. It is the fourth largely cultivated cereal that is primarily used in the form of animal fodder and the barley malt acts as a source for the production of alcoholic beverages (Kaczmarek et al. 2016). The worldwide rapid spread of RLS is thought to be a major threat in barley production resulting in massive yield loss, and not only losses in quantity of the crop but also the fungus affects its quality (Havis et al. 2015). Rcc produces phytotoxins in plants which are known as rubellins. This toxin is responsible for causing foliar necrosis and also reduces the photosynthetic area of plant (McGrann et al. 2017). Apart from barley, *R. collo-cygni* also affects many other cereal crops namely wheat, oats, maize and so on (Kaczmarek et al. 2016). Although the disease was reported at the end of 19th century, but it mostly arose attention of the scientists for last three decades (Havis et al. 2015; McGrann et al. 2017). This lack in recognition may be due to the difficulty in isolation as well as identification of the fungus, separating the symptoms of RLS from other diseases affecting barley crops. Molecular diagnostics in all over the world on seed samples indicated that the disease is widespread across barley cultivating regions even if the chances of disease occurrence is low (Havis et al. 2015). Currently RLS can be controlled to a great extent by the application of fungicides (Walters et al. 2008).

4.1 Manifestation of RLS

Ramularia collo-cygni is often known as the late season fungus of barley with symptoms arising on the foliage after the emergence of ear (Walters et al. 2008). This fungus is mostly observed on dying and dried leaves. It is a rare vision to locate the fungus on green and healthy leaves during tiltering stages and seedling development of the crop (Oxley et al. 2002). The infection starts to spread mostly from the leaf tip and the margins (Walters et al. 2008). Signs of infection appear rapidly on both the top leaves following the head emergence and also the beginning of flowering. The necrotic spots are visible on both the abaxial and adaxial surface of the leaves (Havis et al. 2015). Whereas, the spots are infrequent on the lower leaves (Oxley et al. 2002). The disease is especially characterized by several brown, reddish to blackish brown speckles that are 1-2 mm in length (Salamatiand Reitan2006; Walters et al.2008; Havis et al. 2015). These small rectangular spots remain delineated with the help of leaf veins (Oxley et al. 2002; Walters et al. 2008). These spots then develop into chlorotic halo (Salamati and Reitan 2006) and gradually the surrounding lesions coalesce into a bigger necrotic area (Walters et al. 2008). After the appearance of spots, the remaining portion of the blade shows chlorosis and finally undergoes necrosis (Walters et al. 2008). Apart from the leaf blade, the spots are even visible on the stems and awns as dark spots (Oxley et al. 2002). Some of the renowned scientists have explained about the disease severity along with using a percentage scale that starts from 1-10% and ends with 76-100% of disease severity in barley plants (Havis and Brown 2018). Some of the pictorial representations of disease symptoms with detail manifestation

along with severity percentages are shown in Fig 6. The signs of RLS caused by *R. collo-cygni* are interconnected to light intensity (Walters et al. 2008). Moreover, it has been observed that if barley plants were exposed to low intensity of light before inoculation, the symptoms are less expressed (Makepeace et al. 2008). Makepeace (2006) in his work showed that the intensity of light before inoculation effected the infection rate of Rcc. Before inoculation of pathogen, plants grown under low light intensity showed less disease symptoms. However, with increasing light intensity after inoculation, the gradual decrease in the appearance of signs of RLS were recorded (Makepeace et al. 2006; Walters et al. 2008). Though, the symptoms of RLS can be easily confused with the signs caused by pathogens such as *Pyrenophora teres* f. *teres*, *P. teres* f. *maculate* (Walters et al. 2008).

Fig 6: The pictorial representations of disease symptoms with detail manifestation along with severity percentages. a. Only upper leaves showing lesions (1-10% of severity); b. Upper leaves undergoes a preliminary stages of necrosis and lesions start to appear on middle leaves (11-20% of severity); c. Many lesions with severe necrosis occur on upper leaves and numerous lesions appear in the middle leaves (21-30% of severity); d. Extensive and moderate lesions on upper and middle leaves, respectively (31-40% of severity); e. Severe damage of upper leaves and middle leaves start to necrosis

(41-50% of severity); f. Upper leaves show complete lesions (100%) and about 50-75% necrosis on middle leaves (51-75% of severity); g. Almost all leaves are necrotic with severe damage of plant (76-100% of severity).

4.2 Pathogen biology

RLS is triggered by the deposition and thereafter the adhesion of conidia of Rcc on fronds of the host plant (Stabentheiner et al. 2009). Moisture plays an active role on the germination of conidia and its further development on the host fronds (Walters et al. 2008). This fungus sporulates in large quantities and sporulation can occur even when buried beneath the snow (Salamatiand Reitan 2006). The conidiophores of Rcc which appears to be swan necked shaped (Fig 7) first appeared on the abaxial surface of leaf followed by the adaxial side of the leaf and then development of conidia began. Each conidiumis obovoid, ovoid, or ellipsoid in shape. Spores are warty structures with a verrucose surfaceand the hilum is located eccentrically on the basal portion and are 7-11µm in length, 3-6µm wide. The amounts of conidia are about 4.05×106 per plant. It has been estimated that about 50000 conidia can be produced by a diseased leaf but this data is subjected to change with regard to the various studies being carried out on the amount of conidia produced by the diseased plant. In case of Rcc, more than one germ tube can be observed in a particular conidium. The fungal hypha undergoes branching and gradually forms a dense and clustered network on the surface of leaf. The hyphae then enter into the host via the stomatal pores (Stabentheiner et al. 2009). The stages of conidial growth of Rcc in the host plant leaves are shown in the Fig 8. Infection can be caused by a number of possible ways involving the transmission through seed, volunteers, and crops such as rye, oat, perennial couch grass and wheat. Though it was positively noted that winter barley is the most appropriate inoculant for the spring barley. Spores carried by wind may also be a factor for causing infection (Salamatiand Reitan 2006; Walters et al. 2008). Presence of dew also plays an important role in causing infection (Sachs et al. 2006). In infected seeds, Rcc inhabits the outer layers and are found in lemma and present in scarce quantity in the pericarp and the embryo. Endosperm of seed lacked the RccDNA (Havis et al.2015). From a site in Scotland, an important correlation was detected between spore release and prolonged surface wetness of leaves during the month of July. As the temperature gradually increased to 15ºC, a rise in the dispersal of fungal spores was observed making temperature a factor for the rapid spread of RLS. Some studies even referred high humidity to be a factor in the outbreak of the epidemic in suitable areas. In Czech Republic, increased temperature and less amount of rainfall may lead to reduction in RLS provided the crop is in post flowering stage (Havis et al. 2015). The central region of Norway that experiences *Ramularia* Leaf Spot is characterized by cold and wet summers with an average temperature of 11ºC along with 500mm of precipitation. Studies revealed a correlation between the days experiencing precipitation, the leaf wetness period and the high infection rate of Rcc that will affect the crop yield in the next growing season (Salamati and Reitan 2006).

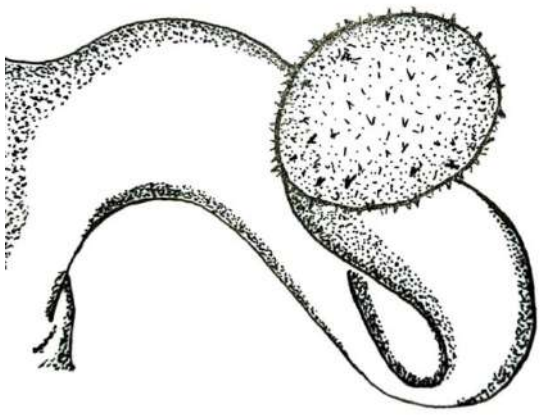

Fig 7. 'Swan neck' shaped conidiophores of *Ramularia collo-cygni.*

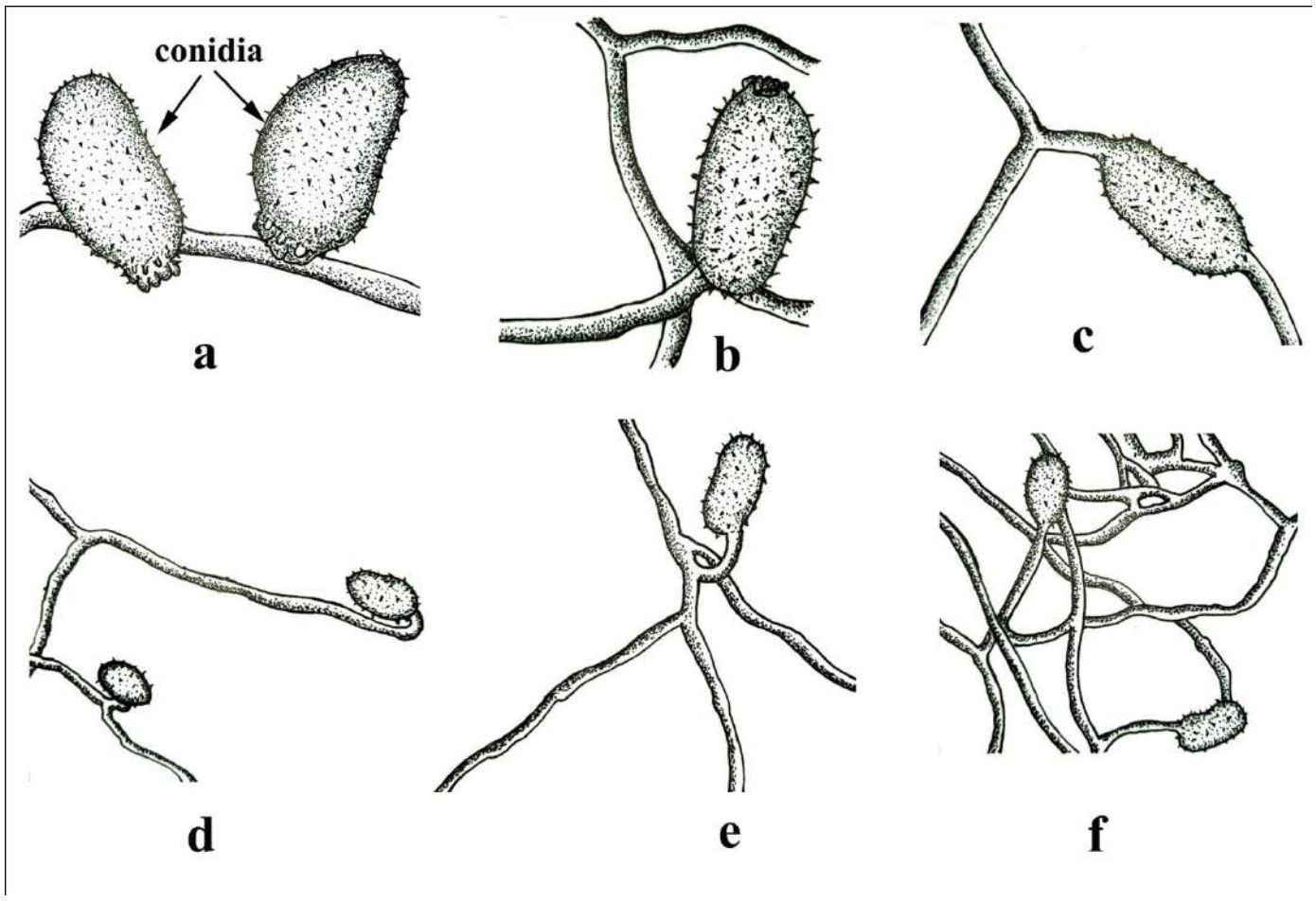

Fig 8. The stages of conidial germination and hyphal branching of *Ramularia collo-cygni* in the host plant leaves. a. Emerging hyphae at the base of conidia (stage 1); b. Germ tube emerging from the tip of conidium (stage 2); c. Two germ tubes emerge at the tip and base of the conidium (stage 3); d. Growing hypha directly enters into stoma (stage 4); e and f. After germination of conidia, hypha branched to form a dense network of hyphae on leaves (stage 5 and 6).

4.3 Disease Cycle of Rcc

The disease cycle begins with the deposition and thereafter the adhesion of conidia of Rcc on the fronds of barley. The infection is thought to occur on the immature winter barley fronds during the autumn season. Pathogen then hibernates on aged barley leaves. In the upcoming spring season, the fungus starts to spread forming loads of conidium thereby spreading the disease into the surrounding crops (Stabentheiner et al. 2009). Studies even stated that the pathogen

could be transferred from the seed to the leaf tissues causing infection even in the absence of external inoculum (Havis et al. 2014). RLS can be caused through various kinds of inoculum including spore dispersal by wind, from previously cultivated crop debris, through infected seeds (Salamati and Reitan 2006). The formation of conidia and the pathogen invasion is not restricted to the vegetation period but can sporulate as well as infest even in winter. The germ tube of the conidium can emerge from either the tip or the base or from both sections of the conidium i.e., more than one germ tube can be observed in a particular conidium. The epidermal wax plates are dissolved in the areas surrounding the hyphae and the spores, and the hyphae then undergoes branching and gradually forms a dense and clustered network on the surface of leaf. The hyphae enter the plant body via the stomatal pores (Stabentheiner et al. 2009) but before penetrating into the stomata a cylindrical or a spherical structure is formed at the tip of the hyphae known as Stomatopodia (Kaczmarek et al. 2016). The hyphae invade the leaf within 24 hours after the germination of conidium (Stabentheiner et al. 2009). After the entry through stomata, stomatopodia branches forming thick basal conidiogenous aggregates within 7 days past the inoculation time (Kaczmarek et al. 2016). During the pre-stages of infection, no symptoms are visible (Kaczmarek et al. 2016). The fungus consists of proteins that are capable of protecting itself from the defense system of the host and also harbours the enzymes that are capable of degrading the cell wall of the host plant. These might be the reason for which the pathogen can harbour inside the host without causing symptoms for a certain period of time (McGrann et al. 2017). Rcc is thought to be an endophyte which can develop into a necrotrophic pathogen when exposed to various conditions amounting to oxidative stress within the host crop (Salamatiand Reitan 2006; McGrann et al. 2017) and this transition is generally visible from 20 dpi (Kaczmarek et al. 2016). Rcc is capable of growing intercellularly after invading the leaf tissues. No intracellular growth of the fungus has been reported. Without rupturing the epidermis, the swan-necked conidiophores grew within the intercellular space pushing the adjoining epidermal cells apart. It has not been determined whether hyphae produce pectic enzymes that may be responsible for losing the middle lamella present between the cells or whether growth of hyphae occurs simply by pushing the host cells (Stabentheiner et al. 2009). The intercellularly developing hyphae within the layer of mesophyll show a ‘brickwork-like’ ornamentation. This is characterized with hyphae stretching along the infected leaf axis connected with the help of lateral branches present in rows of the mesophyll cells. The intercellular hypha neither invades the host cells nor do they traverse the veins and appear to be thick and highly vacuolated. Approximately after 4 weeks of being inoculated the symptoms may appear. Small lesions known as pepper spots were visible from 25 dpi along with reddish discolouration of the tissues surrounding the lesion. This might be due to formation of rubellins. There is no penetration of the vascular bundles at any stage of infection (Kaczmarek et al. 2016). The necrotic spots are visible on the abaxial and adaxial surface of the leaf (Havis et al. 2015). These spots then develop into chlorotic halo (Salamati and Reitan2006) and gradually the surrounding lesions coalesce into a bigger necrotic area (Walters et al. 2008). After the appearance of spots, the remaining portion of the blade shows chlorosis and finally undergoes necrosis (Huss 2004; Walters et al. 2008). Rcc is

even capable of producing secondary metabolites that can be responsible for the expression of the symptoms of RLS (McGrann et al. 2017). Apart from the leaf blade the spots are even visible on the stems and awns as dark spots (Oxley et al. 2002). The life wheel of Rcc in barley plants in favour of forming *Ramularia* leaf spots are clearly shown in Fig 9. After the aged diseased leaves gets detached from the host, they are decomposed by soil inhabiting fungi, bacteria and the fungus live saprophytically (Salamati and Reitan 2006). It has been reported that colonization is more severe when both the external inoculum and seed infection occurs together in the host plant (Havis et al. 2013).

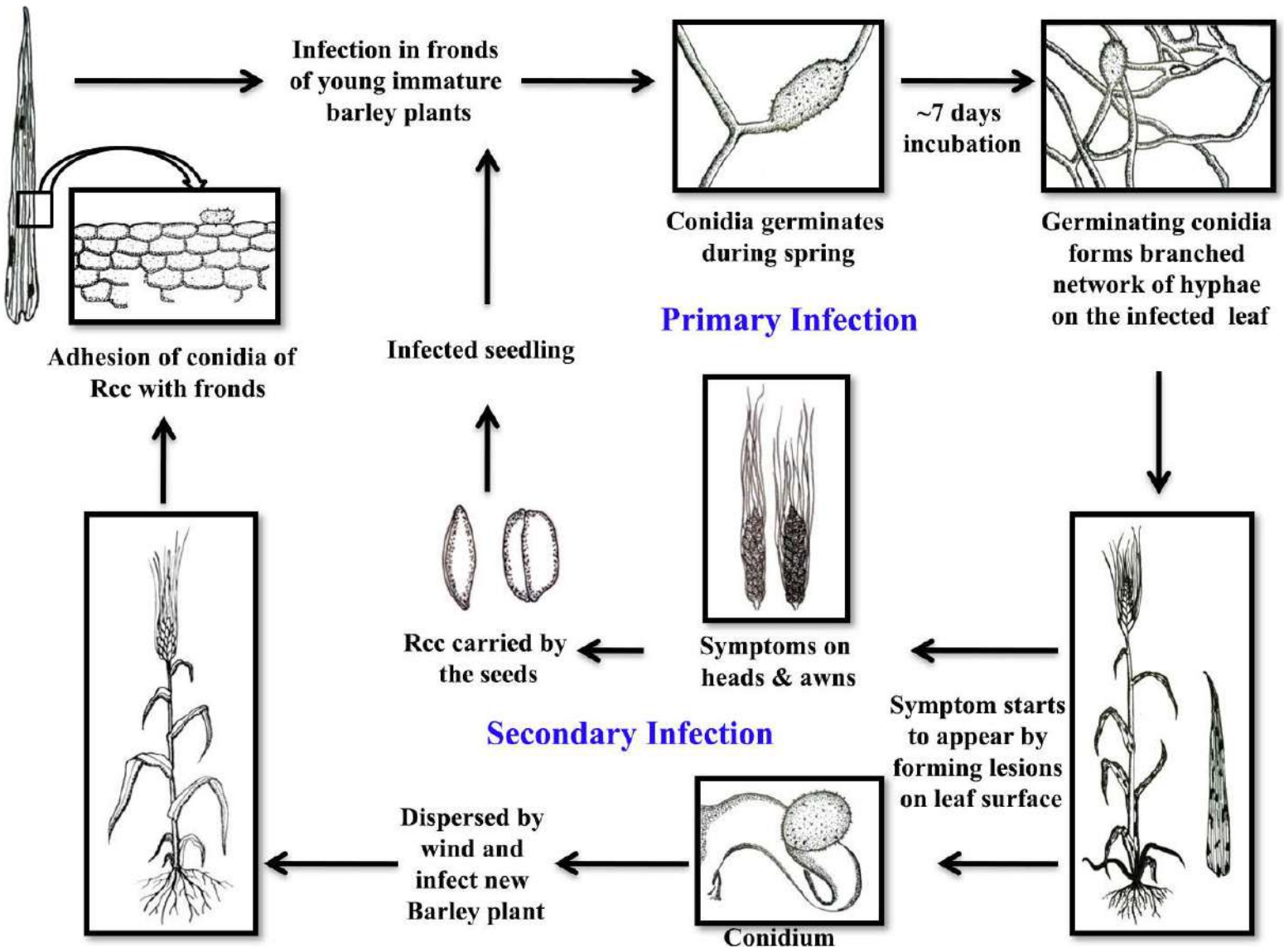

Fig 9. The life cycle of *Ramularia collo-cygni* in barley plants in favour of forming *Ramularia* leaf spots.

4.4 Rapid Nested PCR Based detection of Rcc

The employment of Polymerase chain reaction (PCR) methods has led to proper and rapid detection of the fungus thereby playing a significant role in describing the epidemiology (Walters et al. 2008). In ribosomal RNA the internal transcribed spacer regions are the most appropriate target for performing molecular based detection of Rcc. Internal transcribed spacers (ITS) 1 and 4 are the primers that are used to amplify the ribosomal RNA portion present between 18s and 28s ribosomal subunits. The reaction mixture was made which comprises of dATP, dGTP, dCTP, dTTP, template DNA, DNA Taq Polymerase, buffer and primers in specific amount (ITS 1 and ITS 4). The mixture then

undergoes the PCR cycle. For performing the PCR cycle, initial denaturation was conducted at 94ºC for 4 minutes. The next phase was run for30 cycles (in 94ºC for 1 minute →55ºC for 1 minute→72ºCfor 1 minute). The mixture was finally extended at 72ºC for 10 minutes. The products obtained by this process were then purified and sequenced. The obtained products are of almost similar in size i.e., without any variation in their size. Intraspecific homogeneity within each of the sequences as well as with the earlier published ITS of the Rcc fungus (AF222848) was observed. *R. collo-cygni* 1, 2 and 3 primers were designed to move in the direction of transcription of rRNA, whereas *R. collo-cygni* 4, 5 and 6 were designed to move in the opposite direction. It was detected that all the combinations of primers could amplify the fungus DNA among which *R. collo-cygni* 1 and 5 showed more clarity and intensity in amplification of the segment giving rise to a 426bp fragment that was predicted from the previously obtained data sequence. The second step of nested PCR involves the incorporation of primers *R. collo-cygni* 3 and 4. This resulted in the formation of 256bp fragment that increased the durability and sensitivity of the assay. In order to further increase the robustness, more PCR reactions were done with increased concentrations of the host DNA. It was then observed that all the samples obtained from infected hosts gave rise to distinct bands of about 256bp. The results showed that through nested PCR, Rcc can be detected irrespective of whether conidiophores are present or whether any symptoms are expressed (Havis et al. 2006). With the help of this molecular assay, forecasting of disease as well as its control will become much easier in near future (Walters et al. 2008).

4.5 Disease Management Strategies

Disease management strategies of plants are well introduced and continuously exposed for controlling various kinds of plant diseases caused by both microscopic and macroscopic pathogens. Whereas, focusing on the basic principles like both conventional and advanced improved practices, the plant disease control methods were placed in a highlighting perspective from the historical times (Ul Haq and Ijaz 2020). Among the hazardous diseases, RLS is one of the diseases that is currently a matter of concern. Though Rcc remains latent within the host plant till it reaches the flowering stage and this cause difficulty in the proper detection (Walters et al. 2008). The divergence in occurrence of RLS also hinders early detection of the pathogen making it difficult to forecast the disease (Salamati and Reitan 2006). Various studies have reported that Rcc is capable of causing vast damage to barley crops, destructing about 70% of the yield and resulting in 5-10% of yield loss (McGrann et al. 2017) and 60-100% severity was observed with respect to symptoms (Havis et al. 2015).Table 3 represents yield loss percentages in different countries.

Table 3. Percentages of yield loss caused by RLS in different countries

Country	Yield loss	Reference
Austria	20%	Havis et al. 2006
Switzerland	15-25%	Frei et al. 2007
United Kingdom	0.4t/ha^{-1}	Pinnschmidt et al. 2009
South America	70%	Havis et al. 2015

Apart from barley, Rcc is capable of infecting various other plants including *Brachypodium distachyon* (L.) P. Beauv. (Model grass), *Glyceria fluitans*(L.) R.Br., *Agrostis*spp. L.,*Triticum aestivum*L., *Bromus cartharticus* Vahl, *Dactylis glomerata* L. (cock's foot), *Lolium multiflorum* Lam. (Italian ryegrass) thereby establishing its wide host range (Kaczmarek et al. 2016). Though Rcc was first described ages ago, it arouses interest recently due to its epidemic nature. The detection as well as control of this epidemic has a series of hurdles but molecular patterns and few disease management strategies are being applied (Havis et al.2015) which are as follows:

4.5.1 Physical Control Strategies

4.5.1.1 Crop rotation: *Ramularia* Leaf Spot can also be controlled to some extent by applying the method of crop rotation. It is most effective if the crop field can be entirely rotated rather than just applying the technique in areas where the crop was previously planted so that all the crops that may be susceptible to Rcc gets discarded (Mae et al. 2018).

4.5.1.2 Removal of diseased plants: The removal of diseased volunteer plants is another important strategy that can lead to control of RLS to some extent (Havis et al. 2015).

4.5.1.3 Effect of hot water treatments: Treating the seeds of barley with hot water resulted in reduction of the DNA levels of the fungus. Although the use of this method has a drawback. It was reported that due to the use of hot water, damage to the embryo became unavoidable (Havis et al. 2015).

4.5.1.4 Awareness among public: Persons working in the chemical laboratories, the breeders, the disease controlling team, the plant protection representatives were made aware of the pathogen and trained to identify the fungus and thereby helped in detecting RLS (Sachs et al. 2006).

4.5.2 Biological Control Strategies

4.5.2.1 Seed treatments: Transmission of the disease via seeds can be checked by using healthy seeds that are free from pathogen. Non infected seeds can be obtained by avoiding grains from lands that are heavily infected and also by the usage of various seed treatment procedures (Matusinsky et al. 2011).The dressing of seeds with the help of tebuconazole and triazoxide has been found to be an effective method in blocking transmission of the disease (Matusinsky et al. 2011).Azoles and succinate dehydrogenase inhibitors (SDHIs) fungicides were used for seed treatments to check their function in controlling RLS (McGrann et al. 2017).

4.5.2.2 Use of elicitors: The use of defense elicitors triggers the natural defense system within the host and provides a broad-spectrum control over the widespread infection caused by *R. collo-cygni*. Nevertheless, it was observed that elicitors alone where not capable of causing resistance but showed reduction in the rate of infection when applied at an early stage followed by certain fungicides (Havis et al. 2015).

4.5.3 Biotechnological Control Strategies

4.5.3.1 Utilization of mlo alleles: The use of wild type Mutation-induced recessive alleles (mlo alleles) shows resistance to RLS (McGrann et al. 2017). Nowadays, the use of different germplasm is thought to be an effective approach for breeding of disease resistant variety and it mostly depends on the phenotypic selection (Havis et al. 2015). Varietal resistance can cause decrease in the expression of symptoms along with reduction in the accumulation of fungal biomass (McGrann et al. 2017).

4.5.3.2 Resistance breeding: Breeding may account for an important technique in reducing RLS (Oxley et al. 2008). In order to control the epidemic, many countries like Sweden, Denmark, Austria, and Norway have decided to gain knowledge regarding the effectiveness of resistance breeding. Various strains of barley where subjected to research and studies to check their susceptibility to Rcc fungus (Sachs et al. 2006).

Table 4. Rcc causing yield loss in spring barley varieties

Spring barley varieties	Gradation of resistance to Rcc	Yield loss	Reference
Poker	good	0.27 t/ha	Oxley et al. 2006
Optic	intermediate	0.53 t/ha	
Pewter	poor	0.45 t/ha	
Prestige	poor	0.32 t/ha	

Chariot	poor	0.37 t/ha	

4.5.3.3 Genetic resistance: It was observed that the resistant barley varieties work to control the disease only when kept under controlled parameters but are mostly susceptible to *R. collo-cgyni* in adult stage when kept in the wild under normal field conditions. This suggests that there may be different genes that are capable of resisting the fungus in different plant stages or under different environmental conditions (Walters et al. 2008).

4.5.3.4 Varietal resistance: Since Rcc is a late season fungus, it is hard task to obtain appropriate information on varietal resistance to the pathogen. Data obtained from analysis of a number of spring varieties revealed that Decanter was least susceptible to Rcc whereas Cocktail appeared to be more prone to infection. Doyen reported low level of disease in the initial year but with time showed increasing susceptibility to the pathogen. Flagon is most susceptible to Rcc, whereas Accrue shows a lower level of infection (Oxley et al. 2008). Another study based on the observations obtained from resistance of barley cultivars to Rcc in Denmark revealed that among spring barley Power, Nathalie, Isabella, Helium, Isotta, Cruiser and Modena showed a lower level of infection. Among winter barley varieties Lonni was the least susceptible followed by Lomerit, Nobilia, Chess and Carola. But further study is essential to determine the varieties that may provide complete resistance to RLS (Pinnschmidt et al. 2006).

Table 5. Effect of Rcc on Spring and Winter Barley Varieties

Spring Barley Varieties with Rcc			Winter Barley Varieties with Rcc		
Spring barley varieties	**Percentage of Rcc**	**Reference**	**Winter barley varieties**	**Percentage of Rcc**	**Reference**
Decanter	9.1	Oxley et al. 2006	**Accrue**	5-10	Oxley et al. 2008
Power	10.6		**Saffron**	5-10	
Poker	12.5		**Cassata**	10-15	
westminster	13.3		**Amarena**	10-15	
Wicket	14.6		**Suzuka**	15-20	
Optic	16.7		**Pelican**	15-20	
Cellar	17.7		**Colibri**	20-25	

Doyen	18.9		**Boost**	20-25	
Prestige	19.3		**Wintmalt**	25-30	
Rebecca	19.3		**Flagon**	30-35	
Cocktail	20.4				

4.5.4 Chemical Control Strategies

4.5.4.1 The use of fungicides: SDHIs and azoles combine with chlorothalonil and functions as an effective fungicide. Quinone outside inhibitors (QoIs) could also regulate RLS (McGrann et al. 2017). Though QoIs are highly effective against a number of fungal pathogens but later it lost its sensitivity towards Rcc as the pathogen developed a resistance against it. It was reported that in United Kingdom, efficiency rate of QoI rapidly declined due to G143 point mutation which was observed among the pathogen population. The number of mutations depends on the frequency of SDHI sprays on the diseased hosts (Havis et al. 2015). In Uruguay and Argentina, the continuous applications of strobilurins indicate that they are still effective in that area. Switzerland prefers the use of Chlorothalonil mixed with demethylation inhibitor or SDHI (Havis et al. 2015). Chemical products with distinct actions are found to be effective (Havis et al. 2015). Prothioconazole (Proline) caused an increase in yield of barley and chlorothalonil provided control over the expression of symptoms. A mixture made of triazole (epoxiconazole or prothioconazole), chlorothalonil +/- boscalid is found to be highly effective. Chlorothalonil also works against secondary infections that can be caused by wind dispersed spores. The use of Proline is considered as the best control of the disease, whereas azoxystrobin and fenpropimorph (Corbel) were found to be least effective (Oxley et al. 2008). Moreover, the utilization of bixafen, isopyrazam, fluxapyroxad significantly helps the barley growers in controlling the pathogen. However, various guidelines have been proposed regarding the application of the fungicides as the fungus is highly prone to develop resistance against the chemical compounds. The effectiveness, efficiency of each fungicide is monitored annually and the data is made accessible to the barley growers so that they are aware of the researched facts (Havis et al. 2015). Here Table 6 represents the efficiency of stereo on barley yield after the infection caused by Rcc.

Table 6. Efficiency of fungicide on barley yield after infection of Rcc (Reitan and Salamati 2006)

Fungicide	Variety	Yield loss	Reference
	Olsok	100-110 kg/ha	Reitan and Salamati 2006
	Lavrans	110-115 kg/ha	

Stereo	**Gaute**	120 kg/ha	
	Tirib	110-120 kg/ha	
	Ven	120-125 kg/ha	
	Edel	120-130 kg/ha	

4.5.4.2 Time of application of fungicides: In most cases the symptoms of RLS are observed after the flowering stage. So, the effective fungicides should be sprayed when no symptoms appear on the infected plant (McGrann et al. 2017). Various other cultivation procedures, environmental parameters probably help in controlling the disease but further research work is required to establish it (McGrann et al. 2017).

5. Future aspects

Despite carrying out an extensive research on RLS, various questions still remain unanswered. A number of major issues regarding the epidemic need to be addressed for public welfare. At this scenario it is important to determine the most crucial inoculant which is capable of causing *Ramularia* Leaf Spot in barley (Havis et al. 2015). Often black-grass and rye grass have been found to grow near the barley crop fields. So, there is an assumption that these grasses might be an important source of Rcc inoculum (Mäe et al. 2018). There is another possibility that under particular environmental and climatic parameters external inoculum may be a potential threat to the production of barley (Mäe et al. 2018). The various environmental factors that might play a role in the distribution of the fungus across fields are unknown to us (Salamatiand Reitan 2006). Pathogen biology (Havis et al. 2015), the rate of infection (Kaczmarek et al. 2016) and the reasons behind the recent rapid growth of Rcc graph of worldwide basis is of utmost importance for the researchers to look into. Further research is necessary regarding the various alternate hosts of Rcc. The phases of disease cycle (Kaczmarek et al. 2016) and the evolutionary changes of fungus require additional investigation (Havis et al. 2015). The teleomorph stage of the fungus remains unobserved and that is a mysterious part for the scientists (Stam et al. 2019). During the life cycle, Rcc gradually transfers from an asymptomatic phase to a symptomatic phase but the process that acts specifically as a catalyst in mediating the change is currently unknown (Mäe et al. 2018). Many scientists have explained their views that Rcc is a hemi-biotroph characterized with a prolonged latent phase, whereas, a greater number of scientists believe it to be an endophyte which establishes an endophytic relationship with the host and gradually develop into a necrotrophic pathogen within the host crop (Havis et al. 2015). Study regarding varietal interactions with climatic parameters and the role of abiotic stress in expressing the disease are important, to understand the possibility of barley genetics in controlling RLS (McGrann et al. 2017). Presently there is limited study on the spatial diversity of the

pathogen (Stam et al. 2019). The reason for developing resistance of the host plant against the pathogen is unknown to us and a technique is also in need to determine the degree of resistance. Furthermore, the effect of cultural influences on infection rate needs wider knowledge. Information about the probable effect of the fungus on the quality of animal feed, whisky and beer also remains unanswered (Salamati and Reitan 2006). At this point of view, huge numbers of studies are necessary for the various possibilities which can lead to controlling the disease (McGrann et al. 2017). The development of molecular based technologies has played a crucial role in early detection and thereby helping in better control of the disease. But various other techniques are much in requirement in order to fully control the rise of the disease (Havis et al. 2015).

6. Conclusion

R. collo-cygni (Rcc), a fungus of class Dothidiomycetes was first reported more than a century ago but in recent days it has garnered the interest of a large number of scientists all over the world due its epidemic nature. The difficulty in identification and isolation of fungus from its host is a major issue and thereby has accelerated the spread of Rcc more rapidly throughout the world. Apart from infecting barley, it is capable of causing disease in wheat, rye, oat etc. Winter barley serves as an important inoculum for spring barley. The fungus can also spread across wide areas due to the wind-borne spores. Even barley seed infection adds as a factor for causing the epidemic. The rate of infection, germination of conidia, appearance of symptoms, spread of the fungus are governed by a range of weather parameters including light intensity, moisture, temperature, rainfall, humidity, dew and the amount of precipitation that the area experiences. Sporulation of the fungus can even occur beneath the snow indicating the capability of the pathogen to survive in extreme weather conditions. Studies revealed that fungal DNA can be detected in large amounts in the outer layers, lemma and in scarce amount in the pericarp and embryo of an infected barley seed. Only the endosperm of seed lacks the presence of the Rcc fungus. This late season fungus shows symptoms after the emergence of ear. These symptoms include brown, reddish to blackish brown rectangular spots that gradually develop into chlorotic halo. These lesions then coalesce to form bigger patches of infection all over the foliage, stems and awns. It has been reported that the fungus is capable of living saprophytically and Rcc is even thought to be an endophyte which can develop into a necrotrophic pathogen under various conditions. Though much detailed studies are required to fully shed light on this matter. The life cycle of the fungus involves the penetration of the fungal hyphae which contains a spherical structure called stomatopodia at its tip through the stomatal pores. This stomatopodia then branches forming conidiogenous aggregates. The pre stages of infection do not involve the appearance of symptoms. The conidiophores are ‘swan necked’ and starts to grow intercellularly and approximately after 4 weeks symptoms become visible. Though the life cycle of *R.collo-gyni* is somewhat described in this review but detailed study is urgently required in order to control the epidemic. With due time, the development of molecular techniques has somewhat accelerated the detection of the fungus in hosts thereby

paving its control. Through PCR, the products which were obtained were purified, sequenced and were seen to be of same size. An intraspecific homogeneity was observed among the sequences and the previously published ITS sequence of the pathogen. After performing few PCR reactions with increased concentrations of the host DNA, distinct bands were observed. This determines that with the help of PCR, the presence of Rcc can be detected in the host thereby helping in early detection of the pathogen. Till date no method is available that can completely control RLS from spreading over wide regions. Studies and experiments have revealed that the use of certain fungicides, somewhat resistant barley seed varieties, removal of diseased plants, crop rotation, seed treatments, use of elicitors, the time of application of fungicides play a significant role in controlling the epidemic disease to some extent.

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STUDY ON DIFFERENT SOIL BORNE PATHOGENS CAUSING DISEASES OF RICE PLANTS.

ABSTRACT –

Rice is a staple food for majority of population in the world. Rice crops suffer from infection of many diseases of bacteria, fungi and viruses. The production of rice is increased quantitatively and qualitatively then it will generate additional income sources. Soilborne pathogens include fungi, oomycetes, nematodes, viruses and parasitic plants. They are dependent on soil's abiotic and biotic components. Diseases caused by soilborne pathogens cause heavy losses to many crops in rice. The indigenous plant pathogen of rice is causing lot of damage to the crops. It is necessary to identify plant pathogens. The aim and objective of present study is to find out information about different plant pathogens attacking the rice crop, It is also necessary to find out causes, symptoms and eradications of diseases. Keeping this in mind the study of disease was carried out.

INTRODUCTION -

Rice is a staple food for majority of population in the world. The production of rice is increased quantitatively and qualitatively then it will generate additional income sources. Rice is the seed of the grass species Oryza sativa. Rice crops suffer from infection of many bacterial, viral and fungal diseases. Thus, decreasing the annual production of rice in many countries of the world. The diseases of rice are estimated to cause annually about 10 per cent. loss in rice production. Diseases caused by soilborne pathogens cause heavy losses to many crops; they include seedling, vascular and root rot diseases. Soilborne pathogens include fungi, oomycetes, nematodes, viruses and parasitic plants which causes disease in rice. These pathogens have some similar features related to being soilborne. They live and act in the soil, during part of their lives. Pathogens are heavily influenced by the soil's abiotic and biotic components as well as by the agricultural practices, which are applied to the soil. The agricultural practices include irrigation, tillage, manure application, and fertilization. Abnormalities may also cause by abiotic factors such as low or high temperature beyond the limits for normal growth of rice, deficiency or, excess presence of nutrients in the soil and water, pH and other soil conditions which causes the availability and uptake of nutrients, toxic substances such as H2S produced in the soil, water stress and reduced light. Such disorders are referred to as physiological diseases. A disease is an abnormal condition that injures the plant or causes it to function improperly. Diseases are readily recognized by their symptoms - associated visible changes in the plant. The organisms that cause diseases are known as pathogens. Pathogens invade the plants through belowground organs but may also reaches the upper parts of the plants. Pathogens need common options supported their shut reference to the soil, that features a robust influence on their survival and capability to cause disease. The latter stems from interactions between the infectious agent and also the host, that each successively act with the organic phenomenon and abiotic parts of the surroundings. Soilborne pathogens turn out resting structures that, within the absence of a number, area unit inactive, and area unit so protected against the soil's hostile activities thanks to fungistasis. However, within the presence of root exudates of a vulnerable host within the rhizosphere, or associate degree adequate nutrient supply, they germinate and infect the plant, unfinished appropriate conditions. additionally, soilborne pathogens could colonize the roots of plants that don't seem to be their major host, while not inducement visible symptoms. Soilborne pathogens have several mechanisms for spatial dispersion, e.g., through infected propagation material.

Types of soil-borne diseases -

- Soil-borne diseases within the garden embrace pre-and post-emergence damping-off, like Fusarium, fungus and Rhizoctonia species, root rot, as well as genus Phytophthora, vascular wilts caused by fungi as well as fungus and nematodes.

- Pre-emergence damping-off is wherever young seedlings decay within the soil before they seem higher than the soil surface. this happens once conditions for seed germination area unit poor, like cold, hot or terribly wet soil, poorly-drained soil, compacted soil or within the presence of undecayed organic matter.
- Post-emergence damping-off is wherever stems and roots of tender seedlings area unit attacked at the soil line and also the seedlings go over. High salt concentrations within the soil conjointly cause damping-off.
- Root rots will have an effect on plants on the far side the seed plant stage once the fungi invades-internal root tissue, interfering with the provision of water and nutrients. surface symptoms embrace loss of vigor, leaf yellowing, leaf drop, weakening beginning at the growing tip, twig plant disease, and extra time.
- Vascular wilts area unit defined by plant weakening and discoloration of the system at stems or trunks and branches.
- Nematodes area unit microscopic, nonsegmental worms. They embrace blighter worms just like the root knot nematode that invades the roots and causes them to make gall-like lesions that prohibit water and nutrient uptake that causes weakening.

The loss caused by the major diseases of rice are as follows;

Bakanae – Fusarium fujikuroi Nirenberg.

Stem rot – Sclerotium oryzae Cattaneo.

Sheath blight – Rhizoctonia solani Kuhn.

Sheath rot – Sarocladium oryzae

Brown spot – Bipolaris oryzae.

Blast (leaf, neck [rotten neck], nodal and collar) – Pyricularia grisea.

Narrow brown spot – Cercospora janseana.

DIAGONOSIS OF RICE DISEASES :

1. Disease – **Bakanae**

   Pathogen – Fusarium fujikuroi Nirenberg [telomorph: Gibberella fujikuroi]

   Host – Rice [Oryza sativa]

'Bakanae' is a Japanese word that means, 'Foolish seedling' refers to the abnormal elongation, this disease is also known as "thin noodle seedling", and "stupid rice crop". The disease is caused by Fusarium fujikuroi Nirenberg also known as foot rot or elongation disease is one of the important diseases in all the rice growing countries . Symptoms of elongated seedlings has been identified of Bakanae in California rice fields in 1999. This disease is now widespread throughout the rice growing areas of California and some fields has suffered significant yield losses in 2002.The disease is reported to be caused by one or more Fusarium species and complex of disease symptoms including seedling blight, root rot, crown rot, stunting and, the most classical symptoms of etiolation, excessive elongation of infected plants, foot rot, seedlings rot, grain sterility and the discoloration of the grain with leads to effect on grain yield and seed quality which has been recorded from different regions of the world. It is one of the newly discovered; emerging and increasing problems of rice, particularly on basmati rice in India during recent years.

SYMPTOMS AND SIGNS –

The infected seedlings are frequently identified on the concept of tall and yellow plants. Diseased seedlings appear to be taller, more-slender, and slightly chlorotic, green to pale in colour advanced stage and abnormally elongated primary leaves are seen in fields. Disease occurs in seed-beds, but doesn't kill the seedlings which are attacked immediately. Rice seedlings which grow from the infected seeds, display Bakanae symptoms. The foremost common symptom of the disease is yellowing and excessive elongation of infected plants which are observed generally. The disease starts appearing within the fields; slight yellowish, weak, abnormally elongated plants, which in due course of sometime grow faster than others. Bakanae

infected fields were uneven throughout the season. the event of adventitious roots from the lower nodes of the culms and presence of whitish fungal growth on the lower parts of the plants are observed from Bihar, state, Rajasthan, Uttarakhand and Punjab. Leaves dried up quickly from below and die one after another in an exceedingly few-weeks. In India, infected plants developed adventitious roots from the lower nodes of the culms and presence of white to pinkish fungal growth even are noticed as other diagnostic features of the disease.

Fig.-1 Bakanae disease Rice field.

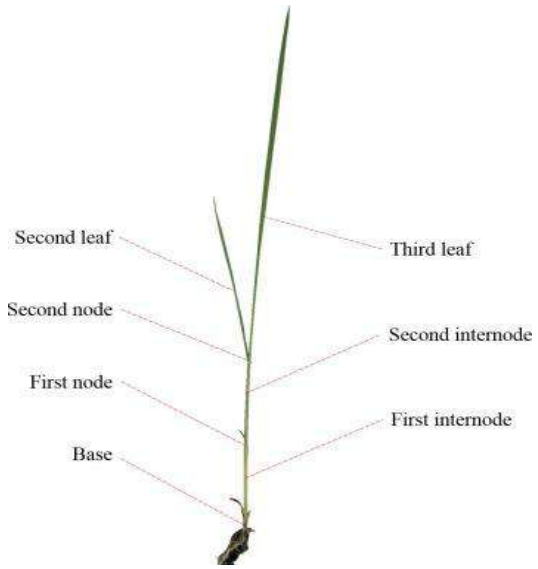

Fig.- 2 Bakanae disease: elongated deformed Rice plants.

Fig.- 3 Secondary root at upper node of Bakanae infected rice plant.

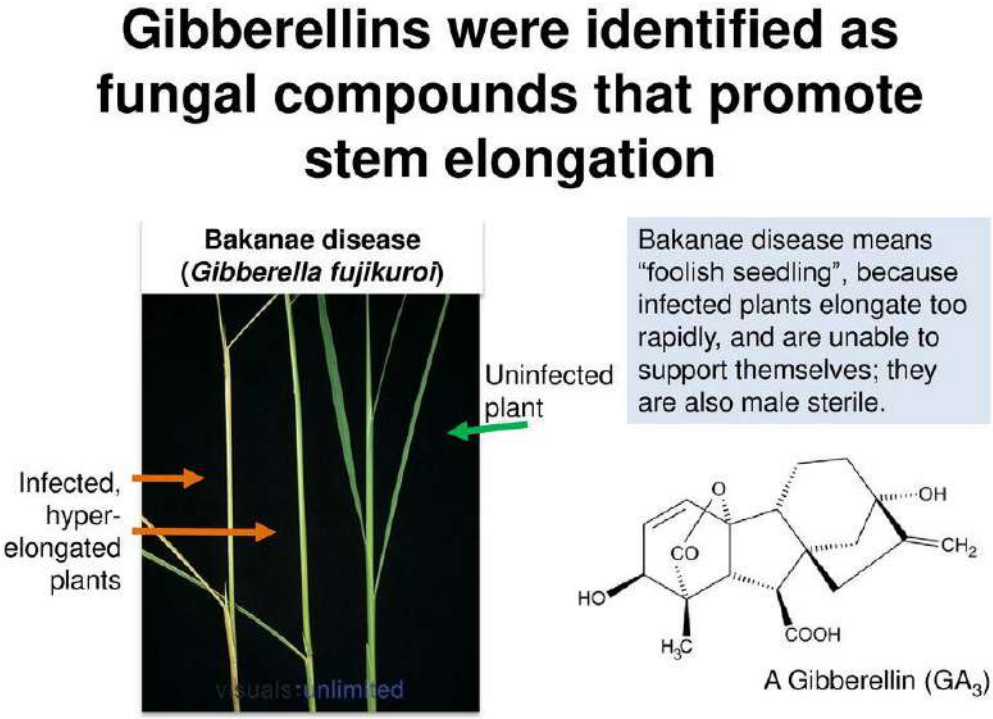

Fig.-4

PATHOGEN BIOLOGY –

The causative agent of this disease Fusarium fujikuroi Nirenberg (sexual stage: Gibberella fujikuroi) belongs to kingdom Mycota, Division Eumycota and class Ascomycotina. Pathogen produce sexual and asexual spores, sexual spores are ascospores that formed within a sac called as ascus. The shape of the pathogen is piston, cylindrical, flattened and are 90-102 x 7-9 µm in size. Pathogen produce two types of asexual spores i.e. macro conidia and micro conidia. Microconidia are hyaline, single celled and oval. While macroconidia are slightly sickle shaped and two to five cell. During the season of dormant, pathogen survives by producing sclerotia which is dark blue colour and spherical in shaped. The size of sclerotia is 80 x 100 µm.

EPIDEMIOLOGY –

Bakanae is primarily as seed borne disease in nature but the pathogens also survive in the debris of the soil and plants. the airborne spore of the pathogens might contaminate the seed during dispersal or during harvest. The main source of primary inoculum encourages the disease in the seeds, which is infected by the pathogens as the inoculum which is present in the soil reduces quickly, as of the host debris in the field. The pathogen doesn't appear to infect the seeds internally but rather contaminates seeds externally through seed coat. In rough conditions, the pathogens survive in the form of spores on seed coat and as macroconidia or, thick-walled hyphae in plant debris in the soil. The survival period for the pathogen in the soil is 100-120 days in the form of macroconidia or, thick-walled hyphae.

Karov et al. (2009) recorded the very low incidence of Bakanae disease when the fields with previous records of disease occurrence were planted with clean and healthy seeds. Puyam et al. (2017) incidence was less in the presoaked seeds as compared to dry seeds.

DISEASE CYCLE -

Bakanae disease spreads mainly through air-borne conidia and the fungus survives like parasite as well as saprophyte in the grains which are already infected, in plant debris and also on other crop debris.

Favourable Conditions -Highly humid and cloudy weather during the heading stage.

Importance of Bakanae disease -

I. Crop losses may reach up to 20% in outbreak cases.

II. A 20−50% loss was observed in Japan.

III. In Thailand, yield loss of 3.7% was reported.

IV. In India yield loss of 15% was observed.

2. Disease - **Stem rot**

Pathogen – Sclerotium oryzae

Host – Rice [Oryza sativa]

Stem rot disease of rice occurs in major areas of the world and is caused by the pathogen Magnaporthe salvinii. The stem decay microorganism is frequently found in its sclerotial state, Sclerotium oryzae, in the field. Pathogen causes black lesions which are generally located around the water level on the stem of the plant and is first noticed around the panicle initiation. Stem rot was first appeared in the MIA in 1995. large number of field were affected, but no major yield losses were experienced. In the year 2017 stem rot occurred and affected, and with a number of properties reported to have stem rot in the particular areas.

SYMPTOMS AND SIGNS –

The primary indications are by and large saw in the field after the mid tillering stage. At first, the disease shows up as little, blackish, irregular lesion on the external leaf sheath close to the water line. The lesion augments as the infection advances with the fungus entering into the internal leaf sheaths. The infected leaf sheaths regularly pass on and swamp off all through the season. In the end, the growth enters and spoils the culm while the leaf sheath is incompletely or totally decayed . Infection of the culm may bring about lodging, unfilled panicles, white grains, and in serious cases, demise of the tiller. Dark lesions show up lastly a couple internodes of the stem decay and breakdown. After opening tainted stem, dark greyish mycelium might be found inside the empty stem and various tiny, dark sclerotia are inserted everywhere on the unhealthy leaf sheath tissues. Dark sclerotia found inside the infected leaf sheaths .Sclerotia and mycelium of the growth are by and large present inside infected culms. The presence of sclerotia is typically a positive and simple method of diagnosing the illness.

Fig.-5 Initial lesion of stem rot on Rice.

Fig.-6 & 7 Progression of Stem rot infection at the water surface.

Fig.-8 & 9 As Stem rot disease progresses the fungus penetrates the culm and may kill the tiller.

Fig.-10 Infection has progressed through all the leaves and has penetrated the culm.

Fig.-11 Round black sclerotia of the Stem rot pathogen develop on or, in infected plant tissue as the rice plant matures.

PATHOGEN BIOLOGY –

Fungus's perithecia are found embedded in the leaf sheaths, and are dark, spheroidal, and 250-650m in diameter, having a short beak that does not protrude from the tissue. Asci are cylindrical, short-stalked, and 104-165 x 8.7-17.7m; they liquify at maturity and contain eight ascospores. Ascospores are fusiform, bit curved, 3-septate, and 35-65 x 8.7m. Sclerotia is black in colour, globose or near globose, smooth, and usually 180-280m in diameter. Conidiophores are generally dark, upright, and septate. Conidia are fusiform, 3-septate, curved, 29-49 x 10-14 m, and produced on pointed sterigmata.

EPIDEMIOLOGY –

The epidemiological process leads to the sclerotinia stem rot (SSR) which can be divided into three stages; Carpogenic, Germination of sclerotia, release and production of ascospore and the plants are being affected by ascospores.

First stage; the sclerotia germinates to produce one or multiple apothecia, each containing 2×10^6 ascospores approximately. Germination of sclerotia takes place in the moist soils and cool temperature (16 and 20°C) and it must be within the top 5cm of the soil level, so that it can form apothecia on the soil surface.

Second stage; ascospores are bound to discharge from the asci in the apothecia and then they are dispersed aerially in the air currents. As primary inoculum source ascospores are considered for the majority crops, including rice [Oryza sativa].

Third stage; viable ascospores are deposited on flowers, then they germinate and colonize the flower petals and as well infects the plant, typically occurs at the nodes. Symptoms are visible early, depending on host susceptibility and weather condition.

DISEASE CYCLE -

The sclerotia resides in the stubbles and straw which are carried through water irrigation. The fungus over winters that survives for long periods as sclerotia within the upper layers (2-3 inches) of the soil-level. The half-life of sclerotia within the field is about 2 years. Sclerotia which are viable are found in fields for up to six years after a rice crop. In the surface of water where sclerotia buoyant and floats comes in contact to the nearest waterline, then germinate as well infects the rice tillers.

Favourable Conditions- Infestation of stem borer and leaf hoppers.

Doses of nitrogenous fertilizers are high.

3. Disease – **Rice Blast**

Pathogen – Magnaporthe oryzae [anamorph: Pyricularia oryzae]

Host – Rice [Oryza sativa]

Rice blast disease is caused by the damage of fungi to rice which in turn causes disease on leaves, stems, peduncles, panicles, seeds and even roots. The potential risk of crop failure due to this disease is so great that it is one of the most serious plant diseases. Other grains, including crabgrass, can be infected with closely related fungi (Magnaporthe grisea, Magnaporthe poae, Magnaporthe rhizophila, and Magnaporthe salvinii), which cause almost the same symptoms in their respective hosts.

SYMPTOMS AND SIGNS –

The symptoms of rice blast embody lesions that may be found on all the parts of the plant, as well as leaves, leaf collars, necks, panicles, pedicels, and seeds. A recent report shows that even roots can become infected. However, the foremost common and diagnostic symptom, diamond formed lesions, of rice blast occur on the leaves, whereas lesions on the sheaths are comparatively rare.

Rice leaves. The symptoms on leaves may vary in keeping with the environmental conditions, the age of the plant, and therefore the levels of resistance of the host cultivars. On vulnerable cultivars, lesions may at first seem gray-green and water-soaked with a darker inexperienced border and that they expand speedily to many centimeters in length. On vulnerable cultivars, older lesions typically become lightweight tan in color with death borders. On resistant cultivars, lesions often stay tiny in size (1-2 mm) and brown to dark brown in color.

Fig.-12 Severe leaf Blast symptoms.

Rice collars. The collar of a rice plant refers to the junction of the leaf and therefore the stem sheath. Symptoms of infection of the collars contains a general space of mortification at the union of the 2 tissues. Collar infections will kill the whole leaf and will extend a few millimeters into and round the sheath. The plant might manufacture spores on these lesions.

Fig.- 13 Collar Blast.

Rice necks and racemes. The neck of the rice plant refers to it portion of the stem that rises on top of the leaves and supports the seed head or panicle. Necks are often infected at the node by the rice blast fungus and infection results in a condition referred to as rotten neck or neck blast . Infection of the necks may be terribly destructive, inflicting failure of the seeds to fill (a condition called blanking) or causing the whole panicle to break down as if rotted. The rice blast plant also can infect the racemes because the seeds kind. Lesions are found on the panicle branches, spikes, and spikelet. The lesions are often grey brown discolorations of the branches of the panicle, and, over time, the branches might break at the lesion.

Fig.-14 Node Blast symptoms.

Rice seeds. The fungus has often been isolated from the pedicels of the seeds. Seeds are not produced when pedicels become infected, a condition called blanking .The symptoms of rice blast on seeds themselves consist of brown spots, blotches , and diamond-shaped lesion often seen on leaves. The process and the time during which infection of seeds by spores of the pathogen occurs has not been much described but recent research shows that the fungus can cause infection in the seeds by infecting the florets while the seeds mature, and by this way seeds gets infected and infection develops.

Fig.-15 Seed Blast symptom.

Table 1.: Symptoms caused by rice blast disease.

Infected plant parts	**Blast symptoms**	**Reference**
Leaves	Lesions grey-green, water-soaking with green border, soft-colored tanning with necrotic borders.	11.
Leaves	On younger leaves violet lesions, spindle formation with a gray center and violet to brown terminal, on older leaves brown spots.	16.

Leaves	Primary lesions are white to gray-green with darker borders, older lesions appeared as white gray, encircled by a red brown end and shaped as diamonds.	25.
Leaves	Lesions on the leaves are usually spindle-shaped, larger lesions form a diamond shape including a grayish center and brown border.	28.
Neck	Neck blast marked by the infection at the base of the panicle and it starts rotting.	9.
Neck	Triangular purplish lesions, expanding lesions on both sides of the neck node, attacked younger nodes create white panicles in color. Infected panicles appear as white and are unfulfilled in part or in whole.	10.
Neck	The lesions are often grayish brown discoloration of panicle branches, triangular purplish lesions accompanied by expanding on either side of the neck. The panicles become white when young necks are infected.	16.

PATHOGEN BIOLOGY –

Rice blast disease is caused by Magnaporthe oryzae, previously named as Magnaporthe grisea or Pyricularia grisea. The Magnaporthe oryzae was considered as the new species, after being separated from Magnaporthe grisea based on geneology and mating experiments and findings. Magnaporthe oryzae is filamentous ascomycetes in nature with the aptitude to produce sexually and asexually. The asci are found within specialized structures called perithecia. The fungus conidia size is 20-22 × 10-12 μm which are translucent, two-septate, and slightly darkened.The growth of mycelia, conidia formation and conidial germination of the conidial of the pathogen can occur the least bit pH level for except 2.35-2.95 with optimal conditions for mycelial growth. The mycelium of Magnaporthe oryzae is septate and the nuclei within the mycelium and spores of this fungus are haploid.

Sexual Reproduction -

Rice cell pathogen sexual or transformation steps form the opposite mating isolate pair, but may be produced in the laboratory if not found in the field in the United States. As for Ascomycetes, it produces hyaline, fusiform shaped (spindle-shaped) ascomycete spores with three septa. Asci is unitunicate [single]. This fungus is considered heterothallic, which has a bipolar mating system (mating controlled by two different alleles at a single locus) along with additional genes to control the sexual cycle. Based on recent phylogenetic, molecular and morphological data, fungi isolated from rice and other grasses like; Eragrostris curvula, Eleusine coracana, Lolium perenne and Setaria spp.. Strains isolated from Digitaria sanguinalis (crabgrass) need to be distinguished and explained to Magnaporthe grisea, whereas they are taxonomically described in Magnaporthe oryzae.

Asexual Reproduction -

Magnaporthe oryzae's overgrowth stage is described by the name Pyricularia oryzae (formerly known as Pyricularia grisea) and is the most common spore-shaped mold in the United States. These spores, called conidia, are abundantly produced by culturing lesions and special trunks, called spores. It is a captive, generally 3 cells, and is produced on the apex of a conidiophore. Sporulating colonies on the agar plate take on a fleecy grayish appearance.

Mold under favorable conditions sporulates from the center of the lesions on susceptible cultivars. It can also form spores in seed lesions. The most resistant varieties rarely form spores. Spores are produced on infected leaf, collar, panicle and seeds on conidiophores that extend beyond the surface of infected leaves, rings, cones, seeds, and lesions. Bunsen spores and spores can collect to give the lesion a dusty gray appearance. Conidia are produced after several hours of high humidity and are easily released or released near noon, especially in windy conditions.

LIFECYCLE AND DEVELOPMENT –

The fungus infects all aerial parts of rice plants at all growth and developmental stages due to its polycyclic nature.

The lifestyle of Magnaporthe oryzae is biotrophic at an initial vegetative stage, then transitioning to a saprophytic stage.

The stage in which plant cells are attacked and suppressed is called the vegetative phase, while the necrotic phase is the stage of cell death.

Different sources of pathogenic bacteria. It can come from rice plant residues or debris, rice grains, soil, work equipment, or other alternative hosts.

The mycelium can survive on plant residues, living plant tissue, and asexual spores called conidia can survive for more than one season in tropical and subtropical regions.

The mycelium of fungus can survive on rice straw for more than three years at 1832 ° C and fungal spores develop when moistened. When spores appeared in the rice, sticky mucus was produced during hydration from a compartment at the top of the conidia's head which helped it adhere to the surface of the plant.

Germination of spores would start whenever humidity on the host plant is favourable. Occurrence germ tubes from the tapered end of conidia grew and spread over the surface of the host plant.

The germ tubes developed and then formed a repressor layer. This buffer layer is formed from the germ tube containing melanin and chitin molecules in the cell wall of the host plant .

The turgor pressure due to the presence of glycerol leads to the penetration pin generated by the appressoria in the epidermis and wall of host plant cells penetrates the epidermis and cell wall of the host plant due to the presence of glycerol, which increases the turgor pressure for easy penetration.

Appressoria enters rice through the stomata of the plant.The development of lesions on the rice plant is the result of the enlargement of the mycelium of Magnaporthe oryzae in the tissues of the plant and colonize the plasma membrane and epidermal cells of the host plant.

The mycelium not only feeds on tissue by taking nutrients from the plant tissue and spreading it to various organs through plasmodesma, but also produces effector molecules to attack host cell immunity and initiate infection.

Magnaporthe oryzae replicates in a very short time by mitosis, nuclei migrate and die spores mark the onset of infection The expression of the pathogen occurs within 3 to 4 days after infection.

EPIDEMIOLOGY –

Conidiophores produced from the autophagic death of conidia are transmitted to other plant tissues or nearby by plants by wind, working tools, water splash or plant contact start a brand new infection cycle.The pathogen conidia can spread within 230 meters from its source when the environment is favorable; high ratio with winds of 3.5 m s-1 or more. Airborne Magnaporthe oryzae conidia exist everywhere the year-round and are liable for epidemics occurrence throughout the year. Longer period of leaves dampness, ratio of about 92-96% and therefore the air temperature around 25-28°C were environmental factors that favour spore growth and lesion development. However, reports from several researches have indicated that a high dosage of nitrogen supply favors heavy Magnaporthe oryzae infection.

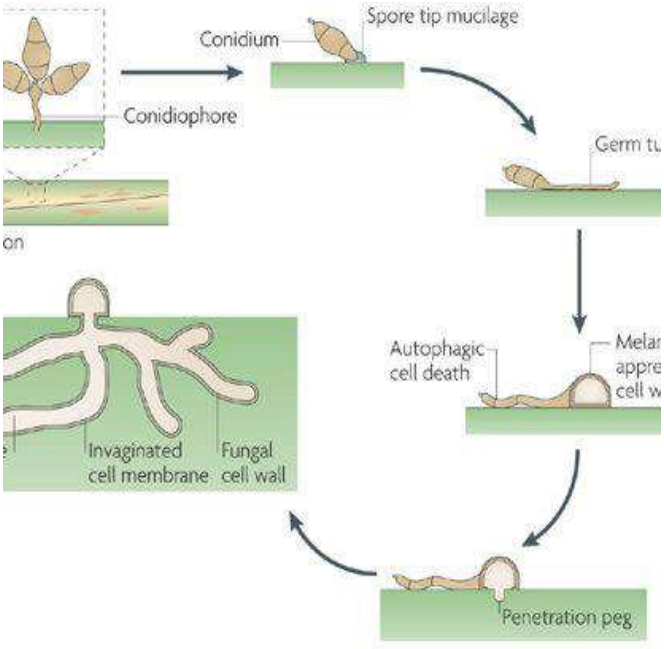

Fig.- 16 Disease Cycle.

4. Disease – **Sheath blight**

Pathogen – Rhizoctonia solani

Host – Rice [Oryza sativa]

Rice sheath blight is caused by Rhizoctonia solani Kuhn (Teleomorph: Thanatephorus cucumeris (Frank) Donk), it is a destructive disease throughout the world that causes significant yield loss and quality degradation. Not only rice, the pathogen also infects many other plant species. A significant amount of achievable rice production is being protected from Rhizoctonia solani by applying protection strategies. In absence of protection strategies, rice Sheath blight disease causes 10-30% yield loss and reaches up to 50% approx. during prevalent years. Cases have been reported in China, that about 15-20 million ha of rice growing area is affected, causing losses of 6 million tons of grains per year.

SYMPTOMS AND SIGNS –

The symptoms on rice initially starts to develop at or above water level, or above soil level. The lesions appears as dull greenish -grey, ellipsoidal which are about 2-3 em long initially and the margin is dark brown in colour, then it coalesce and enlarges in combination with the colour change to off-white or, fawn with a purplish-brown or brown margin. On the leaf sheath lesions may be found anywhere, initially appears on the outer sheath but then extends towards inner sheath, and in humid warm climates which is in favourable conditions on the leaf blade they are being detected.For sheath blight development the favourable conditions are low sunlight, high humidity (about 95%) and high temperatures ranges between 28-32°C. Under these conditions, the fungus spreads rapidly with the help of runner hyphae to upper plant parts as well as to adjacent plants. Usually most rapid is disease development in the early heading and grain filling stages. Rice seedlings might be infected with Rhizoctonia solani in the nursery, if they are planted in infected soil. It is studied that sclerotia are the major source of infection. Sclerotia are found on or near the lesions, and are detached easily, and it survives for longest period in the soil. In rice plant, they germinate and the emerging hyphae produces the infection cushions on the leaf sheath that is exposed. From these infection cushions, growth of the haustoria and penetration of the host tissue occurs and the lesions develop. Generally, after the death sclerotia are formed of the invaded cells and are found on the tissue where fungal growth has been most prolific.

Fig.-17 Rice Sheath blight.

Fig.-18 Elliptical grey spots.

Fig.-19 Late season Sheath blight damage to rice plants.

PATHOGEN BIOLOGY –

The rice Sheath blight fungus produces sclerotia instead of spores, generally measuring 1-3 mm in diameter and relatively spherical. Near the spots or, on sclerotia are formed and easily can be detached from the plant. Under natural conditions, sclerotia occurs singly but might sometimes coalesce for forming larger masses. It is whitish in colour when at young stage and slowly turns brownish or dark brown when at old stage.

Fig.- 20 Sclerotia of rice Sheath blight disease.

Sheath blight disease's first description appeared in Japan in 1910 and the causal organism was identified as Hypochnus sasakii which was previously described by Shirai (1906). In the Philippines, a similar disease was identified by Reinking (1918) & Palo (1926) which they introduce as fungus of the Rhizoctonia group. In Sri Lanka, Park and Bertus (1932) found sheath blight with a Rhizoctonia species, referred to it as Rhizoctonia solani. After two years the disease was identified in China 1934, but after descriptions it appeared in Brazil, Surinam, Venezuela, Madagascar and the USA the initial studies revised that sheath blight occurred only in Asia. Sheath blight is considered as one of the most important disease in Sri Lanka, China, Taiwan and Japan. In Malaysia, 15-20% of the total area where rice were planted has been reported to be infected with the disease. In Korea half of the total cultivated rice yield has been infected with sheath blight and a 90% of loss have been recorded. A sudden increase in sheath blight incidence in Vietnam has been reported where the infection in area has been increased from 21,000 ha in 1985 to about 200,000 ha in 1990 and 1991.

DISEASE CYCLE -

Sclerotia develop on lesions and drop to the soil

↓

The fungus survives in sclerotia in the soil.

↓

The sclerotia float on the water surface during land preparation.

↓

The sclerotia germinate and fungus penetrates the plant.

↓

The fungus grows on the plant.

Favourable conditions - Humidity should be around (96-97%).

Temperature approximately high(30-32˚C).

Planting should be closer.

High doses of nitrogenous fertilizers.

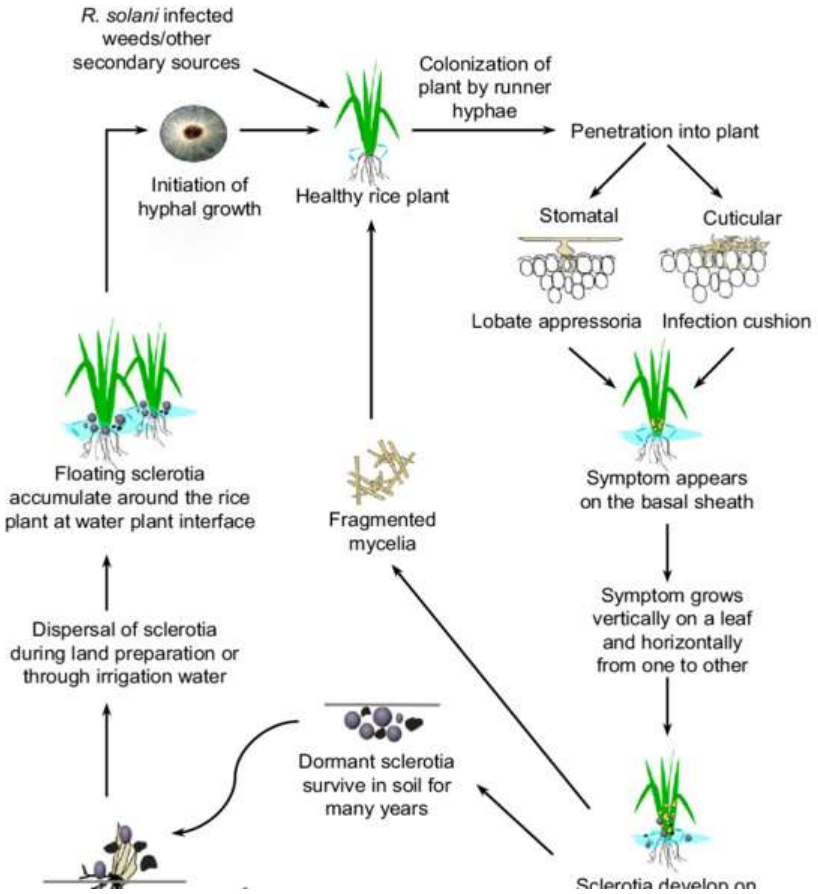

Fig.-21 Disease Cycle.

DISCUSSION –

I have discussed about the soil borne pathogens which causes disease in rice crops like Bakanae, Stem rot, Blast, and Sheath blight disease which causes heavy losses in rice crops where they are being harvested in different states of India. Bakanae disease in Punjab, Haryana and Uttarakhand which caused 15% losses throughout the year. Bakanae disease can be managed by using various chemical, physical and biological methods. Mainly chemical method is preferred because of the complexity of the disease and broader range of host of the pathogen; chemical methods include such as mixture of Phenamacril and ipconazole [2:1 ratio] respectively, it causes synergistic inhibition of the pathogen's mycelial growth. Blast disease was first recorded in Tamil Nadu in the year 1913.Also it was found in Japan causing yield loss (1-100%), in China 70%, Indonesia 921-37%), Bangladesh (30-100%). Different control measures have been adopted for managing the disease includes resistant variants, chemical controls like some fungicides and antibiotics; Tricyclazole and Blasticidin respectively are effective against the blast disease and some integrated disease management strategies. Stem rot disease of rice has widely spread in the North-Eastern Karnataka in the year 2014 and 2015. The disease had has caused 5-80% losses in grain yield. It has been found through studies that the increased incidence of stem rot disease is due to the high use of nitrogenous fertilizer. Sheath blight disease generally occurs in temperate and tropical production areas. This disease was first recorded in Japan in the year 1910. It caused 10-30% yield loss and might reach upto-50% during prevalent years. Sheath blight disease includes cultural control, chemical control – Fungicides widely used for management of the disease. It provides better management than non-systemic products.

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Content

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Shalini Ganguly

Role of Silicon in Plant Defence against Pathogenic Fungi

I. Introduction

Si comprises up to 70% of soil mass being the second most abundant element on the earth's crust (27.7% by soil weight) after oxygen (Epstein, 1994; Savant et al., 1997; Ma and Yamaji, 2006). It rarely occurs as a pure element but as its oxides: Silica and Silicate. Although, Silicon is abundantly found in soil, only Monosilicic acid (H_4SiO_4) is up taken by the plants. Si uptake generally takes place through plant roots as silicic acid [$Si(OH)_4$] (Ma and Yamaji, 2006), and is passed through the plasma membrane via two Si influx and efflux transporters, Lsi1 and Lsi2 (Ma et al., 2006; 2007; 2008).

Several studies have shown the beneficial effects of Si in plants, especially in Gramineous plants such as rice, sugarcane and also in some plants of Cyperaceae (Epstein, 1994, 1999; Liang, 1999; Liang et al., 2005). Absorbed Si is deposited mainly in the cell wall of plants, and helps in signalling during stress conditions (Fauteux et al., 2005). Si improves the mechanical as well as physiological properties of plants and help overcoming several biotic and abiotic stresses (Epstein, 1999; Richmond and Sussman, 2003; Ma, 2004; Ma and Yamaji, 2006; Tripathi *et al*., 2020). For instance, Si enhances resistance to diseases caused by fungi, bacteria, and pests (Fauteux *et al*., 2005; Marschner, 2012). Silicon prevents pathogen penetration by structural reinforcement (Epstein, 1999; Epstein, 2001; Rodrigues *et al*., 2015), inhibiting pathogen colonization through stimulating systemic acquired resistance through production of antimicrobial compounds (Fauteux *et al*., 2005; Datnoff *et al*., 2007; Fortunato *et al*.,2012; Van *et al.*, 2013), and through increasing plant resistance by activating multiple signaling pathways and defense-related gene expression (Fauteux *et al*., 2005; Chen *et al*., 2014; Vivancos *et al*., 2015). The beneficial effects of Si towards disease includes accumulation of Si in the epidermal tissue, formation of organic complex compounds in cell walls, induction of phenolic compounds, production of phytolexin or glucanase or peroxidase enzymes, and regulating pathogenicity or stress-related gene expression to limit pathogen invasion and colonization in plants (Belanger *et al.*, 2003; Brunings *et al*., 2009; Chain *et al*., 2009; Sakr, 2016). Several studies have revealed that Si is effective in enhancing the

resistance to diseases and pests. In this review the role of Si in defence against plant pathogenic fungi has been discussed. Besides biotic stress, Si also protects plants against various abiotic stresses like drought stress, salt stress, water logging, metal toxicity, nutrient imbalance, etc. (Epstein, 1994, 1999; Savant *et al*., 1997; Ma, 2004; Ma and Yamaji, 2006; Liu *et al*., 2014; Coskun *et al*.,2016).

II. How do plants uptake, translocate and accumulate silicon?

➢ Si UPTAKE

Silicon is an essential nutrient for humans, animals and plants. It is available in the form of silicon dioxide (SiO_2 or Silica). At room temperature Si exists in two different forms, one crystalline or Silica crystal (mono and poly forms) and another amorphous or Silica glass. Chemically active and available from of Si in soil are Monosilicic acid and Polysilicic acid. Plants take up Si in the form of Monosilicic acid and transports it from the roots to the shoots and when the concentration is over a critical level (approximately 100 ppm at biological pH) it gets polymerized as opaline phytoliths (Jones and Handreck, 1967)which constitutes the bulk of a plant's Si content. Though whether Si is taken up through the leaves or not is still controversial (Gu´evel *et al*., 2007). Si deposition in the epidermal cells help in protection of plants against insect herbivores and plant diseases (Ma, 2004).

Si is said to be present in the cytoplasm and subcellular structures of plants. Si in plants has a tendency to polymerize inside the cell walls, cell lumen, intercellular spaces and in the subcuticular layer (Sangster *et al*., 2001; Singh *et al*., 2020). This process is called opal formation and it does not occur in random. Plant species differ with respect to their size and shape of the phytoliths that they accumulate. The growth and nucleation of these phytoliths are under the control of specific proteins (Perry *et al*., 2000; Harrison, 1996).

In soil, Si is available for plants in the form of uncharged monomeric monosilicic acid (H_4SiO_4), under common pH conditions (Sommer *et al*., 2006). The concentration of Si in soil ranges from 0.1 to 0.6 mM (Richmond and Sussman, 2003). Recently it has been noticed that alkaline pH may lead to decrease in plant available Si, given an increase in adsorption of Si on mineral rich surfaces (Haynes *et al*., 2018). The study of Si mobility in soil-plant

systems is very essential for optimizing its benefits towards plant protection. There are three different modes of uptake of Si by plants, active, passive and rejective (Takahashi *et al*., 1990). Plants with active mode of Si uptake take up Si faster than water, thus depleting the Si present in the uptake solution. Plants with passive mode of Si uptake take up Si at a rate similar to the uptake of water, resulting in no significant changes in the concentration of Si in the uptake solution. The rejective mode of uptake has a tendency to eliminate Si from the uptake solution. However, all the three mechanisms differ from each other and are not completely understood (Takahashi *et al*., 1990). For the past few years huge efforts have been put together to know about the mechanisms involved in uptake and transport of Si by vascular plants. Recently different transporters have been identified (Pontigo *et al*., 2015). Si uptake in vascular plants is quite complicated due to the selectivity of transport and accumulation in specific tissues so these studies have been considerably improved over the last 15 years and the progress is reviewed by Mandlik *et al*., 2020.

- **Transporters Involved In Si Uptake**

The uptake of Si in plants involves influx and efflux transporters (Ma and Jian, 2009).

❖ **Influx Transporters**

Low Silicon 1 or Lsi1 was the first identified Si transporter from Rice (Ma *et al*., 2006). Rice has a capability of accumulating Si over 10% in its shoots. An experiment was conducted by mutation approach in which the Lsi1 gene was isolated by map-based cloning method (Ma and Jian, 2009). Lsi1 gene is a part of the Nod26 like major intrinsic protein (NIP) subfamily, like that of the aquaporins. Recent studies revealed that aquaporins belongs to NIP III family and it plays a major role in Si uptake (Deshmukh *et al*., 2020).In an experiment using a *Xenopus* oocyte assay system, Lsi1 showed positive influx activity for silicic acid and the transport was not even affected by low temperature (Mitani *et al*., 2008). Lately, it was also discovered that Lsi1 is permeable to arsenite too (Ma *et al*., 2008).

The Lsi1 gene is expressed constitutively in the roots. Near the root tip region comprising of both the apical meristem and elongation zone the expression of Lsi1 is much lower, ranging between 0-10 mm than in the basal regions (>10 mm) (Yamaji and Ma, 2007). These observations proved that the site of Si uptake in plants is near the mature region of the roots

and not in the root tips. An old study revealed that 67% of total Si was taken up during the reproductive stages of rice from panicle initiation to heading in rice (Ma *et al*., 1989). Thus, deficiency of Si causes significant reduction in grain yield.

Lsi1 is found in the main and lateral roots but not in the root hairs (Ma *et al*., 2006). Thus, root hair has no role play in Si uptake but the lateral roots do help in significant uptake of Si (Ma *et al*., 2001). The Lsi1 protein is found in both exodermis and endodermis where the Casparian strips are present. Following the Lsi1 identification in rice, Si influx transporters are identified in maize (ZmLsi1) and barley (HvLsi1) (Chiba *et al*., 2009; Mitani *et al*., 2009). Both ZmLsi1 and HvLsi1 are present at the epidermal, hypodermal and cortical cells (Chiba *et al*., 2009; Mitani *et al.*, 2009). At amino acid level ZmLsi1 and HvLsi1 show 82% identity with OsLsi1. There transport activities are same but the cell-type specificities of localization and expression patterns are different. Lately, proteins and channels involved in membrane permeability have been recorded in many monocot and dicot species (Yamaji *et al*, 2012; Bokor *et al*., 2019; Zellner *et al.*, 2019). Si uptake transport system also plays a huge role in agricultural purpose (Noronha *et al.,* 2020).

- **Efflux Transporters**

Lsi2 is the efflux transporter gene of Si that was cloned using a novel rice mutant (Ma *et al*., 2007). Lsi2 is defective in Si uptake (Ma *et al.*, 2007) and is predicted to encode a membrane protein with 11 transmembrane domains that belongs to a putative anion transporter with no similarity with the silicon influx transporter Lsi1. In contrast to the Si influx transporter Lsi1, Lsi2 is an efflux transporter of Si that is capable of transporting Si out from the cells. The efflux of Si can be inhibited by low temperature treatment and by three protonophores 2, 4-dinitrophenol (DNP), carbonylcyanide 3-chlorophenylhydrazone (CCCP) and carbonylcyanide*p*-(trifluoromethoxy)penylhydrazone (FCCP). Also, the Lsi2 efflux activity can get increased at lower external pH values (Ma *et al*., 2007). All of the results prove that Si transport by Lsi2 is an energy dependent active process that is driven by the proton gradient. Lsi2 is expressed near the roots of the plants similar to that of Lsi1. Similar to that of Lsi1, Lsi2 is also localized at the exodermis and the endodermis cells of the roots. Though the location of Lsi1 is on the distal side and Lsi2 is on the proximal side of the exodermis and endodermis cells of the roots.

- **Coupling Of Lsi1 And Lsi2 In Rice**

As previously been said, both the Si transporters Lsi1 and Lsi2 are present in the exodermis and endodermis where the Casparian strips exist. This Casparian strip prevents the passage of any solutes from the external solution to the stele. Both Lsi1 and Lsi2 are essential for the transcellular transport of Si to the stele (Vaculik *et al*., 2020).

In rice, Si is transported by Lsi1 into the endodermis cells and by Lsi2 the Si is released into the stele. The coupling of Lsi1 and Lsi2 in the cells of the Casparian strips is essential for efficient transport of Si across the cells into the stele.

➢ Si TRANSLOCATION

Translocation Transporter

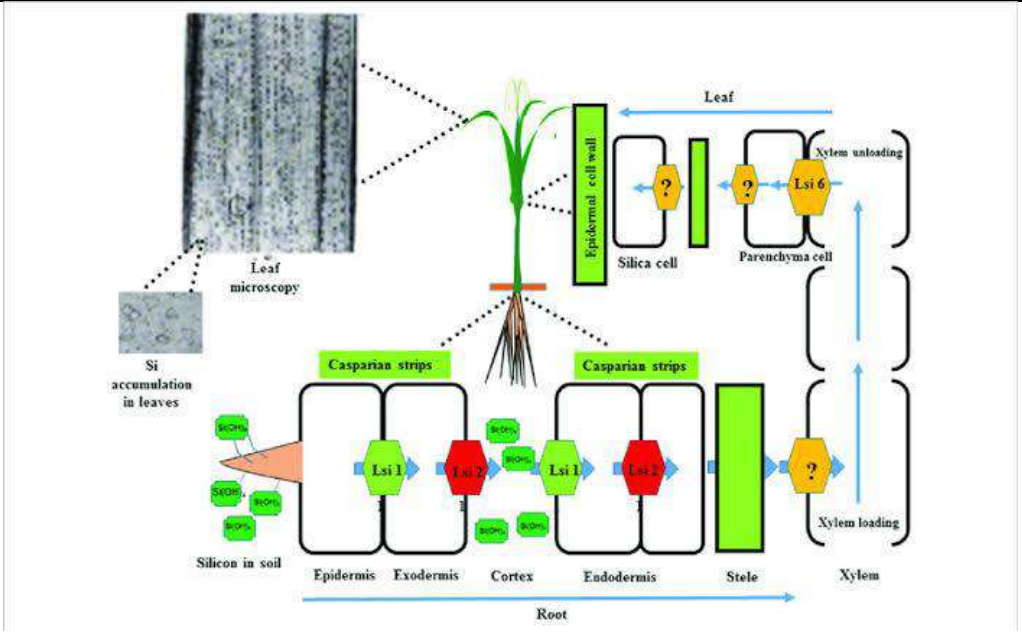
Si is transported to the stele by Lsi1 and Lsi2 and then translocated to the shoot through xylem. A transporter Lsi6 has been observed that is said to export silicic acid from the xylem.Lsi6 is expressed in the leaf sheath, leaf blades and root tips unlike Lsi1 and Lsi2 (Yamaji *et al*., 2008). Lsi6 is present in the adaxial side of the xylem parenchyma cells in the leaf sheath and leaf blades. Knockout of the Lsi6 gene does not affect the Si uptake by the roots but it does affect the deposition pattern of Si in the leaf blades and leaf sheaths. Furthermore, the knockout of the Lsi6 gene causes an increase in excretion of Si in the guttation fluid. All these results suggest that the knockout of the Lsi6 gene may result in alternation of the Si pathway to the specific cells. A very similar transporter has been identified from maize, ZmLsi6 (Mitani *et al.*, 2009). Si accumulation in the shoots greatly differ among plant species, ranging from 0.1% to 10% of dry weight (Hodson *et al*., 2005; Ma and Takahashi, 2002). Though, the molecular mechanisms for these are still not known.

➢ Si ACCUMULATION

Si deposition in the shoots varies from 0.1% to 10% in dry weight (Ma and Takahashi, 2002). In an experiment it is observed that plants of Gramineae and Cyperaceae show a high accumulation of Si, whereas plants that belong to Cucurbitales, Urticales, and Commelinaceae show intermediate Si accumulation and most other plants species show low accumulation of Si. This

difference in Si accumulation in plants depend on the ability of the roots to take up Si from soil (Takahashi *et al*., 1990). According to the ability to accumulate Si in the shoots, plants are classified into three types, Si accumulators, intermediate and non-accumulator species (Takahashi *et al.*, 1990). Equisetaceae, Gramineae and Cyperaceae families accumulate up to 100 g/kg of Si on dry weight basis, on the other hand most dicotyledonous species accumulate less than 1 g/kg of Si on dry weight. Also, Si tissue concentration can also vary among genotypes of the same species like that of rice (*Oryza sativa*) (Deren, 2001; Ma *et al*., 2007), sugarcane (*Saccharum officinarum*) (Deren, 2001) and barley (*Hordeum vulgare*) (Ma *et al*. 2003).

Silicic acid, a non-charged molecule is taken up by the roots and transported from cortical cells to stele. This Si is released into the xylem and translocated to the shoots. Here due to water loss by transpiration, Si gets transformed into amorphous Si by polymerization. The amorphous Si is accumulated in the leaves, cell wall, stem etc. of the plant (Ma and Yamaji, 2006). Si can also be deposited in cells of roots, tubers and inflorescence of certain plant species (Hodson and Sangster, 1988, 1989; Lux *et al*., 1999; Chandler-Ezell *et al.*, 2006). About 90% or more Si is taken up by the roots and translocated to different parts of the shoot (Ma and Takahashi, 2002). High concentrations of Si is present in the xylem that forms monosilicic acid (Casey *et al*., 2003; Mitani *et al*., 2005).



A Schematic Representation Of Si Uptake, Transport And Accumulation In Rice (Khan *et al*., 2019)

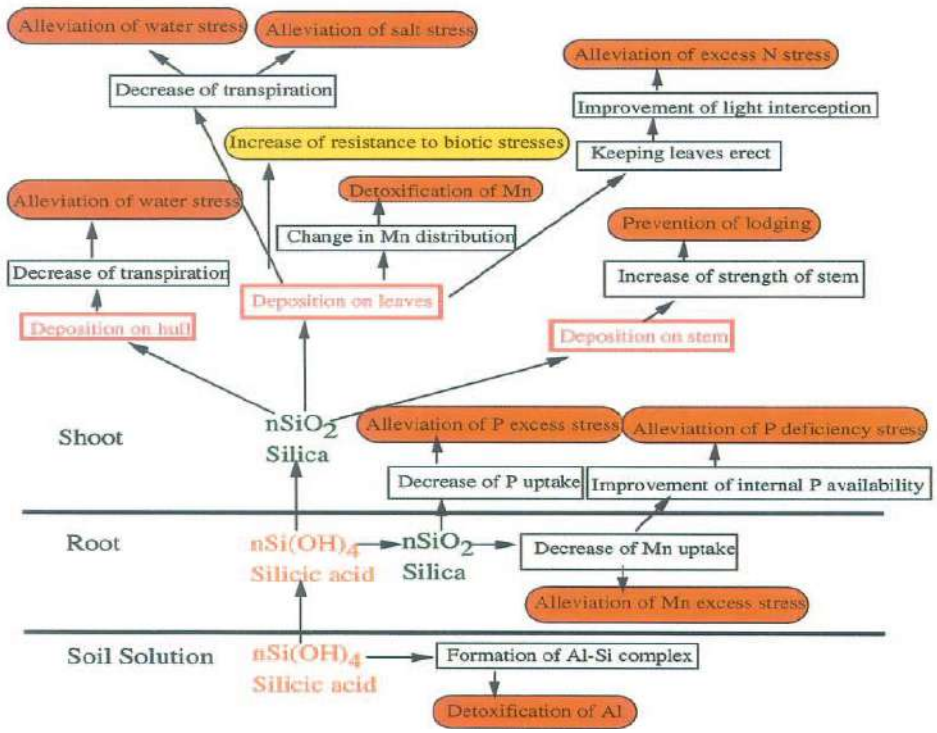
III. Silicon mediated resistance against abiotic and biotic stress

Si provides mechanical strength and resistance to a plant by silicification of epidermal cells, formation of thick layer of silicaunder in the cuticle, double cuticular layer formation, thickened cellulosic membrane by Si, papilla formation, and deposition of complex organic barriers inhibiting the pathogen penetration. This makes the plant cells less susceptible to enzymatic degradation due to pathogen invasions (Inanaga *et al.*, 1995; Fauteux *et a*l., 2005; Datnoff *et al.,* 2007; Van *et al.,* 2013). In most cases, Si is cross-linked with hemicellulose in the cell wall of plants, which improves the mechanical support and regeneration of the plant during stress conditions (He *et a*l., 2015; Guerriero *et al.,* 2016). Si also affects the synthesis of plant defense enzymes during stress conditions and helps in the integration of secondary metabolites (Ahanger *et al*., 2020). Si mediated defense mechanism includes morphological, biochemical, and molecular (Singh *et al.,* 2020).

- **ABIOTIC STRESS**

Si is widely known to help the plant against all kinds of biotic stresses, but there are also a number of studies that shows how Si alleviates during physical stresses, including radiation, low and high temperature, wind, drought, waterlogging, low and high light, etc. (Ma, 2004). For example Si alleviates water stress by decreasing transpiration. Since, transpiration occurs from the leaves through stomata and partly through the cuticle, Si deposition occurs beneath the cuticle forming a double layer and decreasing the plant's transpiration through the cuticle (Ma, 2004). Si reduces transpiration rate by 30% in rice that has a thin cuticle Under water-stress of low humidity conditions, effect of Si on rice growth was more noticeable than that of the ones cultivated under non-stress or high humidity conditions (Ma *et al*. 2001a). The positive effect of Si under salt stress was seen in rice (Matoh *et al*., 1986; Yeo *et al*. 1999), wheat (Ahmad *et al.*, 1992) and barley (Liang *et al.,* 1996). In rice, the root and shoot growth was inhibited by 60% for about three weeks in the presence of 100 mM NaCl. But after introducing Si there was a significant alleviation of salt-induced injury (Matoh *et al*., 1986). Si also effectively alleviated toxicity of heavy metals like Zinc (Neumann and zurNieden, 2001), Iron (Okuda and Takahashi, 1962), Manganese (Horst and Marschner, 1978; Iwasaki and Matsumura, 1999) and other chemicals like Aluminium (Cocker *et al*., 1998), Nitrogen

(Morimiya, 1996) etc. Si application in rice showed alleviation in the damage caused by climatic stresses like low temperature, typhoons and insufficient sunshine during the summer season (Ma *et al*., 2001a). Si was also seen increasing rice plant's tolerance to heat stress (Agarie *et al*., 1998).



Beneficial Effects Of Si Under Various Abiotic Stresses (Meena *et al*., 2014)

- **BIOTIC STRESS**

Several studies have revealed that Si is effective in enhancing the resistance to diseases against plant pathogens and pests. This review highlighted an extensive role of Si in plant defence against phyto-pathogenic fungi and the physical and biochemical mechanisms involved in disease resistance have been elucidated.

IV. Mechanism of Si Mediated Resistance in plants against fungal plant pathogens

- **PHYSICAL MECHANISM**

Si acts as a physical barrier against plant fungal infections in the form of cell wall rigidity and reinforcement, papillae formation and callose deposition.

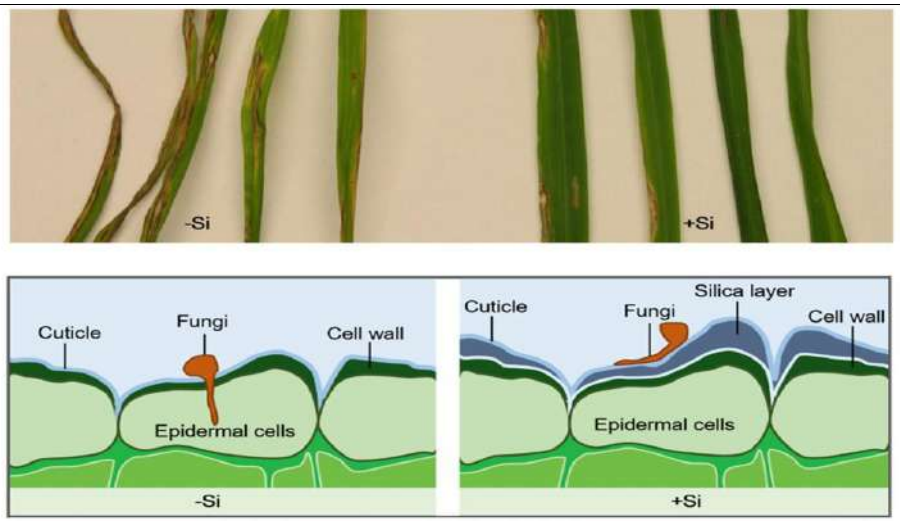
- **Silicon Layer Formation**

Si provides mechanical strength to a plant by enhancing its resistance through silicification of the epidermal cells, double cuticular layer formation, deposition of silica under the cuticle, thickened Si cellulosic membrane, papilla formation, and deposition of complex organic compounds in epidermal cell walls. These physical barriers inhibit the pathogen penetration and make plant cells less susceptible to enzymatic degradation caused by fungal infection (Inanaga *et al.,* 1995; Fauteux *et a*l., 2005; Datnoff *et al.,* 2007; Van *et al.,* 2013). Si accumulates beneath the cuticle forming a double layer on the epidermal cell of plants which prevent pathogen penetration, thus inhibiting the pathogen to enter host plant to cause diseases and decreasing disease incidence (Ma and Yamaji, 2006; 2008). In the cell walls, most Si is cross-linked with hemicellulose which improves the mechanical properties and regeneration (He *et a*l., 2015; Guerriero *et al.,* 2016). A successful penetration occurs in case of *Magnaporthe grisea* in rice plants where Si is not treated, but if the plant is treated with Si the pathogen shows an unsuccessful penetration inside the cell.

- **Papillae Formation**

Si also stimulates papillae formation during pathogen infection. Si accumulation occurs in the haustorial neck and collar area of fungus as well as in the papillae which prevents the invasion of the pathogen (Samuels *et a*l., 1994). In barley when infected by *Blumeria graminis f.sp.hordei*, Si application produces papillae in the epidermal cells (Zeyen *et a*l., 1993). A similar result is seen in rose when infected by *Podosphaera pannosa*, Si supply increases the number of papillae in the leaf cells (Shetty *et a*l., 2012). The formation of papillae after application of Si increases the resistance of rice against blast disease (Cai *et al*., 2008), wheat and barley against powdery mildew (Zeyen*et a*l., 1993). During the infection of barley by *Blumeria graminis f. sp. Hordei*, the application of Si enhances the deposition of callose and phenolics causing the formation of

effective papillae and inhibiting the fungal growth by trapping the penetration peg inside the papillae (Chowdhury et al., 2014).



Symptoms Of Leaf Blast Disease In Rice After Inoculation With *Magnaporthe grisea* And The Effect Of Si In Providing Resistance To The Fungal Infection By Physical Barriers (Wang *et al*., 2017)

- **BIOCHEMICAL MECHANISM**

Si enhances the plant defense by enhancing various defense related biochemical mechanisms. This enhances the defense relatedactivity of enzymes such as polyphenoloxidase, glucanase, peroxidase, and phenylalanine ammonia-lyase (PAL); increases the production of antimicrobial compounds such as phenolics, flavonoids, phytoalexins and pathogenesis related (PR) proteins; upregulates several defense signalling pathways such as salicylic acid (SA), jasmonic acid (JA) and ethylene signalling pathway (ET) (Fauteux *et al*., 2005; Datnoff *et* al., 2007; Fortunato *et* al., 2012; Van *et al.*, 2013).

- **Signal Transduction Cascade Triggered By Si**

Plants develop a very complex immune system to prevent pathogen infection, which consist of multiple layers of constitutive and inducible defense mechanisms which are found in induction of various signal transduction pathways (Grant *et al*., 2013). On the basis of observations done on cucumber, a model was suggested to explain the role of Si in induced plant resistance (Fawe *et al*., 2001). According to that model, Si bioactivity was compared to that of the activators or

secondary messengers of systemic acquired resistance (SAR). However there is a difference between SAR activators and Si and that is the loss of activity when the Si feeding is being interrupted since polymerization of Si causes inactivation as an inducer of resistance.

Plants fed with Si, naturally translocate silicic acid throughout all tissues. Upon pathogen attack, these infected tissues synthesize antimicrobial compounds and other defense reactions along with systemic stress signals such as salicylic acid, jasmonic acid and ethlylene. Silicic acid is said to modulate the post-elicitation intercellular signalling system activities. Silicic acid being a secondary messenger itself, plays a positive role in both systemic and local resistance. The post-elicitation intracellular signalling causes the expression of defense genes leading to hypersensitive responses, structural modifications of the cell wall, synthesis of stress hormones, synthesis of antimicrobial compounds and PR proteins. Si is also involved in other responses to the accumulation of phytoalexins. The target of plant signalling on elicitation of pathogen, is the cell nucleus that receives information for de novo protein and antimicrobial compound synthesis. Gene expression control by phosphorylation of transcription factors and their inhibitors is a major plant stress response. Signals leading to the expression of plant defense responses are transmitted through the activation of specific kinases or phosphatases cascades, into the nucleus. Biotic stress responses are largely dependent on Mitogen Activation Protein (MAP) kinases (Nu¨rnberger and Scheel, 2001; Zhang and Klessig, 2001; Tena *et al*., 2001; Morris, 2001). These transmit information to the nucleus by phosphorylation of the hydroxyl group on amino acid residues.

- **Signalling Pathways**

The signalling pathways include SA, JA and ET signalling that play an important role in plant immunity and regulate plant defense responses (Clarke *et al*., 2000; Devadas *et al*., 2002). SA induces the plant defense mechanisms mainly against the biotrophic and hemibiotrophic pathogens, while JA and ET mediates the plant defense mechanisms against necrotrophic pathogens (Pieterse *et al*., 2012). Several studies have revealed that Si regulates the plant stress responses by regulating phytohormone homeostasis and enhancing various signaling pathways (Zhang *et al*., 2004; Fauteux *et al*., 2006; Iwai *et al*., 2006; Brunings *et al.*, 2009; Chen *et al.*, 2009; Ghareeb *et al*.,2011; Reynolds *et al*., 2016). In Si treated plants, accumulation of plant phytohormones was seen in response to pathogen attack and wounds that were caused by them

(Fauteux *et al.,* 2006; Ye *et al.,* 2013; Kim *et al*., 2014); for example JA accumulation was seen in Si induced rice against insect herbivores (Ye *et al*., 2013) and it was also observed that the wounding induced the JA biosynthesis (Kim *et al*., 2014). In Si treated *Arabidopsis* plants, it was seen that during infection with powdery mildew pathogen (*Erysiphe cichoracearum*), there was an increased resistance due to enhanced biosynthesis of SA, JA and ET in the leaves (Fauteux *et al.,* 2006). A similar event was seen in tomato infected with *Ralstonia solanacearum,* where Si induced the activation of JA and ET signalling pathways (Zhang *et al*., 2004; Chen *et al.,* 2009; Ghareeb *et al*., 2011). In rice, an inducing effect of Si on the JA and ET signalling pathways showed that Si enhances the signalling activity which increases the plant's resistance against the blast disease caused by *Magnaporthe oryzae* (Iwai et *al*., 2006; Brunings *et al*., 2009).

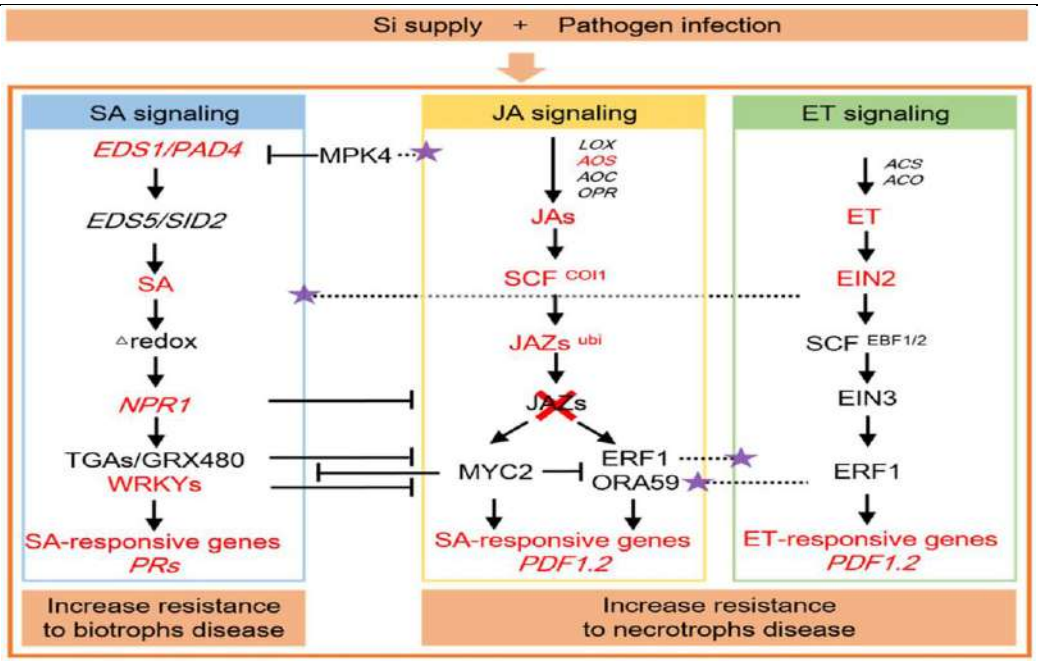
- **SA Mediated Pathway**

In case of infection caused by *Cochliobolus miyabeanus* in rice plant, it was observed that Si induced the plant's resistance independent of the SA and JA signalling pathways (Van *et al.,* 2015). In powdery mildew of *Arabidopsis*, Si increased the gene expressions encoding enzymes that were involved in the SA pathway. The expressions of EDS1, EDS5, PAD4 and SID2 genes were induced that regulate the SA biosynthesis (Shah, 2003). In *Arabidopsis* it was observed that Si caused higher expressions of the EDS1 and PAD4 genes along with the NPR1 and three pathogenesis related (PR) defense genes PR1, PR2 and R5. The regulatory protein NPR1 triggered the activation of PR gene expression in response to SA and NPR1 itself got positively regulated by some SA inducible WRKY proteins whose activity was enhanced by Si application (Li *et al.,* 2004). In tomato plant when infected with *R. solanacearum*, the expression of defense gene was induced by WRKY1 transcription factor and the activity was upregulated by the application of Si (Ghareeb *et al.,* 2011). Si thus enhances the expression of several defense genes and also increases the activity of several transcription factors and upregulates many signalling pathways. There was a certain increase observed in the level of endogenous SA and the subsequent PR expressions by Si treatment (Durrant and Dong, 2004; Kurabachew *et al.,* 2013).

- **JA And ET Mediated Pathway**

Si induces expression of several defense responses by serving as a priming agent for the JA pathway like the enhanced production of defense related enzymes and proteins (Johnson *et al.,*

2020), enhanced expression of transcription factor inducing the expression of proteins involved in JA signalling. JA also promotes increased leaf silicification and maturation of phytolith bearing silica cells by increasing the Si accumulation (Fauteux *et al.*, 2006; Ye *et al.*, 2013). For fine tuning of JA signalling pathway ubiquitin-protein ligase is involved, which causes degradation of the JA negative regulator JAZ1 domain (Thines *et* al., 2007). In an experiment it was seen that the application of Si in plants after the infection caused by the pathogen enhanced the defense signalling pathway by up regulation of ubiquitin protein ligase (Dreher and Callis, 2007).Among the ET and JA signalling pathway associated marker genes JERF3, TSRF1 and ACCO are important in view of disease resistance. The transcription factor JERF3 activates the response of ET and JA signalling pathways, while ACCO helps in ethylene biosynthesis and TSRF1 acts as an ET-responsive transcription factor (Pirrello *et al.,* 2012). In tomato plants it was observed that the application of Si upregulated the expression of JERF3, TSRF1 and ACCO genes supporting the fact that Si induced resistance are mediated via ET and JA signalling pathways (Ghareeb *et al.,* 2011). When infected with pathogens, ET and JA pathways help in regulating the expression of defense related genes, such as PDF 1.2 (Pieterse *et al.,* 2009). The Si regulated signalling pathways in plant defense response are demonstrated below:



Crosstalk Between Signalling Pathways In Plant Defense Originating From The Action Of SA, JA And ET Along With Their Defensive Response Regulations Are Demonstrated (Wang *et al*., 2017)

V. Defence responses in plants induced by Si against fungal pathogens

Si is known to reduce fungal diseases by deposition in the leaf apoplast, preventing penetration of pathogenic fungi. It is also seen to trigger a faster and more extensive deployment of the plant's natural defenses. This hypothesis was first proposed in case of the powdery mildew disease in cucumber (Fawe *et al*., 2001). In cucumber and powdery mildew pathosystem, it was observed that within a short period of time after the application of Si, all the prophylactic effects were lost (Samuels *et al.*, 1991). Si treated cucumber showed more effective resistance against *Pythium* (Che´rif *et al.*, 1992). In cucumber, Si enhanced the activity of chitinases, peroxidases and polyphenoloxydases, and increased accumulation of phenolic compounds to provide defense against *Pythium* (Samuels *et al*., 1991; Che´rif *et al*., 1992). Furthermore, increased production of flavonoid phytoalexins in Si applied plants was demonstrated in cucumber infected with powdery mildew (Fawe et al., 1998). In tomatoes early blight disease caused by *Alternaria solani* results in a huge loss worldwide. But it was observed that Si-treated tomato plants showed better resistance to the pathogen by delaying and having reduced disease severity than non-Si-treated plants (Gulzar *et al.*, 2021).

Mostly on the basis of experiments (Fawe et al., 2001), it was seen that Si played an active role in reinforcing plant disease resistance in cucumber by stimulating the natural defense reactions. Though this hypothesis was seen to be true only in the case of dicotyledons, many research groups also carried out experiments to see whether it is applicable in monocot-pathogen interactions. In wheat – *Blumeria graminis* f.sp. *tritici* (Bgt) system, after histological and ultrastructural analysis it was observed that the epidermal cells that was pre-treated with Si reacted to the pathogen attack with specific defense mechanisms like the papilla formation, callose production and release of electron-dense osmiophilic material. These results proved that the Si mediated defences in wheat are same as that found in cucumber (Be´langer et al., 2003).

Later, more experiments with the rice blast disease (Rodrigues et al., 2003) showed cytological evidences that Si-mediated resistance to *Magnaporthe grisea* in rice was correlated with specific leaf cell reactions that interfered with the development of the fungus. In rice infected with *Magnaporthe grisea*, Si was associated with higher accumulation of antimicrobial compounds at infection sites, including diterpenoid phytoalexins (Rodrigues et al., 2004). All these studies with Si and monocots brought further support that Si plays an active role in protecting plants against pathogens and this role is not only specific to dicots but also generalized to the entire plant kingdom.

The application of Si enhanced disease resistance by producing phenolic compounds and delaying the growth of invading pathogens (Dallagnol *et* al., 2011; Fortunato *et al.*, 2015). Si application also enhanced the production of flavonoids, another phenolic compound whose production provides resistance to rose plant against *Podosphaera pannosa* (Shetty *et* al., 2012), and wheat against *Pyricularia oryzae* (Silva *et* al., 2015). The strawberry powdery mildew disease caused Podosphaera aphanis and two-spotted spider mite caused by Tetranychus urticae were both causing serious destruction to the strawberry production worldwide. Si treatment to the plants showed decreased severity of both these diseases (Liu *et al*., 2020). Phytoalexins play a major role in plant defense against pathogen infection. Si application in plants showed enhanced production of phytoalexins that reduced incidence of powdery mildew caused by *Podosphaera xanthii* in cucumber plants (Fawe *et* al., 1998) and blast disease caused by *M. grisea* in rice (Rodrigues et al., 2004; 2005). It was reported that in rice Si application induced the production of phytoalexins such as momilactones A and B (Rodrigues *et al.*, 2004; 2005) which caused resistance to the plant against fungal pathogens. In perennial ryegrass

(*Magnaporthe oryzae*), the application of Si induced the production of phenolic acids including chlorogenic acid and flavonoids and also a magnification of the genes encoding for PAL and lipoxygenase were observed that provided resistance against the gray leaf spot disease (Rahman *et* al., 2015). Polyphenol oxidases (PPO) were found to be involved in the synthesis of lignin and it showed antibacterial ability inside the host plants (Song et al., 2016). Si application also caused an increase in the peroxidase (POD) and chitinase (CHT) activities that played major roles in pathogen interactions. POD was involved in a variety of plant resistant activities like cell wall reinforcement, biosynthesis of lignin and crosslinking of cell wall proteins (Brisson et al., 1994). CHT being one of the important PR proteins helps in cell wall lysis of several phytopathogenic fungi (Pan and Ye, 1992; Shewry and Lucas, 1997).

Si is associated with a series of physiological and biochemical reactions that lead to the induction of signalling pathways and enhancement of disease resistance in plants from pathogen attacks (Fauteux *et al.*, 2005; Vivancos *et al.*, 2015). Si also enhances the activity of WRKY transcription factor, induces production of resistance response protein ferritin and increases the activity of trehalosephosphatase to provide resistance. In tomato, it was observed that Si increased the activity of trehalosephosphatase to provide resistance to the plant against *Ralstonia solanacearum (*Ghareeb *et al.*, 2011).

Thus Si enhances several signalling pathways starting from the expression of defense related genes to structural modification of the cell wall leading to production of hypersensitive responses, PR proteins, defence related enzymes, hormone and other antimicrobial compound synthesis.

VI. Some Examples of Si induced resistance against fungal pathogens

Sl. No.	**HOST**	**DISEASES**	**PATHOGEN**	**DEFENSE-RELATED ENZYMES/ PROTEINS**	**REFERENCE**
1	Bean	Anthracnose	*Colletotrichum lindemuthianum*	Superoxide dismutase, ascorbate peroxidase,	Polanco *et al.*, 2014

				glutathione reductase	
2	Cucumber	Crown and root rot	*Pythium spp.*	Chitinase, peroxidases, polyphenoloxidases	Chérif *et al.*, 1994
3	Melon	Pink rot	*Trichothecium roseum*	Peroxidase	Bi et al., 2006
		Powdery mildew	*Podosphaera xanthii*	Chitinases, superoxide dismutase, b-1,3-Glucanase	Dallagnol *et al.*, 2015
4	Chinese Cantaloupe	Pink rot	*Trichothecium roseum*	Peroxidases, phenylalanine ammonia-lyase	Guo *et al.*, 2007
5	Pea	Leaf spot	*Mycosphaerella pinodes*	Chitinase, b-1,3-glucanase	Dann and Muir, 2002
6	Perennial Ryegrass	Gray leaf spot	*Magnaporthe oryzae*	Peroxidase, polyphenol oxidase	Rahman *et al.*, 2015
7	Rice	Blast	*Magnaporthe oryzae*	Glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase	Domiciano *et al.*, 2015
		Brown spot	Bip*olaris oryzae*	Chitinase, peroxidise	Dallagnol *et al.*, 2011
		Sheath blight	*Rhizoctonia solani*	Phenylalanine ammonia-lyases, peroxidases, polyphenoloxidases, chitinases	Schurt *et al.*, 2014

8	Soybean	Target spot	*Corynespora cassiicola*	Chitinases, b-1-3-glucanases, phenylalanine ammonia-lyases, oxidases	Fortunato *et al*., 2015
9	Wheat	Blast	*Pyricularia oryzae*	Chitinases, peroxidises	Filha *et al.*, 2011

VII. Conclusion

Silicon (Si) is the second most abundant element on earth's crust after oxygen. It is present abundantly in the soil and is taken up by plant root in the form of silicic acid. It is most prevalent in plants of Gramineae and Cyperaceae families. A number of translocators such as LSi1, LSi2, and LSi6 are involved in Si uptake by root and its translocation in shoot. Si generally helps the plants to combat against a number of biotic and abiotic stresses. It plays a very effective role in plant defence by enhancing resistance against diseases and pests. In this review an extensive study has been carried out to evaluate the role of Si in plant defence against pathogenic fungi. Si can induce resistance against fungal pathogens either through physical mechanisms by silicification of epidermal cell, double cuticular layer formation, thickened Si cellulose membrane, papilla formation and deposition of organic complex compounds in cell wall. These physical barriers inhibit the fungal pathogen penetration. Si can also enhance plant defence by activating a biochemical signal transduction pathway inside the plants. Plants treated with Si, can translocate Silicic acid in all the tissues which can trigger systemic signals like SA, JA or ET inside the plant cells. This post elicitation intercellular signalling system causes expression of defence related genes leading to hypersensitive response, structural modifications of cell wall, synthesis of antimicrobial substances like phytoalexins defence related enzymes, phenolics and PR proteins, This review thus highlights the potential mechanisms involved in Si induced resistance against fungal pathogens. This study indicates the positive role of Si in plant defence response and also emphasizes on the use of silicon in fungal disease management.

VIII. References

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Abbreviations

NMs- Nanomaterials

NP/NPs- Nanoparticle/Nanoparticles

AuNP- Gold Nanoparticle

AgNP- Silver Nanoparticle

Au-Ag NPs: Gold-silver alloy nanoparticles

TEM: Transmission electron microscopy

SEM: Scanning electron microscopy

EDX: Energy dispersive X-ray

FTIR: Fourier transform infrared spectroscopy

XRD: X-ray diffraction

Introduction

Synergy between technology and medical sciences has opened new arenas in the ever-growing domain of nanotechnology aimed at synthesis, application and use of nanomaterials (NMs) to integrate with biological research. Nano-biotechnology involves the fabrication of nanoscale particles by use of biological moieties that regulate the characteristics of nanoparticles (NPs). Green synthesis is a significant process for nontoxic metal nanoparticle production employing plant materials (Khanna et al.,2019).

Nanotechnology involves the manipulation and synthesis of materials ranging in size from 1 to 100 nm in size (Rajeshkumar et al.,2019). The nanoparticles can play a huge role in nano-medicines and can help in health care sector and for screening purposes, drug delivery, antisense and gene therapy, tissue engineering and nano-robots configuration(Malarkodi et al.,2019).

Physicochemical synthesis of NPs is costly and releases harmful by-products posing a very high risk to living organisms (Khanna et al., 2019). Biosynthesis of NPs using microorganisms, plants, and algae is an alternative to physical modes of synthesis. The main focus is on selecting compounds which are harmless and commercially viable. In the last few years, Phyco-Nanotechnology has become an upcoming area with wider scope in the synthesis of algae-based NPs. Algae are the largest photo-autotrophic group of microorganisms and have the potential source for a number of secondary metabolites, pigments and proteins, which can serve as nano-factories for metallic nanoparticles (Ali et al., 2011; Khanna et al.,2019). Another promising field related to nanoparticles is the production of nano-alloys. They are used to test binding affinity, DNA-protein interaction, drug-liposome interaction, antigen-antibody interaction etc. (Rich et al., 2001; Pal et al., 2015).

Nanomedicines using Silver nanoparticles have also proved effective as anti-cancer agents (Pal et al.,2016; Huy et al., 2020). Anti-proliferative activity of silver nanoparticles have also proved to be effective after being screened against three leukemic cell lines viz. K562, MOLT-3, and REH through MTT assay(Pal et al.,2016).

A classification of nanoparticles has been given below (Ahmad et al., 2019; Table 1).

Table 1: Classification of NPs is provided below (Ahmad et al., 2019):

Basis of Classification of NPs	**NP classes**
Functionalization	**Bare NPs**: aluminum oxide, iron oxide **Capped/Coated Nps** : nanowire, nanorod, nanotube **Functionalized NPs**: in-situ functionalized NPs core-shell Nps ,post functionalized NPs
Surface morphology	**High aspect ratio: nanowire**, nanorod ,nanotube **Low aspect ratio: suspension**, colloids
Chemical nature	**Organic NMs**: dendrimers, liposomes, micelles **Inorganic NMs**: nanocomposite, metal and alloy, metal oxide, semimetal oxide
Physicochemical properties	**Lipid based NMs**: triglycerides based, **Carbon based NMs**: single, double and multi-walled Carbon nanotubes **Metallic NMs**: alkali , noble metal NPs, quantum dots **Semiconductor NMs**: SiO_2 **Polymeric NMs**: nano-Composites, nano capsules, nanospheres

	Ceramic NMs : zirconium oxide, aluminum oxide based
Dimension	**Zero dimensional**: atomic clusters, quantum dots, hollow spheres **One dimensional**: nano-wire, nanotube, nanofiber, nanorod **Two dimensional**: nanofilm, nanodisc, nanolayer **Amorphous NMs** :glasses
Origin/source	**Natura**l: C-containing NPs, **Inorganic NPs** (SnS, Poly vinyl pyrrolidone) **Anthropogenic**: engineered
Magnetic properties	**Paramagnetic**: iron oxide , zinc sulfide , cadmium sulfide **Diamagnetic**: titanium oxide, magnesium ferrite
Crystalline Nature	**Crystalline NMs**: single extended domain structures **Polycrystalline NMs**: multi extended domain structures

Algae have emerged as “bio-nano factories” in the past couple of decades. They serve an advantage in the fact that both the living and dead biomass can be used to synthesize nanoparticles. The technology is cost effective and environment friendly and is advantageous in having higher metal uptake capacity (Rajeshkumar et al.2014).

Algae and cyanobacteria can hyper-accumulate heavy metal ions and convert these into malleable forms (Chaudhary et al., 2020). NPs can be biosynthesized by incubating algal tissues with precursor metal extracts. The different biochemical compounds present in algae

and cyanobacteria then reduce the charge of the metal ions into zero valent state. The process of nanoparticle biosynthesis involves three steps:

- Activation phase- It involves reduction of metal ion and nucleation due to the synthesis of enzymes by algal cells. This is evident from the change in color of solution.
- Growth phase- Here the nucleated metal elements amalgamate with one other resulting in NPs of different shapes and sizes that are thermodynamically stable.
- Termination phase-The ultimate shape of the NPs is obtained in this phase.

Factors like temperature, pH, time, substrate concentration and stirring control the physical nature of NPs (Chaudhary et al., 2020).

Several species of algae and cyanobacteria have proved to promote production of NPs (Parial and Pal, 2015). For e.g. *Spirogyra insignis* has been used in the biosynthesis of Au-NPs and Ag-NPs(Li et al., 2015). *Anabaena flos-aquae*, *Calothrix pulvinata* and *Leptolyngbya foveolarum* have been used in the biosynthesis of nanoparticles of Au, Ag, Pt and Pd(Brayner et al., 2007)**.** *Euglena gracilis, Euglena intermedia* (Jena et al., 2015), *Amphora* sp. (Patel et al., 2015), *Anabaena* sp., *Cylindrospermopsis* sp., *Lyngbya* sp., *Limnothrix* sp., *Synechocystis* sp., *Synechococcus* sp. (Barwal et al., 2011) have been used for synthesis of AgNPs.

In this current study, an attempt has been made to review the green synthesis of silver nanoparticles using algae and cyanobacteria.

Materials and Methods:

Silver Nanoparticle synthesis from algae and their application: Silver nanoparticles can be synthesized by different species of algae and cyanobacteria.

- The first example is of AgNP biosynthesis by ***Caulerpa racemosa*** as demonstrated by **Kathiraven et al.,2015**. The specimens were collected from the Gulf of Mannar, Southeast coast of India. Thereafter for the synthesis of AgNP, seaweed filtrate was added to biosynthesis of Ag nanoparticles 10 ml seaweed filtrate was added in 90 ml of 10-3 M aqueous $AgNO_3$ solutions at room temperature. Silver nitrate was reduced to silver nanoparticles. The nanoparticles were further characterized using UV-VIS Spectroscopy, XRD, FTIR and TEM. The synthesized nanoparticles of silver were then tested for antibacterial activity against human pathogens of *Staphylococcus aureus* (ATCC 29123) and *Proteus mirabilis* (ATCC 25933) using the agar well diffusion test technique.
- Gold-Silver Nano-alloy using pure strain of *Lyngbya majuscula* demonstrated by **Roychoudhury et al., 2016a.**

  Pure strain of *Lyngbya majuscula* was exposed to equimolar conc. of Au (III) and Ag (I) solution (1 mM, pH 4) for 72 hours. Nanoparticle was extracted from the algal biomass by sonication for 30 minutes at 60 % amplitude with 7.5 mM sodium citrate solution followed by centrifugation. Thereafter UV–Vis spectrum of the nanoparticle extract was recorded from 200nm to 1100 nm. The extract was further characterized by XRD analysis, SEM and FTIR.
- Silver nanoparticles derived from *Oscillatoria limnetica* **(Hamouda et al. 2019)**

Oscillatoria limnetica biomass was used by the authors for biosynthesis of Ag NPs. The synthesized AgNPs were characterized by UV-Vis Spectroscopy, FTIR, SEM and TEM. Furthermore the synthesized AgNPs were tested against human pathogens for their antimicrobial activity as well as hemolytic and cytotoxic effects against human breast cell line (MCF-7) and human colon cancer (HCT-116).

Results and Discussion:

- AgNP biosynthesis by ***Caulerpa racemosa***(**Kathiraven et al.,2015):**

  Silver nanoparticles synthesized by *C. racemosa* after incubation with $AgNO_3$ were first characterized using UV-VIS spectroscopy. The synthesized AgNPs exhibit a yellow-brown color. The UV-VIS absorbance spectra obtained is demonstrated in figure 1:

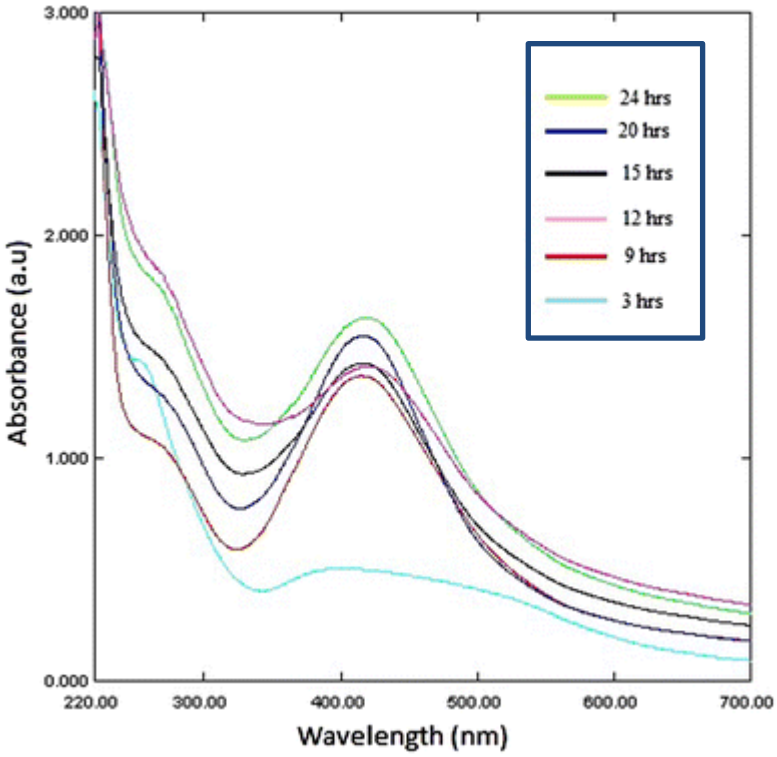

Figure 1: UV Vis spectra of the AgNP solutions incubated with *C. racemosa* as a function of time of reaction. (**Kathiraven et al.,2015)**

FT-IR spectra for *C. racemosa* extract and that of synthesized AgNPs were recorded so as to identify the possible biomolecules connected with the reduction of $AgNO_3$ into AgNPs. FT-IR spectrum showed peaks at 3416, 2924, 2854, 1631, 1389, 1061, 1019 and 660 cm^{-1} (Figure 2).

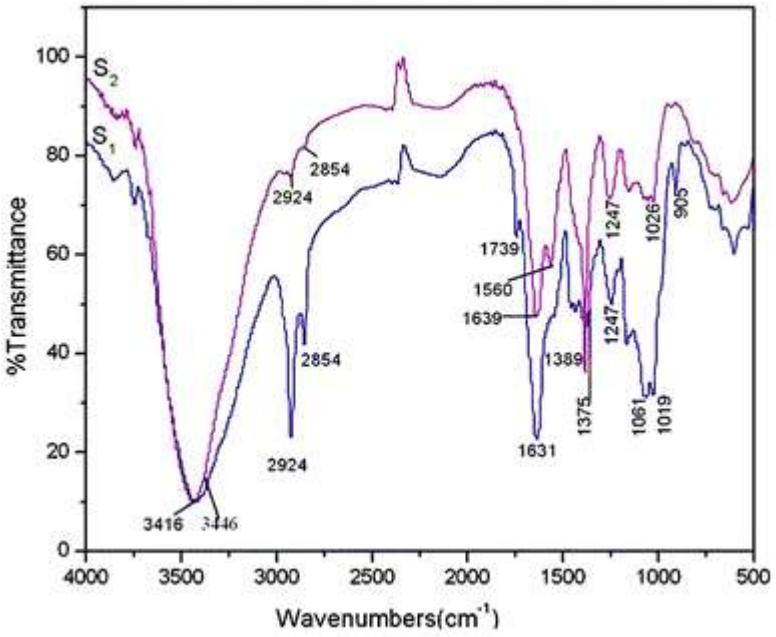

Figure 2: FTIR spectra of S1-*C. racemosa* extract and S2- biosynthesized silver nanoparticles using *Caulerpa racemosa* (modified from **Kathiraven et al.,2015)**

In S1 the peak at 3416 cm^{-1} could be due to O–H group of polyphenols or proteins or polysaccharides, the peak at 1631 cm^{-1} can be due to the stretching vibration of the (NH)=O group. . The FT-IR spectrum of the AgNPs showed the presence of peaks at 3440 and 1639 cm^{-1}. These are associated to OH–stretching vibrations and stretching vibration of the (NH)=O group. Peptides may play a role in the reduction of $AgNO_3$ into AgNPs and thus shifting of the band from 1631 to 1639 cm^{-1}.

XRD pattern of AgNPs was observed. It was compared with the standard powder diffraction card JCPDS. Diffraction peaks of AgNPs were clearly observed at 38.24°, and 44.42°, 64.44° and 77.40° matching with earlier reports on AgNPs .

The TEM analysis revealed spherical with a few triangular nanoparticles with size of 10 nm (Figure 3).

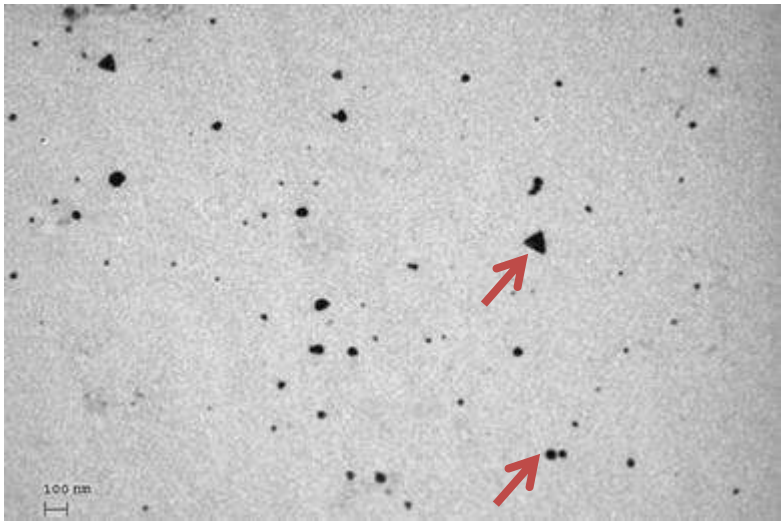

Figure 3: TEM image of silver nanoparticles synthesized using *C. racemosa*

(Kathiraven et al.,2015)

Anibacterial activity against *P. mirabilis* and *S. aureus* showed that in *P. mirabilis (*14 mm for 15 µl) maximum level activity was seen and minimum level antibacterial activity was present in *S. aureus* (7 mm for 5 µl). Thus it can be concluded that *C. racemosa* is capable of synthesizing AgNPs and these nanoparticles can be used as antibacterial agents against pathogens like *P. mirabilis* and *S. aureus.*

- Gold-Silver Nano-alloy using pure strain of *Lyngbya majuscula* demonstrated by **Roychoudhury et al., 2016a (Figure 4).**

  After exposure to $AuCl_4$ and $AgNO_3$, the filaments turned brownish after 24 hours and the sheath turned dark pink in color after 72 hours. The typical coloration pointed towards Au-Ag alloy formation at 24h which completed by 72h. The UV-Vis spectrum also proved the same. The XRD analysis showed the 2θ values or Bragg reflections at 38.2°, 44.5°, 64.8°and 77.8°. The peaks of individual monometallic nanoparticles could not be distinguished and as a result it can be concluded that Au–Ag bimetallic nanoparticles were formed. The dimensions of the nano-alloy were determined by SEM and HR-TEM (Figure 4) which showed that particle size ranged

from 5 to 25 nm. Shape was either spherical or irregular. FTIR analysis showed N-H, C=C, C–O, C=O stretching. The nanoalloy FTIR spectra showed N-H stretching matching primary amine, methylene vibrations, C–O stretching and C=C stretching (Figure 4). It can be concluded that *Lyngbya majuscula* can be used for synthesis of Au-Ag nano-alloy which can then be used in different biotechnological applications.

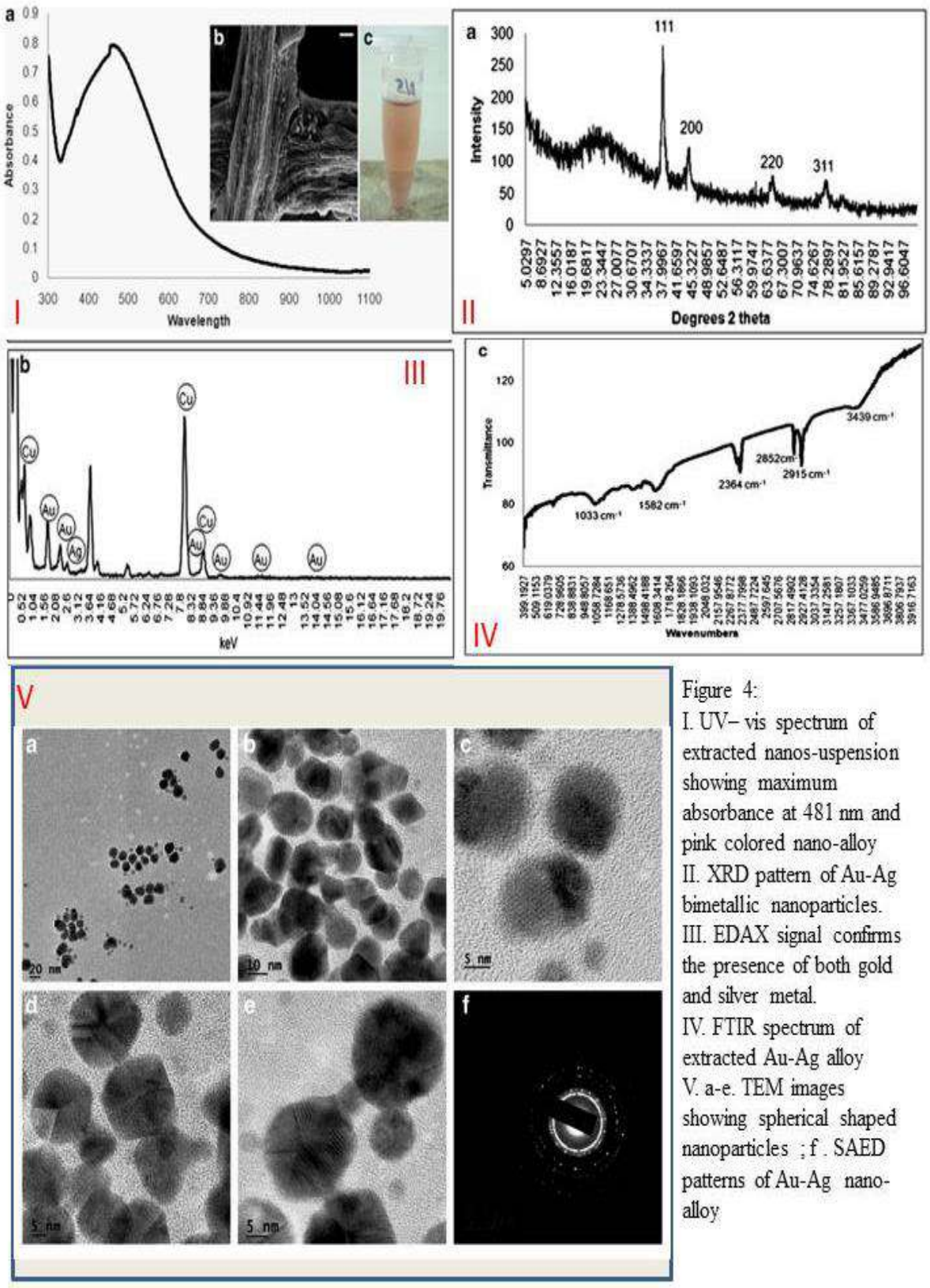

Figure 4:
I. UV– vis spectrum of extracted nanos-uspension showing maximum absorbance at 481 nm and pink colored nano-alloy
II. XRD pattern of Au-Ag bimetallic nanoparticles.
III. EDAX signal confirms the presence of both gold and silver metal.
IV. FTIR spectrum of extracted Au-Ag alloy
V. a-e. TEM images showing spherical shaped nanoparticles ; f . SAED patterns of Au-Ag nano-alloy

Figure 4: I. Figure reference- Roychoudhury et al., 2016a

- Silver nanoparticles derived from *Oscillatoria limnetica* **(Hamouda et al. 2019)**

  The color of nanoparticle extract changed from green to brown implying the biotransformation of Ag^{+} ion to Ag^{0}. This proved the synthesis of silver nanoparticles. The UV-Vis spectrum showed a surface plasmon peak at 426nm (Figure 5).

  TEM (Figure 5) illustrated the quasi-spherical morphology of the bio-fabricated AgNPs. SEM micrographs illustrated roughness of the synthesized nanoparticles. The FTIR spectrum showed several peaks corresponding to O-H stretching, N-H stretching vibrations, C-H stretching vibrations, N-H bending vibrations etc.

  The peak between the range 1648–1621cm^{-1} could be due to amides (N-H) stretching along with peptide bond and C=C stretching vibrations related to stabilizing nanoparticles by proteins. The nanoparticles synthesized also showed antimicrobial activities against *E. coli* as well as *B. cereus* which was observed by disc-diffusion method (Figure 6). Inhibitory zones were profound against both the pathogens. The inhibition zone around disc was found to be directly proportional with concentration of AgNPs and can be considered as a function of toxicity. The AgNPs showed hemolytic activity. At low concentrations Ag^{+}induced hemolysis in RBC cells. AgNPs showed cytotoxic effect against MCF-7 and HCT-116 cell lines. Cytotoxic effect was achieved at 6.147 µg/ml concentration against MCF-7 and 5.369 µg/ml for HCT-116 cell lines. It can be thus concluded that *Oscillatoria limnetica* can be made to biosynthesize AgNPs by reduction and stabilization of the NPs. Furthermore these nanoparticles can be used for their bactericidal and hemolytic properties as well as cytotoxic effects against cancer (MCF-7 and HCT-116) cell lines.

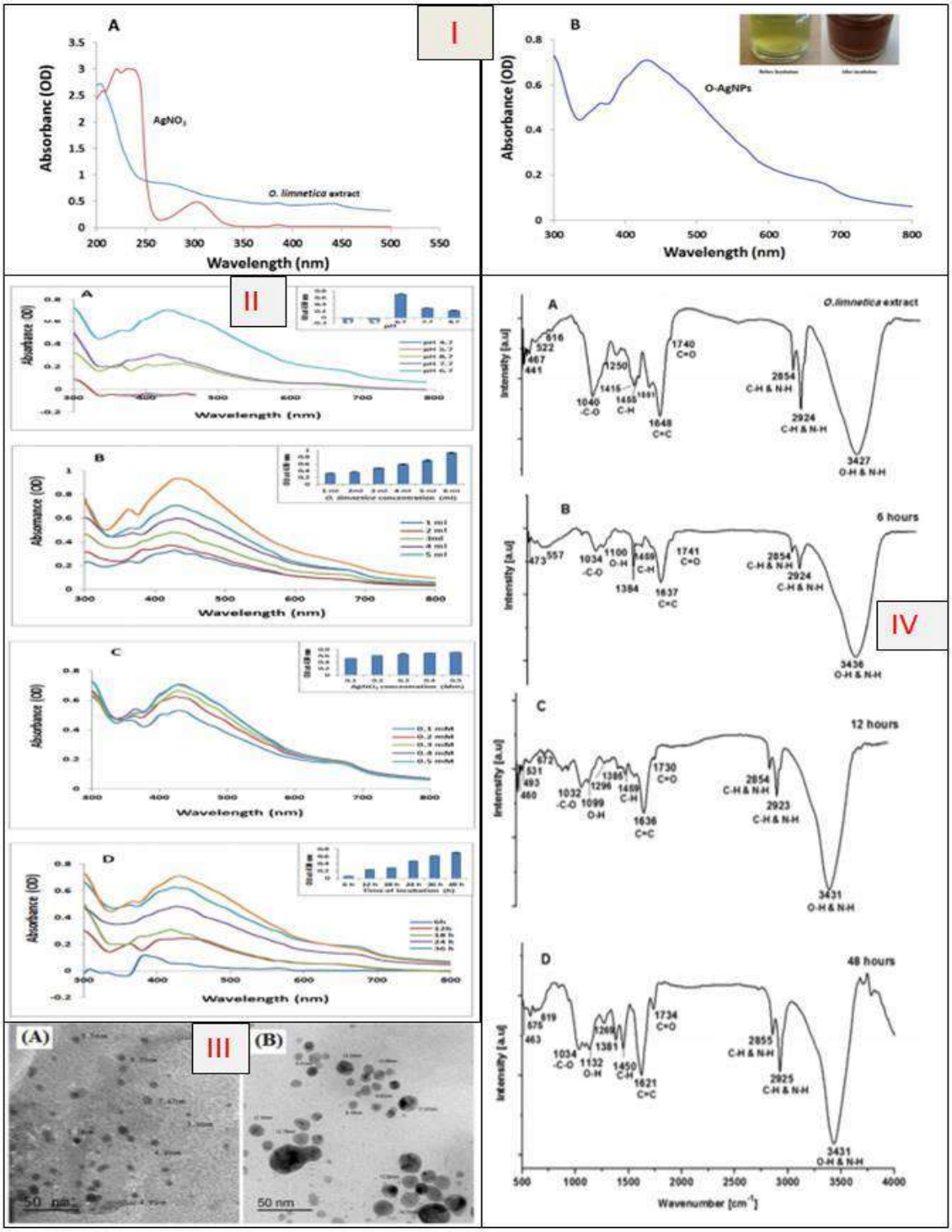

Figure 5: I. UV-Vis spectrum of *O. limnetica* extract & AgNO3 (A) and AgNPs(B), II. UV-Vis spectra of AgNPs biosynthesized at various pH values, III. TEM micrographs of AgNPs biosynthesized, IV. FTIR spectra of *O. limnetica* extract and O-AgNPs(**Hamouda et al. 2019)**

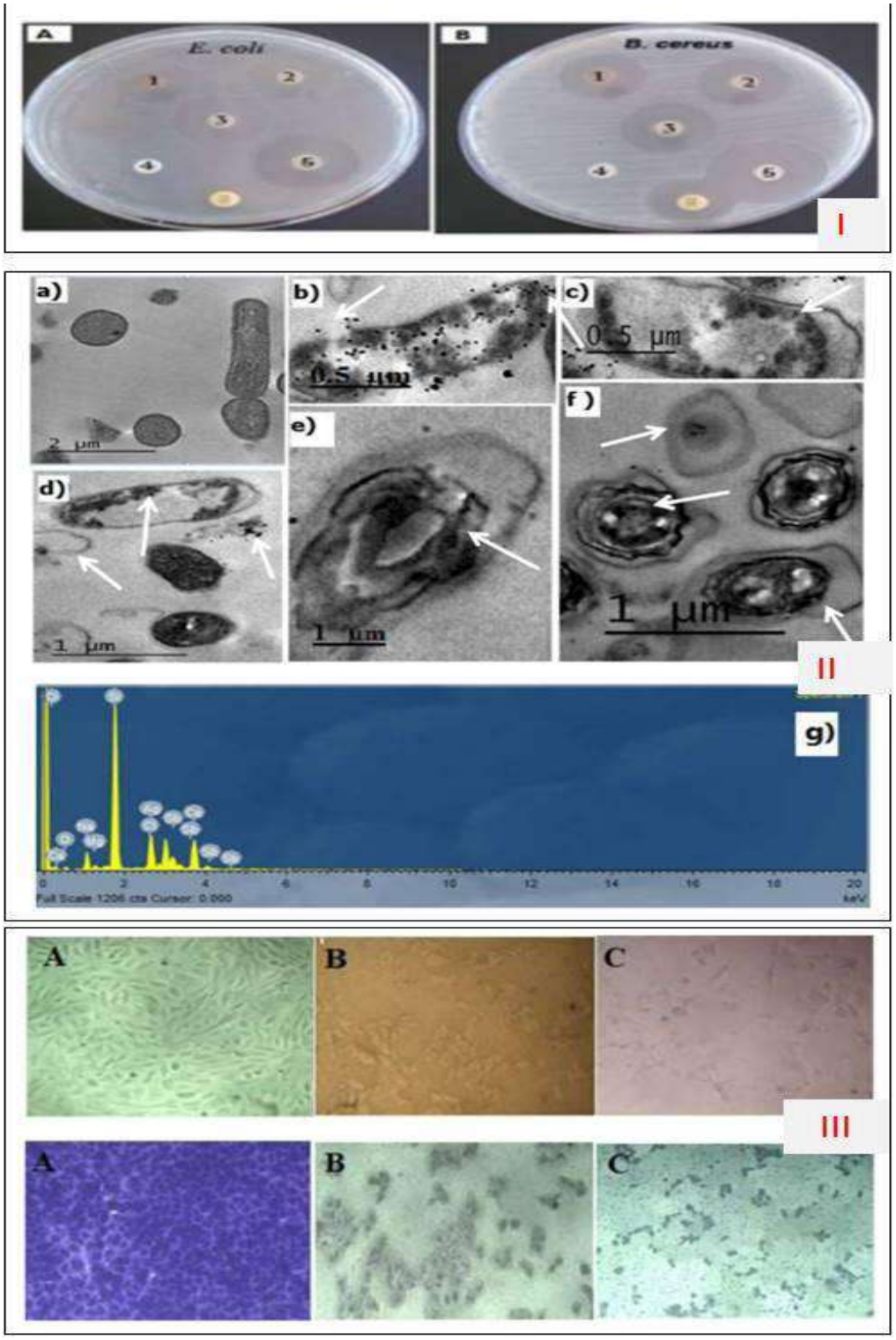

Figure 6. I. Antibacterial activity (zone of inhibition) of O-AgNPs against two human pathogenic bacteria *E. coli* (A) and *B. cereus*(B), II. TEM morphological changes of *B. cereus* as cell structure with or without O-AgNPs. (a)Structure of intact cell (control), (b) damaged cell membrane and releasing of cytoplasm (g) EDX spectrum of *B. cereus* cells with AgNPs, III.Effect of the *O. limnetica* extract (A), O-AgNPs (B) as well as the silver nitrate alone (C) on MCF-7 cells (concentration of 120 µg/ml) (Hamouda et al. 2019).

Conclusion:

From all the above-mentioned methods and their subsequent observations, it can be concluded that how important green nanotechnology plays a role in production of Silver nanoparticles (SNPs). Not only are the processes eco-friendly and low priced over the physical and chemical techniques, but also labor friendly and not that time consuming. The bio hazards are a lot less than other means. Green technology, as a branch of nanotechnology, contributes as one of the significant ways of nano-products and nanomaterials production promoting a sustainable environment. They have huge application in almost all fields starting from drugs, cancer therapy, antimicrobials, optics, drug delivery, etc. (Figure 7). Cancer therapy and anti-leukaemic therapies have become a milestone for life-saving applications of AgNPs. Green nanotechnology is enlightening us towards a sustainable future, serving a great alternative in recent days, via curbing the use of our chemical sources over the green and environment friendly ones. The results we have obtained in various fields like medicine have been really impressive and very soon we may totally depend on the algal origin for nanoparticles synthesis.

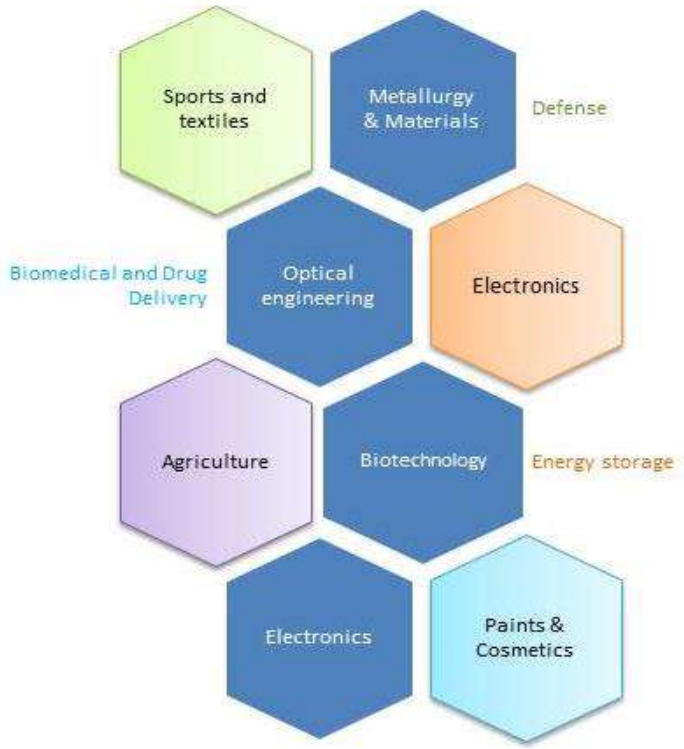

Figure 7: The various applications of nanoparticles.

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Introduction

Global warming has elevated global temperatures at an alarming rate. Concentration of the most important GreenHouse Gas(GHG) i.e, CO_2 has reached formidable levels.(**Dhar et al.,2019**). For human beings a critical issue in the form of global warming has been raised and it has been seen that the temperature of earth rose by around 0.85^{o} C from 1880 to 2012 (**De Silva et al,2015**). This in turn has caused changes in phytoplankton populations, rise in sea level and abnormal weather conditions. To control high levels of CO_2 it needs to be captured and trapped which is done by CO_2 sequestration. Carbon sequestration or carbon-dioxide removal is the long term removal or sequestration of carbon-dioxide from the atmosphere to lessen global warming or slow down atmospheric carbon-dioxide pollution (**Sengupta et al.,2017**). Sequestration of atmospheric carbon-dioxide by algae has its own benefits that can be emphasized by the fact that the process is cost-effective along with the capability of algae to use CO_2 as carbon(C) source (**Anguselvi et al.,2019**).

Sequestration of the CO_2 using algae is the biological method of Carbon sequestration (**Kumar et al.,2011**). There are also chemical as well as technological methods and all of these involve two types of strategies: adaptive and mitigative (**Lal et al.,2008**). Associated benefits with this process are that extraction of algal biomass can yield amino acid rich feed, fatty acids, Vitamin A and different types of dietary supplements (**Ramanan et al.,2010**). Increase in concentration of CO_2 in the atmosphere is contributed by the usage of fossil fuels. Dependency on fossil fuels or their demand can be reduced by using algal biofuel as an alternative (**Anguselvi et al.,2019**). Around 54.9-67.7 tonnes of carbon-dioxide can be annually sequestered from raceway ponds (**Kumar et al.,2011**). Algal species such as *Spirogyra, Oscillatoria, Oedogonium* can be used

for CO_2 removal (**Anguselvi et al.,2019**). Algae cultivation for CO_2 sequestration can be both in open as well as closed systems (**Kumar et al.,2011**). Thus these algae associated mitigation methods for Carbon-dioxide can provide a solution to problems related with global warming. Fixation of CO_2 is linked to cell density and light utilization efficiency of microalgae. (**Chiu et al.,2008**)

Algae and cyanobacteria are chosen for sequestration studies since carbon concentrating mechanisms play a role in enhancement of higher growth rates for these organisms (**Ramanan et al.,2010**). They have the ability to convert solar energy to fuels with higher photosynthetic efficiencies than the terrestrial feed stocks (**Beer et al.,2009**). Atmospheric CO_2 content is mainly contributed by electricity generating, cement manufacturing as well as iron manufacturing industries accompanied with common practice of burning of municipal solid wastes **(Kumar et al.,2011**). According to the report of Carbon-dioxide Analysis Center(CDIAC), CO_2 emissions have increased from 3 metric tons in 1751 to 8230 metric tons in 2006 (**Kumar et al., 2011**). Sequestration of carbon is not only the prime reason for dramatic reduction of CO_2 from the atmosphere but also possesses an important aspect of assisting in maintenance of the natural carbon cycle.

Algal biomass has the potential to generate first, second, third and fourth generation biofuels (**Chakrabarti et al.,2014**). The production of biofuel is considered carbon neutral biofuel as the Carbon-dioxide produced from the combustion of biomass can again be used by microalgae in culture during its growth phase. Open raceway ponds as well as bioreactors can serve as tools for algal biomass cultivation. Along with biofuels, biogas can also be produced by microalgae by anaerobic digestion (**Moreira and Pires,2016**). Advantage of microalgal biofuel system is that it is independent of soil fertility and thus pressure is not exerted on arable land or forest ecosystems (**Stephens et al.,2010**).

CO_2 sequestration process is affected by various factors. The factors are listed below:

1. **Temperature-** Possibility of CO_2 sequestration from flue gas depends on installation of a heat exchanger system or use of thermophilic species. Flue gas emitted from power plants has a temperature around 120^oC (**Kumar et al.,2011**).

2. **pH-** CO_2 and SOx from flue gas influences pH of culture medium. There has been a report of a drop of pH to 2.6 with higher SOx concentrations. pH drops down to 5 with elevated CO_2 concentrations (**Westerhoff et al.,2010; Kumar et al.,2011**)

3. **NOx and SOx** – Growth of microalgae is influenced by NOx and SOx and tolerance to them varies widely among species (**Kumar et al.,2011**).

4. **Light**- Optimum light intensity is required for CO_2 fixation. Below that level, light becomes a limiting factor (**Kumar et al.,2011**). Exposure of cells to long periods with high intensity results in photoinhibition due to damage of repair mechanisms and leads to inactivation of other systems. (**Rubio et al.,2003**).

5**. Proper mixing**- This helps both in uniform mixing of nutrients and better distribution of light over cells, minimizing the Io and taking advantage of the flashing light effect.

Productivity is increased to 40% in a tubular bioreactor with the help of the flashing light effect **(Ugwu et al.,2002; Kumar et al.,2011**).

6. **Culture density**- It has two functions- productivity and light utilization efficiency . For efficient CO2 sequestration, selection of optimal cell concentration is required. When concentration is below the optimal, not all light energy is captured by cells. When above, a large proportion of cells is in dark due to leaf shading (**Zhang et al.,2001; Kumar et al.,2011**).

7. **CO_2 concentration**- HCO_3^- is easily absorbed by cells but is poor source of carbon in comparison to CO_2 (**Carvalho et al., 2006**),

8. **Culture strain**- CO_2 mitigation involves a very important aspect of culture strain selection (**Kumar et al.,2011**). It has been reported that *Scenedesmus* is appropriate for mitigation due to its high biomass productivity and ability of carbon fixation (**Yoo et al.,2010**).

9. **CO_2 mass transfer**- A characteristic of the bioreactor that determines its capability to sustain optimum cell growth is the volumetric mass transfer coefficient (K_La) (**Kumar et al.,2011**). A conclusion was obtained from a comparison of different types of photobioreactors that decreasing K_La value results in increase of CO_2 concentration. (**Zhang et al.,2002**).

In this review, we are going to discuss different takes on CO_2 sequestration using algae and cyanobacteria by different groups of scientists.

Methods and Methodologies

Microalgae contain about 50% carbon by dry weight. This carbon is fixed from atmospheric CO_2.A 100 t of algal biomass fixes around 183 t of carbon dioxide. This biomass can be used for biofuel production. For this purpose large scale production of biomass is required which in turn can sequester enough CO_2 and can be used for biofuel production (**Chisti ,2007)**

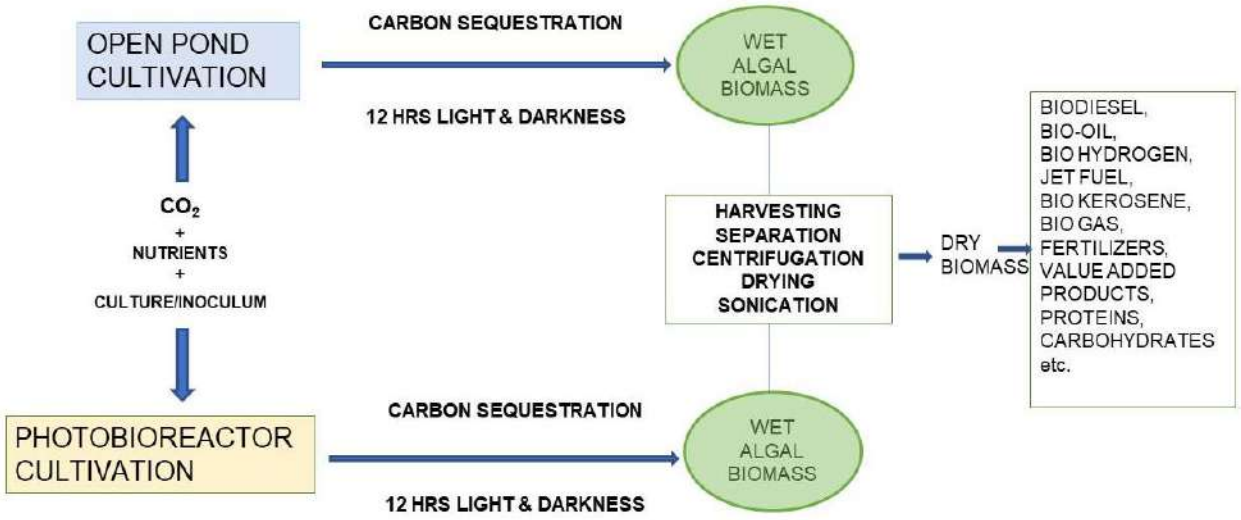

Fig1: Diagram showing the total scheme of carbon sequestration by algal biomass

(figure modified from the scheme presented by **Eloka-Eboka and Inambao**., 2017)

Raceway ponds and photobioreactors are the two practicable methods for large scale production of microalgae for CO_2 sequestration (**Chisti ,2007;Chakrabarti et al.,2014;**).(figure 2).

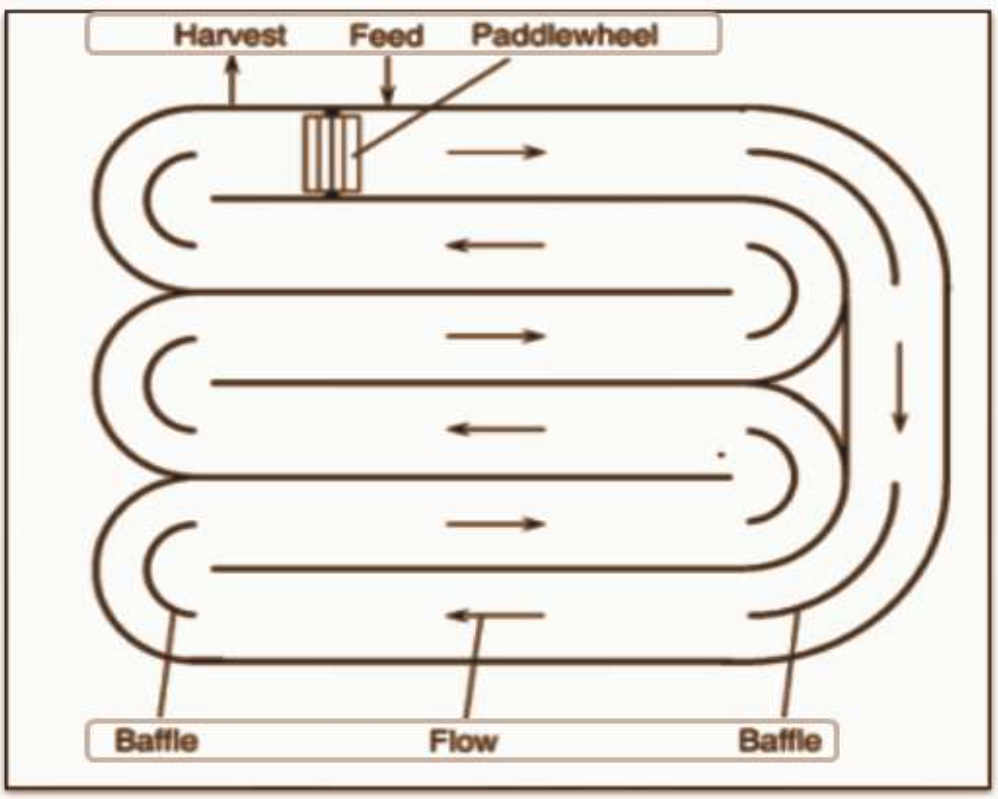

Fig.2. Top view of Raceway pond (modified from **Chisti,2007)**

A closed loop, oval-shaped reticulation channel makes up the raceway (**Chakrabarti et al.,2014;** figure 3). The channels are between 0.2-0.5m deep and require mixing and circulation for stabilization of growth and productivity **(Brennan and Owende,2010)**. These channels are built in concrete and even compacted earth (**Chisti,2007**). Paddlewheels help in mixing and circulation (**Brennan and Owende,2010).** Algal broth and nutrients are fed continuously in front of the paddlewheel that are circulated through the loop to the harvest extraction point. Sedimentation is prevented by continuous operation of paddlewheels (**Brennan and Owende,2010; Chisti ,2007)**.

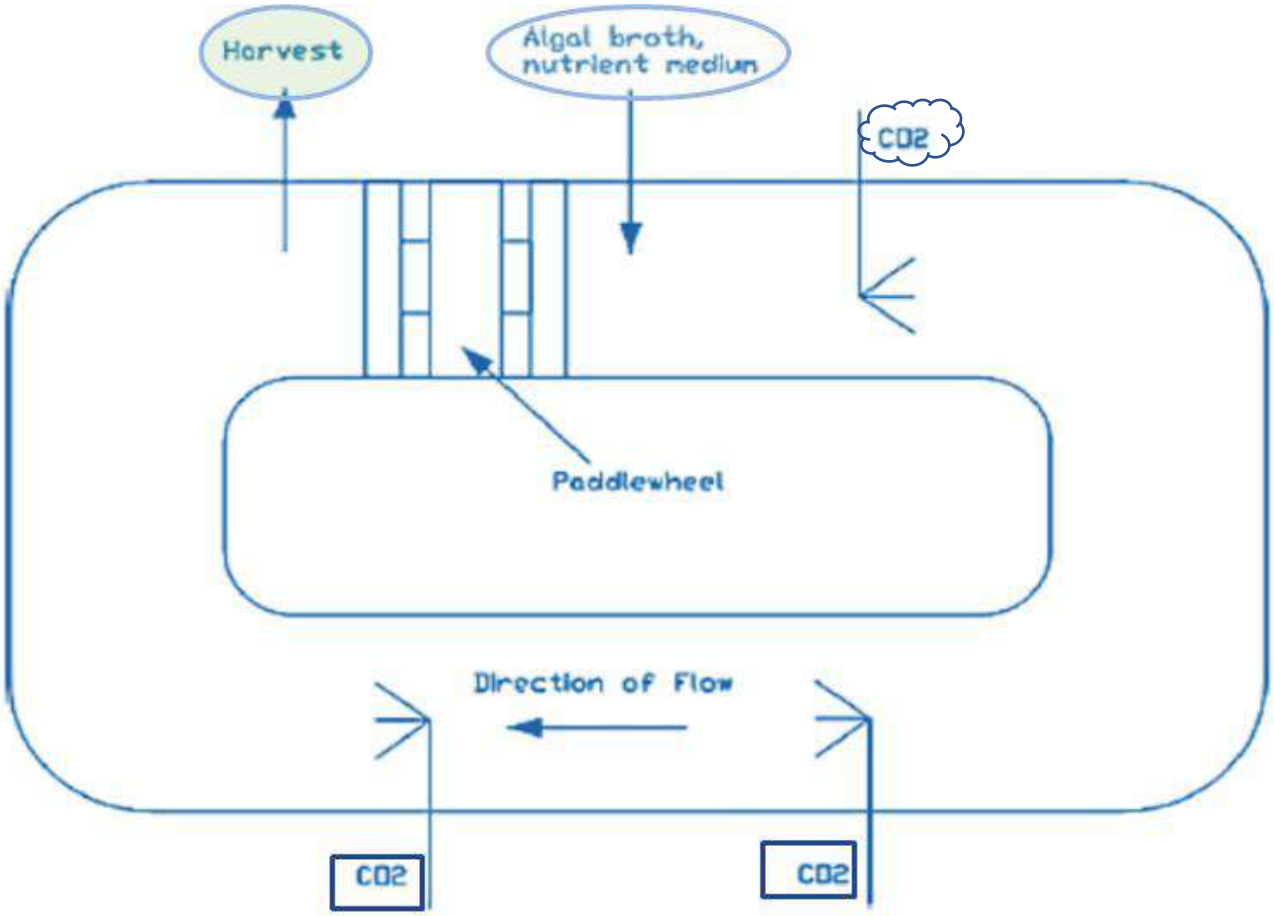

Fig 3: Diagram showing a raceway pond consisting of closed loop, oval-shaped circulation channels (figure modified from **Chakrabarti et al.,2014**)

In comparison to photobioreactors, raceways are less expensive. In the raceway method, cooling is achieved only by evaporation and evaporative loss may be significant. Fluctuation of temperature occurs within a diurnal cycle and seasonally. This allows the algae or cyanobacteria to grow like it would in its natural habitat. This also allows maximal capture of CO_2 naturally (**Chisti,2007**).

A further example of raceway ponds being used for Carbon sequestration involves use of green algal samples like *Scenenedesmus*, *Dunaliella*, *Chlorella vulgaris* . that are used as inoculum for mass cultivation and subsequent carbon fixation within the biomass. (**Eloka-Eboka and Inambao, 2017)**).

Another mass cultivation unit for carbon sequestration involves the open pond tanks. Open pond tanks made of thermos plastic are used with 8.5L of broth agar (**Eloka-Eboka and Inambao, 2017)**. In these types, vigorous and continuous stirring is done for homogeneity. Moreover to mix the culture, pressurized PE hoses are used daily throughout the entire period of growth. Growth of the system is allowed from inoculation until 16(log phase) up to 20 days (lag phase). Accumulated biomass during the growth period is then monitored for absorption rate, optical density, pH, salinity, sequestered CO_2, total and dissolved organic/inorganic carbons(TIC,TOC) at different periods (**Eloka-Eboka and Inambao, 2017**; figure 4**).**

Fig 4: Diagram showing open pond tanks made of thermos-plastic material (**Eloka-Eboka and Inambao, 2017)**

A number of photobioreactors which can be used for carbon sequestration such as the Bubble column photobioreactor, Airlift photobioreactor, Flat panel bioreactor, Horizontal tubular bioreactor etc. are available. (Ugwu **et al., 2008; Kumar et al., 2011**).

In one study conducted, factors monitored in photobioreactor based algal cultivation are (i) pH and fatty acid configuration, both of which have been analyzed with respect to daily growth, (ii) cell density and (iii) total organic/inorganic/dissolved carbon that has been sequestrated (**Eloka Eboka and Onunka ,2016**).

The horizontal tubular photobioreactor has a tubular array of straight glass or plastic tubes that make up the photobioreactors. (**Chakrabarti et al.,2014**; figure 5). The design is such that it helps capture sunlight maximally, favors constant growth of cells and minimizes contamination. A tubular photobioreactor, contains an array of plastic or glass made transparent tubes. Sunlight is captured in the tubular array that are generally 0.1m or less in diameter. To ensure a high biomass productivity light need not penetrate too deeply in the culture broth and therefore tube diameter is limited (**Chisti.,2007**).

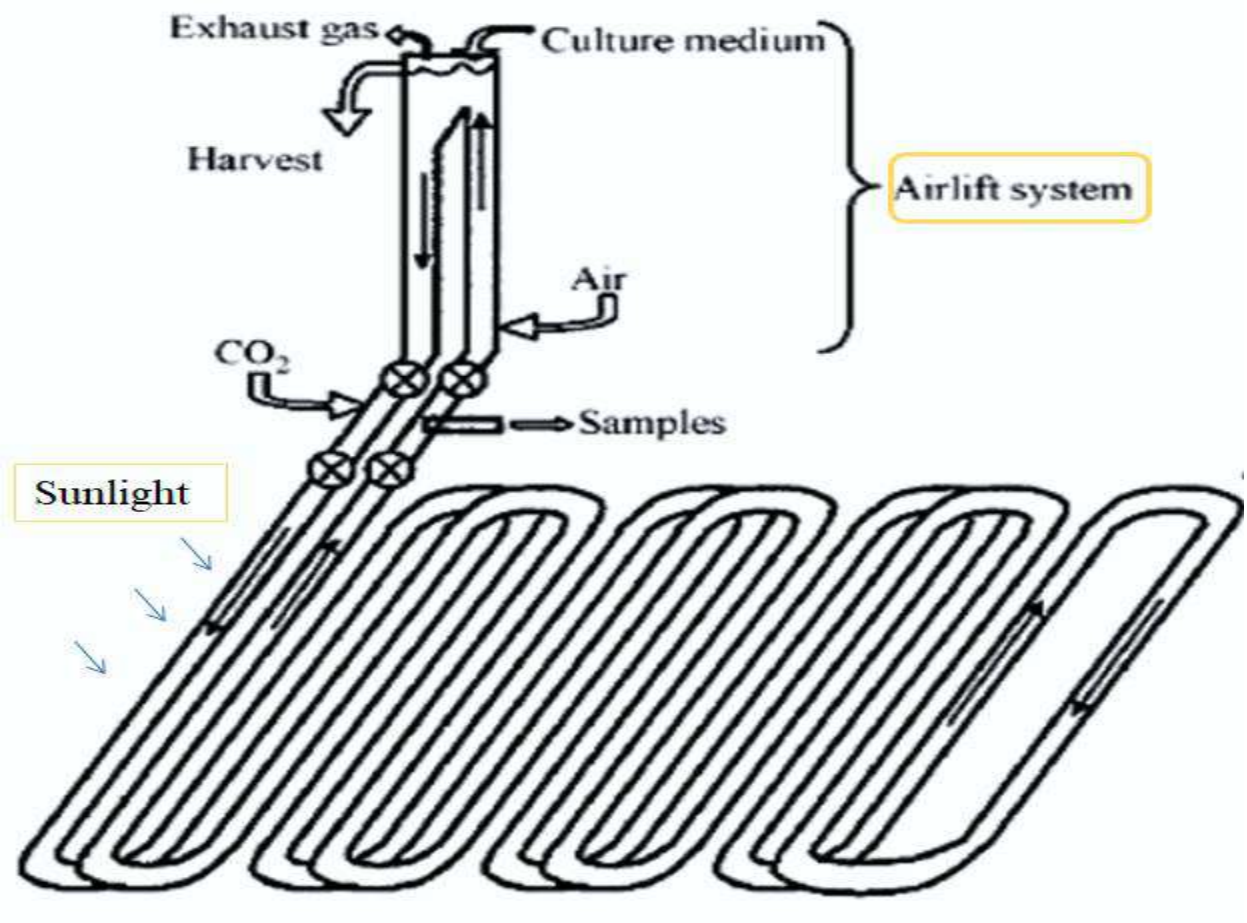

Fig 5: Design of a typical horizontal tubular photobioreactor (figure modified from **Chakrabarti et al., 2014).**

Another type of study has been conducted By **Anguselvi et al., 2019** using the flat panel photobioreactor. It has been used for experimentation with freshwater cyanobacterial and algal species of *Oscillatoria* and *Spirogyra* collected prior from ponds in the coal mining area of Dhanbad, Jharkhand, India. Of these,blue-green microalgae *Oscillatoria* has been used for CO_2 capture study. BG11 medium has been used for the culture. In 500 ml Erlenmeyer flask with 200 ml of BG11 medium, they have been pre cultured aseptically and the inoculum has reached an exponential growth phase after pre-cultivation. Collection of a gram of algal cells has been done using centrifugation. After washing with distilled water they have been inoculated in growth media. Optimization of

parameters such as pH, inoculum concentration, nutrient media have been done. Analysis of exhaust gas from the natural gas processing industry etc has been done followed by inoculation of selected species of microalgae in a bioreactor to study CO_2 capture. CO_2 has been injected at a rate of 500ml/30 minutes continuously for 48 hours.

After culture**, the** dried biomass can be obtained by draining, centrifugation and drying them after their growth. (**Eloka-Eboka and Inambao,2017**). For total lipid extraction, biomass is subjected to solvent extraction whereas for biodiesel production it is subjected to non-polar solvent. (**Eloka-Eboka and Inambao,2017**). The biomass can then be used for production of biodiesel, bio gas, bio ethanol, bio-butanol etc. (**Singh and Dhar, 2019**).

Results and Discussion

- Chisti, 2007 has demonstrated how different types of mass cultivation units can serve for CO_2 capture. He has concluded that low cost biodiesel production requires improvement of algal biology and engineering. He has also pointed out that tubular photobioreactors are more efficient than raceway ponds in terms of carbon capture.
- Chakrabarti et al., 2014 has shown designs of different types of mass cultivation units. A number of cyanobacterial and algal species have emerged as potential strains for Carbon capture. Some of them are enlisted below (Table 1).

Table 1: Overview of recent studies on microalgae for CO_2 sequestration (Chakrabarti et al., 2014)

Sr. No.	Microalgae	CO_2 fixation rate ($g^{-1}L^{-1}d^{-1}$)
1	*Scenedesmus dimorphus*	1.27
2	*Chroococcus cohaerens*	0.78
3	*Chlorella sp.*	1.38-1.62
4	*Spirulina platensis*	0.92
5	*Dunaliella tertiolecta*	0.27
6	*Haematococcus pluvialis*	0.14

Spirulina sp , Chlorella sp., Haematococcus pluvialis, Scenedesmus obliquus, Scenedesmus dimorphus etc. have been recognized as potential microalgal strains for assimilation of carbon dioxide.

- Eloka- Eboka and Onunka 2016 (figure 6) have used *Chlorella vulgaris(BA1), Scenedesmus obliquus (BA2). Synechococcus spp(BA4)* and *Duneliella spp(BA3)* and have produced biodiesel using open pond cultivation and photo-bioreactor model BF-115 Bioflo/celli Gen. The biodiesel produced has been mixed with conventional fuel (AGO) and it has been deduced that the mixture of biodiesel with AGO proved better for engine's performance. Microalgae derived biodiesel is oxygenated, hence allows full combustion. The study has proved that microalgae based fuels are feasible and can change the biodiesel industry.

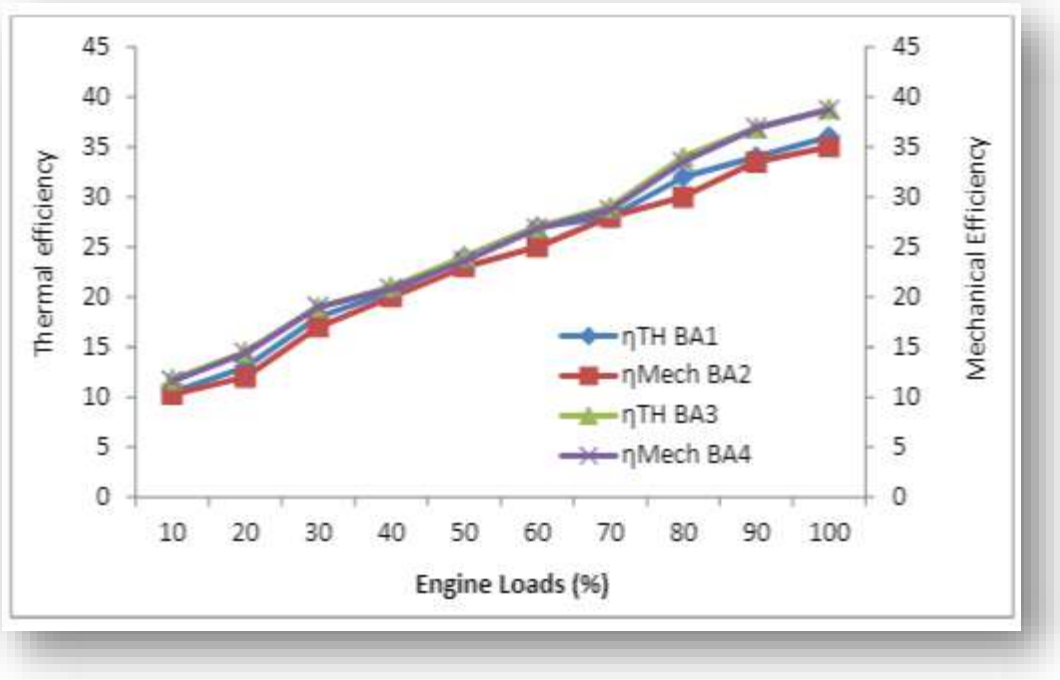

Figure 6: Graph showing effects of engine loads on thermal and mechanical efficiencies (Eloka- Eboka and Onunka, 2016).

- Eloka- Eboka and Inambao, 2017 (figure7,8) have studied *Chlorella vulgaris*, *Dunaliella*, *Scenedesmus quadricauda* and *Synechococcus* spp for their carbon dioxide sequestration capacities. It has been seen that the maximum rate at which carbon (IV) oxide has been removed, r_{max} ranged from 11.73 mg L^{-1} min^{-1} to 18.84 mg L^{-1} min^{-1} from *Chlorella vulgaris* to *Synechoccocus* spp. The ratios of rate of CO_2 absorption constant to the constant for the CO_2 desorption rate i.e. k1/k2 is highest in *Dunaliella*.

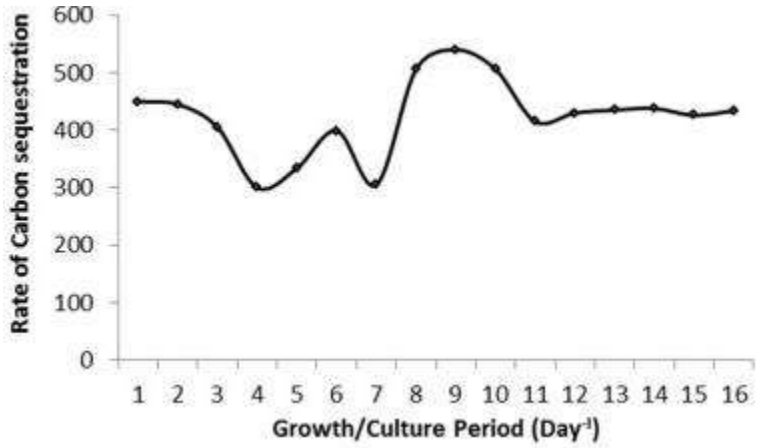

Figure 7: Rate of Carbon sequestered during algae cultivation

Moreover excellent quality MUFAs have been synthesized by all four strains. These can act as great precursors to biodiesel. They have also concluded that CO_2 is the force behind maximal biomass production and lipids in algal production.

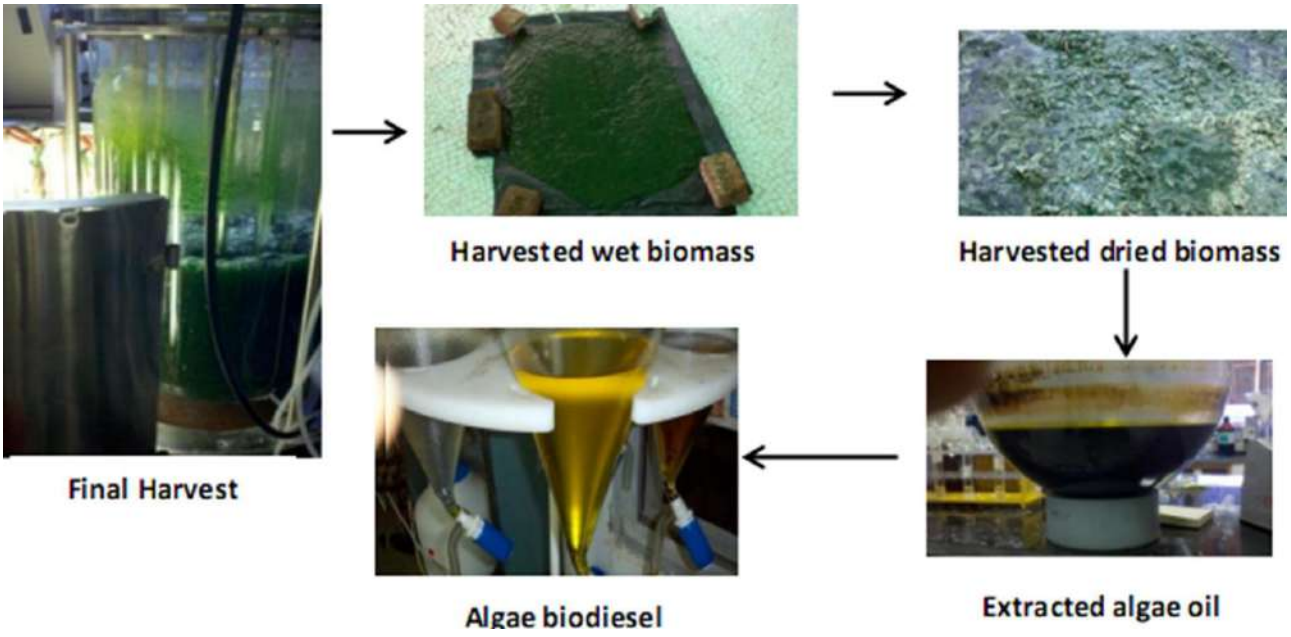

Figure 8: Figure showing biomass sent for processing into biodiesel (Eloka- Eboka and Inambao, 2017)

- **Anguselvi et al., 2019** have used *Oscillatoria* cultures to obtain high carbon dioxide capture capacities. They have used Photo-bioreactor FMT 150 and modified culture medium with high Fe, Mg, vitamins, and surfactants to cultivate it. Highest CO_2 capture has been observed in *Oscillatoria* between 16 to 32 h. After carbon capture, biomass has been used to extract products like amino acid rich feed, algal oil, algal pellets etc.

Conclusion

In the end it can be concluded that carbon-dioxide sequestration by algae is an effective way to lessen the pollution caused by carbon-dioxide. In the current situation, it is of utmost importance to keep a check on the level of harmful CO_2 in the atmosphere. Microalgae based carbon-dioxide removal has gained popularity over the years and this is justifiable only because of the advantages that the method provides over others. Not only rapid growth and richness of various constituents in microalgae, it is also the value-added products (such as PUFA , beta-carotene etc.), that add to its popularity. Biodiesel obtained as a product of this method holds the capacity to compete and displace fossil biodiesels which is very much necessary if one wishes to reduce the CO_2 pollution.

However the method needs improvement in terms of production management, harvesting, extraction of the products as well as utilization and refinement of the residues. Along with the ongoing projects more and more experiments and works related to this topic are required in future to manage the problems associated with the method. If the hurdles can be overcome then microalgal biodiesel surely has the potential to be an alternative for fossil biodiesel.

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Title: Diversity of Green Algae from Sundarban, West Bengal - A Review

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INTRODUCTION

The Chlorophyta have chlorophyll a and b and form starch with the chloroplast, usually in association with a pyrenoid and thus differ from the rest of the eukaryotic algae in forming the storage product in the chloroplast instead of in the cytoplasm. The chloroplast endoplasmic reticulum is absent around the chloroplast (Lee, 2008). Phylum Chlorophyta consist of about 7,000 species, most of which occur in fresh water, although some others are marine as well as terrestrial (Guiry and Guiry, 2021). Apart from chlorophyll a and b; beta-carotene and various characteristic xanthophylls are also present. Food reserves are in the form of starch, some fats or oils like those of higher plants (Lee, 2008).

The different species of Chlorophyta are mostly found in fresh water, brackish water and marine water conditions as well as in terrestrial habitats. The water body housing them might be lentic (stagnant) or lotic (constantly streaming). The lentic climate might be described by a static lakes, more steady lasting pools and pools that serve as regular man-made sources of water. Lotic frameworks include a wide range of streaming water from a little stream to rivers to gigantic waterways. Other than these, any wet surface like wet soil, rocks, tree trunks, dividers of old structures can likewise uphold development of green algae. The algal thallus can range from unicellular forms to multicellular forms which show significant variety in structure (Krishnamurthy, 2000).

In the present study an attempt has been taken to review the floristic work done on unicellular and multicellular green algae (chlorophyta) from Sundarbans of West Bengal.

One of the major spots that have been explored time and again for green algal assemblage is the Sundarbans. The Sundarbans represent the biggest mangrove in the world and contain in excess of 400 islands interconnected with little waterways, brooks and trenches, mud banks or sandy sea shores and ridges along the shoreline of every island. The land is continually formed and adjusted by flowing action of tides along rivers and backwaters, with disintegration along estuaries and ample residue from seawater (Sanyal and Bal, 1986). An assessment of the creation and construction of the mangrove vegetation including algal abundance of the Indian Sundarbans have been made by Mandal and Naskar (1994), Sen et

el. (2003), Mukhopadhyay and Pal (2002) and Chowdhury and Pal (2008). The study of green algal flora from Sundarbans conducted by Satpati et al. (2011, 2012, 2013) and Satpati and Pal (2015, 2016) has been reviewed here. In the studies conducted, a total of 70 green algal taxa were reported.

Materials and Methods:

- The study of green algal flora from Sundarbans conducted by Satpati et al. (2011, 2012, 2013), Satpati and Pal (2015, 2016) has been reviewed here.
- From the study it was observed that the taxonomic samples were collected from Sundarbans (Figure 1) by the authors during four distinctive season's viz. Summer (March-May), Monsoon (June-September), Post monsoon (October-November) and Winter (December-February) from the brackish waters of the fisheries, the bark of mangrove plants, pneumatophores, other airborne root frameworks, wooden and bamboo shafts, mud plain (observed in low tide), on the sides of boat and dispatches, from shells of various aquatic animals and samples were gathered from their normal sources and manmade regions(Satpati et al.,2011, 2012; Satpati and Pal,2013, 2015,2016).
- The phytoplanktons were gathered from the waterways, rivulets and trenches during the floating stages with the assistance of phytoplankton net of 25µm. Test samples were additionally gathered in plastics bottles and were brought to the research facility for additional assessments.
- The taxa collected in this way were completely washed under running faucet water or saline water and afterward with twofold refined water to eliminate soil particles and different materials.
- The samples were then preserved in 4% (v/v) formalin for future examination. The slides were prepared and computerized photos were taken under Carl Zeiss Axiostar microscope using Cannon Power Shot 500D camera.
- Scanning Electron Microscope(SEM) study was conducted on some of the samples. The SEM images were taken under Carl Zeiss EVO 18 (EDS 8100) microscope having Zeiss Inca Penta FETX 3 (Oxford instruments) attachment. The sample was washed with phosphate buffer saline 2-3 times, dried at room temperature with ethanol (30%-90%) for total dehydration. After total dehydration, the samples were put in Quorum (Q 150 TES) gold coater and coated with gold. The microphotographs were taken at different magnification. Thereafter they were identified.

- Identification of taxa was finished by the authors using standard monographs of Prescott (1962); Randhawa (1969); Anand (1998) and Krishnamurthy (2000).

- Their investigation region lies between 21º31' to 22º53'N and 88º37' to 89 º 09'E of the south-eastern piece of Bay of Bengal in the Sundarbans. The examination region covers 14 islands with thick mangrove backwoods and internal island area (Figure 1).

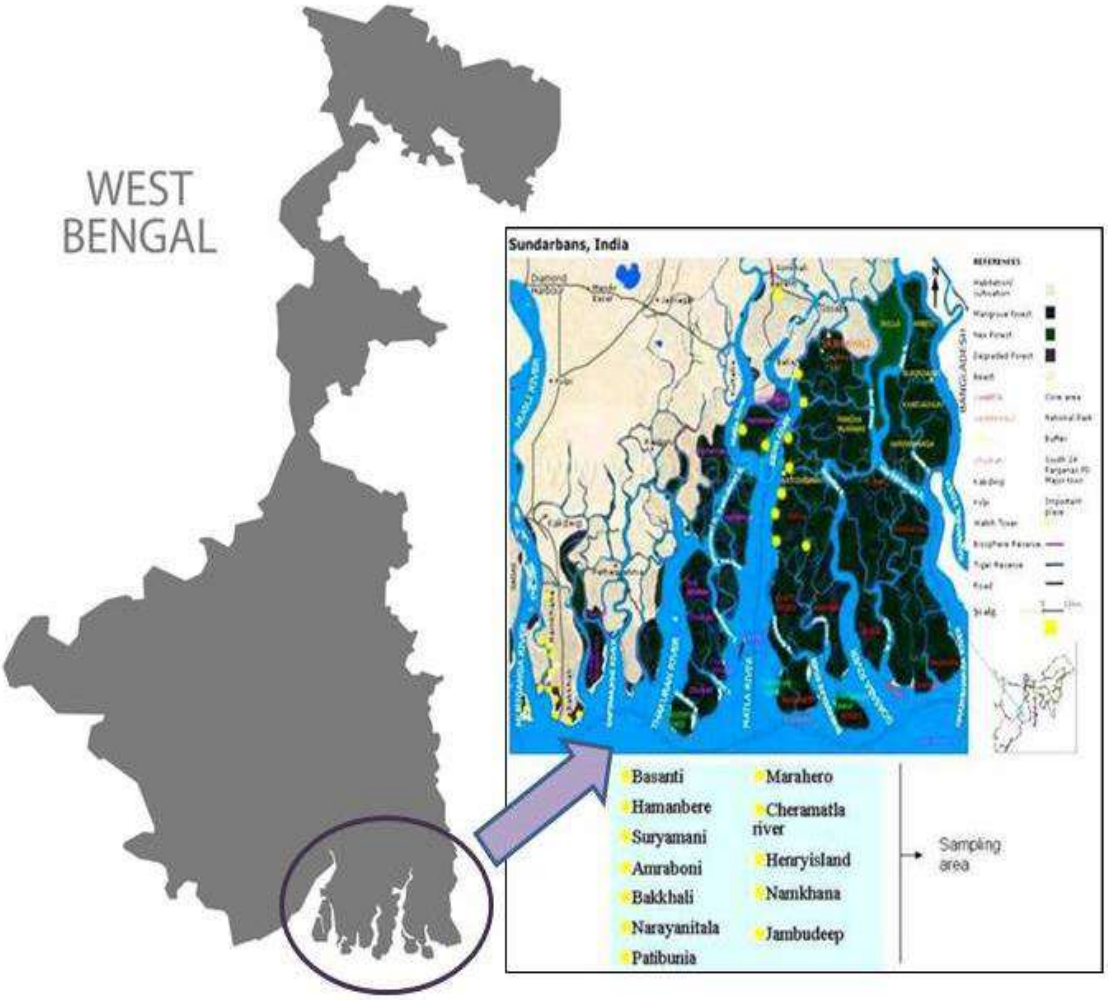

Figure 1: Figure showing algal sampling sites from Indian Sundarbans West Bengal (modified from Satpati et al. 2011, 2012, 2013; Satpati and Pal, 2015, 2016)

Results:

Sundarbans show a dense flora of green algae. The following table enlists the green algae collected by the authors, Satpati et al. (2011,2012, 2013), Satpati and Pal (2015,2016) A total of 70 taxa were reported (Figures 2-5). The data shows a dominance of different species of *Rhizoclonium, Cladophora, Spirogyra* and *Oedogonium.* The salinity ranged from 0.6 to 25.5 ppt (Parts per thousand) in the collection sites. The salinity and pH fluctuated between the different seasons. Filamentous algae dominated in the brackish water habitat with the exception *Spirogyra.*

Among the filamentous, the most dominant species recorded were *Spirogyra* (11 species) followed by *Rhizoclonium* (9 species), *Cladophora* (7 species), *Oedogonium* (5 species), *Ulva* (3 species) and *Trentepohlia* (4species).

Table 1: Table showing taxa reported by the authors from Sundarbans, West Bengal (table modified from Satpati et al. 2011,2012, 2013; Satpati and Pal, 2015,2016)

Serial No.	**Taxa Collected**	Family	**Site Of Collection**	**Reference**
1	*Cladophora nitellopsis*	Cladophoraceae	Hamanbere Island	Satpati et al., 2011
2	*Ulva lactuca*	Ulvaceae	Bakkhali Sea Beach	Satpati et al., 2012
3	*Ulva intestinalis*	Ulvaceae	Fraserganj	
4	*Rhizoclonium riparium*	Cladophoraceae	Jharkhali	Satpati et al., 2013
5	*R. fontinale*	Cladophoraceae	Basanti	
6	*R. hieroglyphicum*	Cladophoraceae	Lothian Island	
7	*R. tortuosum*	Cladophoraceae	Morahero	

8	*R. pachydermum*	Cladophoraceae	Narayanitala Island
9	*R. africanum*	Cladophoraceae	Cheramatla
10	*R. crassipellitum*	Cladophoraceae	Basanti
11	*Pithophora polymorpha*	Pithophoraceae	Suryamani Island
12	*P. cleveana*	Pithophoraceae	Sushni Island
13	*Cladophora crystallina*	Cladophoraceae	Jharkhali Island
14	*C. glomerata*	Cladophoraceae	Jaigopalpur
15	*C. nitellopsis*	Cladophoraceae	Cheramatla
16	*S. variable*	Zygnemataceae	Kala Jangal
17	*Trentepohlia thevalliensis*	Trentepohliaceae	Suryamoni Island
18	*T. abietina*	Trentepohliaceae	Morahero
19	*T. torulosa*	Trentepohliaceae	Lothian Island
20	*Chlorococcum infusionum*	Chlorococcaceae	Jharkhali Eco-Park
21	*Spirogyra orientalis*	Zygnemataceae	Jaigopalpur

22	*S. hymerae*	Zygnemataceae	Bhagabatpur	
23	*S. occidentalis*	Zygnemataceae	Jharkhali Jetty	
24	*S. punctulata*	Zygnemataceae	Lothian Island	
25	*Scenedesmus quadricauda*	Scenedesmaceae	Matla River	
26	*S. dimorphus*	Scenedesmaceae	Matla River	
27	*S. bijuga*	Scenedesmaceae	Matla River	
28	*Pediastrum tetras*	Hydrodictyaceae	Bidya River	
29	*Crucigenia tetrapedia*	Chlorococcaceae	Bidya River	
30	*Chaetomorpha gracilis*	Cladophoraceae	Bakkhali Sea Beach	
31	*Lola capillaris*	Cladophoraceae	Kala Jangal	
32	*L. tortuosa*	Cladophoraceae	Suryamoni Island	
33	*Closterium tumidium*	Desmidiaceae	Matla Canal	
34	*Chlorella vulgaris*	Chlorellaceae	Jaigopalpur Pond	
35	*Oedocladium prescottii*	Oedogoniaceae	Jharkhali Island	
36	*Geminella minor*	Chlorellaceae	Sarberia	Satpati and Pal, 2016
37	*Microspora willeana*	Microsporaceae	Lothian Island	

38	*M. floccosa*	Microsporaceae	Sushni Island
39	*M. abbreviata*	Microsporaceae	Suryamoni Island
40	*Oedogonium hindustanense*	Oedogoniaceae	Rajbari, Malancha
41	*O. mexicanum*	Oedogoniaceae	Rajbari, Malancha
42	*O. anomalum*	Oedogoniaceae	Fraserganj
43	*O. pringsheimii*	Oedogoniaceae	Patibunia Island
44	*O. crispum*	Oedogoniaceae	Fraserganj
45	*Ulothrix zonata*	Ulotrichaceae	Namkhana
46	*U. tenuissima*	Ulotrichaceae	Namkhana
47	*Chaetomorpha ligustica*	Cladophoraceae	Dabu
48	*C. aerea*	Cladophoraceae	Hamanbere
49	*Rhizoclonium fontanum*	Cladophoraceae	Kala Jungle
50	*R. hookeri*	Cladophoraceae	Basanti
51	*Cladophora glomerata* var. *crassior*	Cladophoraceae	Lothian island
52	*C. prolifera*	Cladophoraceae	Hamanbere island
53	*C. fracta*	Cladophoraceae	Fraserganj
54	*Pithophora roetlleri*	Pithophoraceae	Amarboni island

55	*Ulva patengensis*	Ulvaceae	Minakha	
56	*Enteromorpha gujratensis*	Ulvaceae	Dabu	
57	*E. clathrata*	Ulvaceae	Jharkhali	
58	*Spirogyra maravillosa*	Zygnemataceae	Bhagabatpur	
59	*S. brunnea*	Zygnemataceae	Bhagabatpur	
60	*S. daedalea*	Zygnemataceae	Bhagabatpur	
61	*S. plena*	Zygnemataceae	Bhagabatpur	
62	*S. hyalina*	Zygnemataceae	Lothian island	
63	*S. setiformis*	Zygnemataceae	Lothian island	
64	*S. wabashensis*	Zygnemataceae	Henry island	
65	*Temnogyra liana*	Zygnemataceae	Dobanki camp	
66	*Zygnema collinsianum*	Zygnemataceae	Sudhanyakhali	
67	*Z. oudhense*	Zygnemataceae	Sudhanyakhali	
68	*Nitella mirabilis*	Characeae	Basanti	
69	*Chara braunii*	Characeae	Basanti	
70	*Trentepohlia sundarbanensis*	Trentepohliaceae	Cheramatla Island	Satpati and Pal, 2015

Table 2: Seasonal variation of green algal taxa (- absent, + present, ++ abundant, +++ dominant; Satpati and Pal, 2016)

Name of taxa	Summer (March-May)	Monsoon (June-September)	Post monsoon (October-November)	Winter (December-February)
Geminella minor	-	++	+++	++
Microspora willeana	-	+	++	+++
M. floccosa	+	-	+	++
M. abbreviata	-	+	-	+++
Oedogonium hindustanense	+	-	++	+++
O. mexicanum	-	+	+	++
O. anomalum	-	+	++	+
O. pringsheimii	+	-	++	+++
O. crispum	+	+	++	++
Ulothrix zonata	+	-	++	+++
U. tenuissima	-	+	++	+
Chaetomorpha ligustica	-	+	++	-
C. aerea	+	+	++	++
Rhizoclonium fontanum	+	-	+	++
R. hookeri	-	+	-	+
C. glomerata var. crassior	+	-	++	+
C. prolifera	-	++	+++	+++
C. rivularis	-	+	++	+++
C. aegagropila	+	-	+	++
C. fracta	+	+	++	+
Pithophora roetlleri	+	-	+	++
Ulva patengensis	-	+	+	++
Enteromorpha gujratensis	-	+	++	++
E. clathrata	+	-	+	++
Spirogyra maravillosa	-	-	++	+++
S. brunnea	-	-	+++	++
S. daedalea	+	-	++	++
S. plena	-	+	++	+++
S. hyalina	-	++	+++	++
S. setiformis	-	-	++	++
S. wabashensis	+	-	+++	++
Temnogyra liana	+	-	++	+++
Zygnema collinsianum	-	-	++	+++
Z. oudhense	-	+	+	++
Nitella mirabilis	+	-	+	+
Chara braunii	-	+	+	++

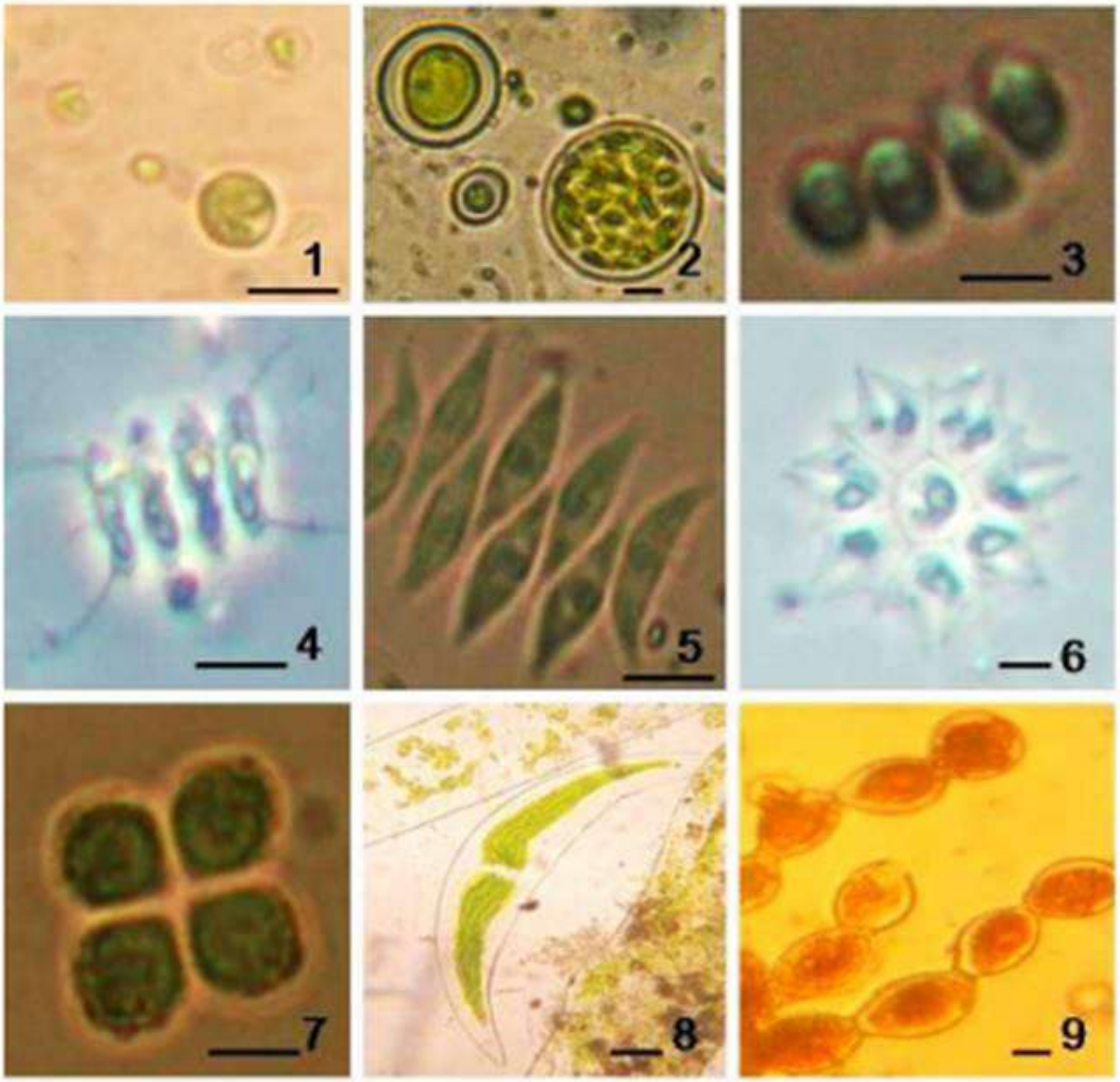

Figure 2. (Scale bar 10 µm) showing microphotographs of 1. *Chlorella vulgaris, 2. Chlorococcum infusionum, 3. Scenedesmus bijuga, 4. S. quadricauda, 5. S. dimorphus, 6. Pediastrum tetras, 7. Crucigenia tetrapedia, 8. Closterium tumidium, 9. Trentepohlia torulosa*

Figure 2.Figure modified from Satpati et al., 2013

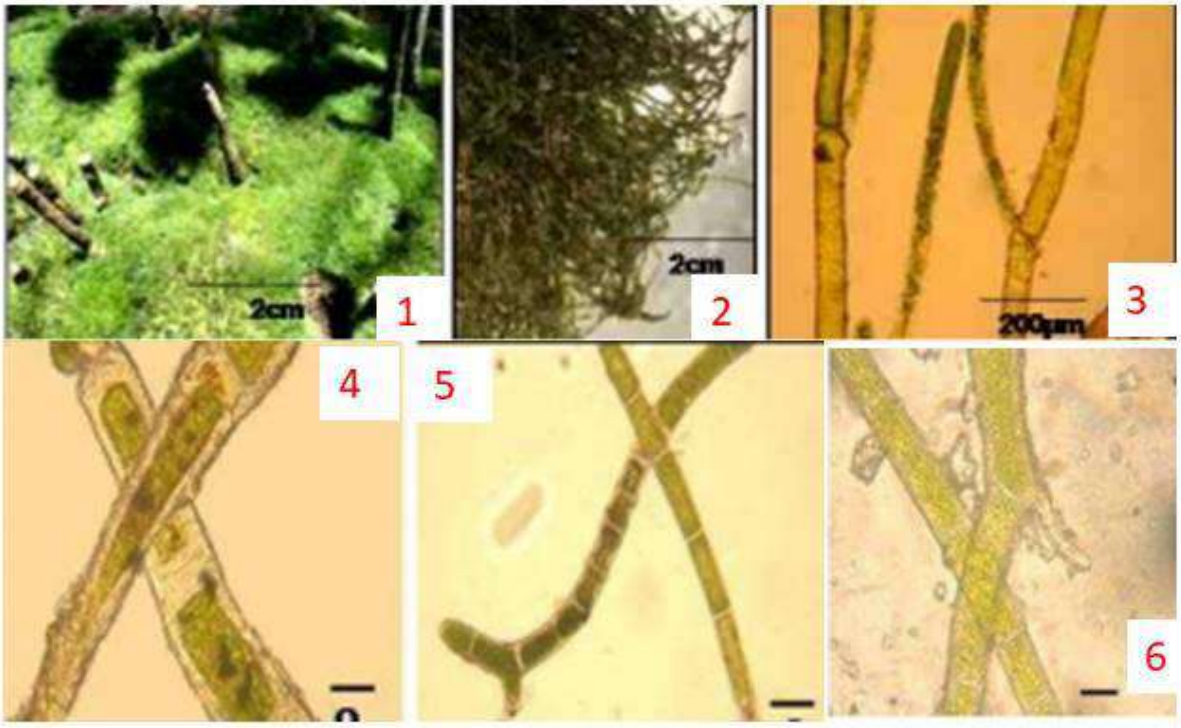

Figure 3. showing microphotographs of 1. *Cladophora nitellopsis* in Hamanbere Island, Sundarban (Scale 2cm), 2. Showing grass like mat under simple microscope (Scale 2cm), 3. Figure showing branching pattern of *Cladophora nitellopsis* under compound microscope (200µm) 4. . *R. riparium*, 5. *R. tortuosum* 6. *R. hieroglyphicum* (Scale 10 µm)

Figure 3: Figure modified from Satpati et al., 2011 and 2013

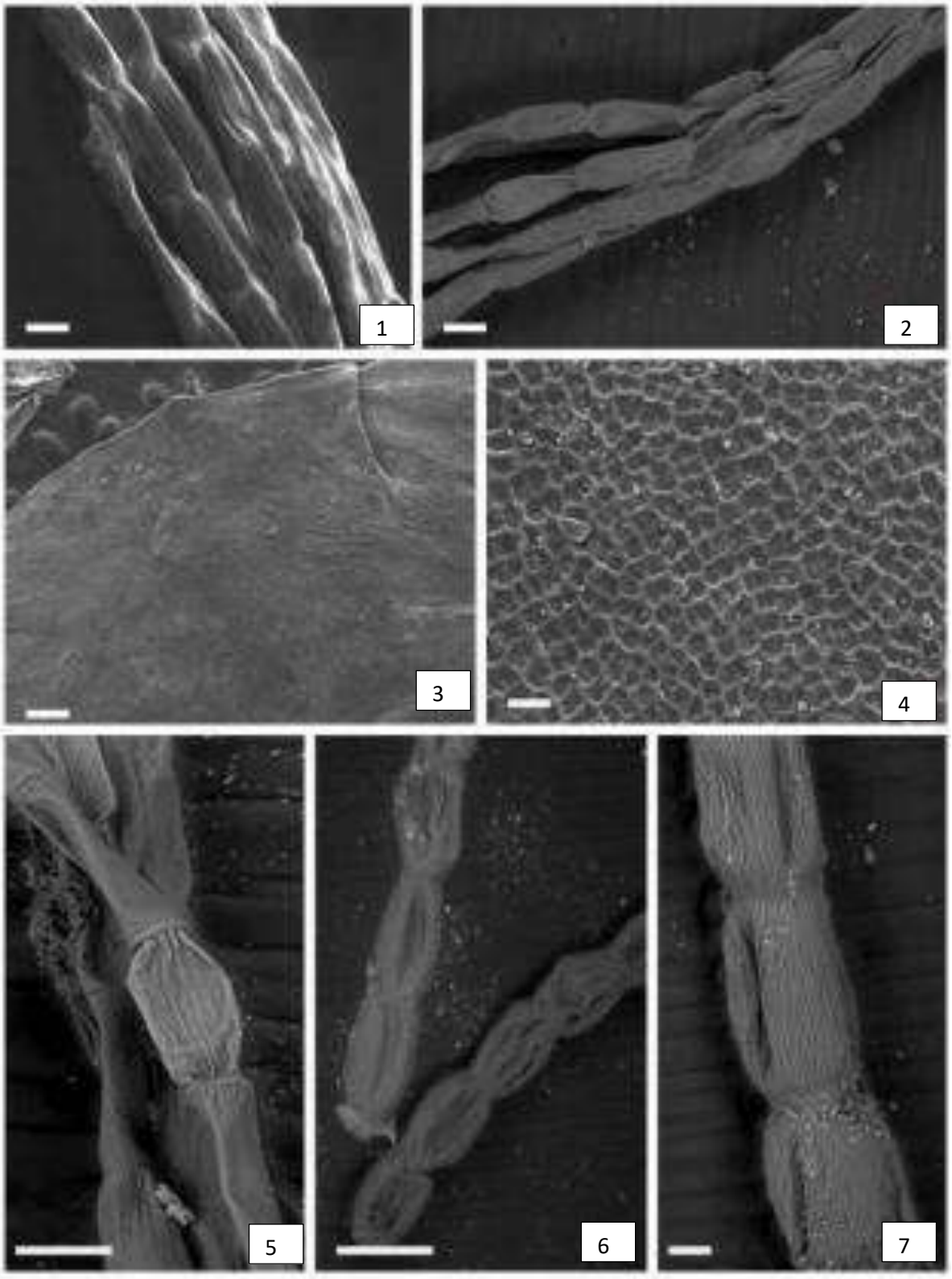

Figure 4. SEM photographs of 1. *Rhizoclonium fontanum* (×1.00KX); 2. *R. hookeri* (×500X); 3. *Ulva patengensis* (×100X); 4. Cellular details of *U. patengensis* (×1.00KX); 5. *Pithophora roettleri* (×1.00KX); 6. *Chaetomorpha aerea* (×250X); 7. *C. ligustica* (×500X)

Scale bar: 1, 5- 30 μm; 2, 3, 6- 100 μm; 4- 10 μm; 7- 50 μm (Satpati and Pal, 2016)

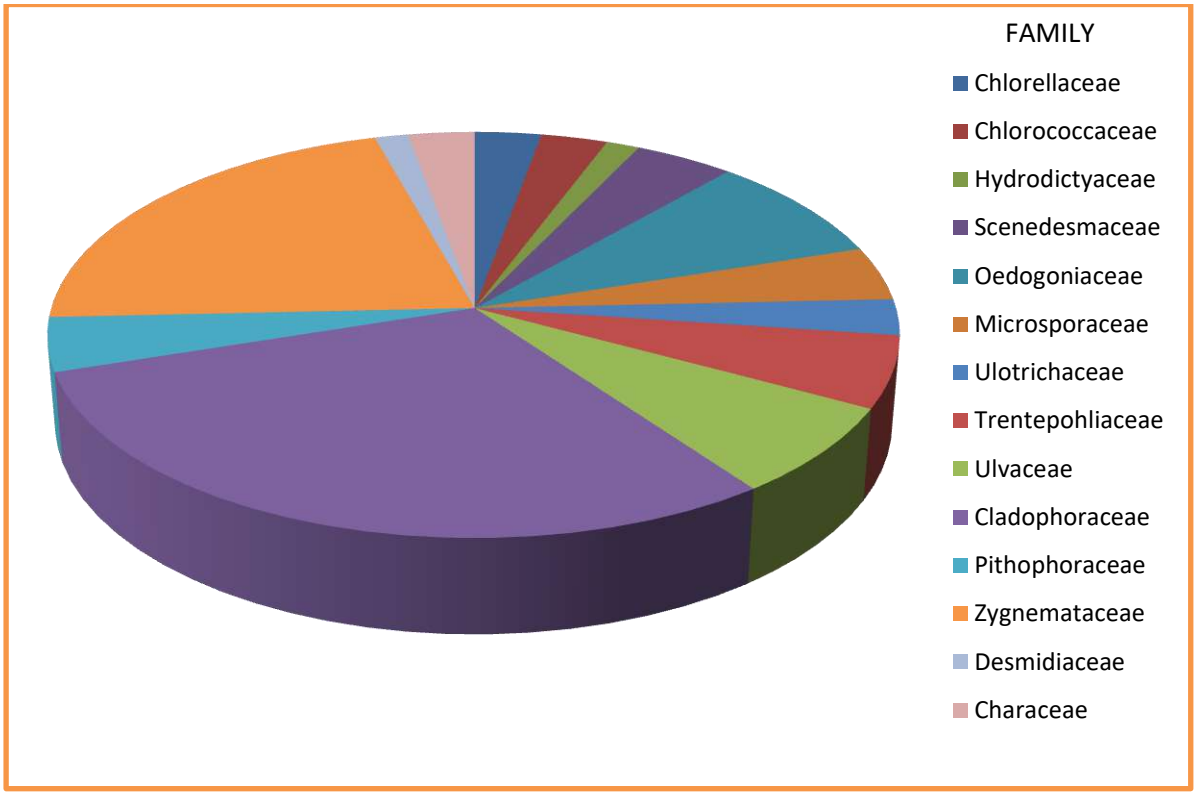

Figure 5: Pie chart showing the abundance of different families of Chlorophyta as collected by Satpati et al. (2011, 2012, 2013) and Satpati and Pal (2015, 2016). The Cladophoraceae family accounts for almost 30% of the total taxa collected by the authors followed by Zygnemataceae.

Discussion:

A total of 70 taxa belonging to Chlorophyta(Lee, 2008) were recorded by the authors, Satpati et al. (2011,2012, 2013), Satpati and Pal (2015,2016). The Cladophoraceae family accounted for almost 30% of the total taxa collected by the authors followed by Zygnemataceae (Figure 5). The data points towards dominance of filamentous forms of green algae like *Spirogyra* (11 species) followed by *Rhizoclonium* (9 species), *Cladophora* (7 species), *Oedogonium* (5 species), *Ulva* (3 species) and *Trentepohlia* (4 species) etc. Among the planktonic forms, *Scenedesmus* dominated followed by *Pediastrum*, *Chlorococcum* etc. *Trentepohlia sundarbanensis* was reported as a novel species by Satpati and Pal(2015) from Cheramatla Island of Sundarbans. It was observed that some of the filamentous forms like *Rhizoclonium hookeri* and *R. fontanum* grew epiphytically on the pneumatophores of different mangrove plants like *Avicennia alba* and *Bruguiera gymnorrhiza*. Thus it can be concluded that different species of green algae particularly the filamentous and parenchymatous are well adapted to the high salinity of brackish waters of Sundarbans.

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Dissertation

Title:**Review on chlorogenic acid synthesis pathway and its significance in *Solanum melongena* and related species**

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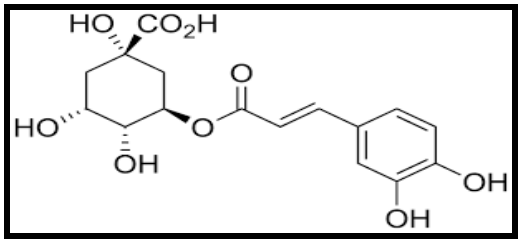
Review on chlorogenic acid synthesis pathway and its significance in *Solanum melongena* and related species



Review on chlorogenic acidsynthesis pathwayand its significance in*Solanum melongena* and related species.

Abstract

Phenylpropanoid accumulation is one of the major defence mechanisms in plants. The eggplant fruits accumulate significant amount of phenylpropanoids in form of CGA, around 70-90% of total phenolic content in flesh. A review of CGA biosynthesis pathway and the genes involved in *Solanum melongena* and allied species of Solanaceae is still lacking. Some crucial information in the available reports and databases have been addressed in this review. It is found that HQT gene, the last one in the pathway to CGA, forms one of the most important targets to study phenylpropanoid accumulation in *Solanum melongena*. Considering the significance of CGA biosynthesis, the utility of bioinformatics databases (NCBI and others) harbouring the HQT gene and protein sequences, has been reviewed. The approach may help in identification of HQT and other such genes in unexplored wild resources of eggplant (reportedly having high CGA content)for future introduction in breeding system. This review along with the in-silico tools is aimed to benefit future research on phenylpropanoid sources in alternative wild species having potency to advance resistance and productivity in the field of eggplant breeding.

Keywords: *Solanum melongena*, CGA, HQT gene, phenylpropanoid, protein, defence

Introduction

Solanaceae plants are medium-sized angiosperms, constituting the largest group of vegetable crops and the third largest group of economic plants. The taxa in the Solanaceae family are abundant and diverse, with 90 genera and 3,000–4,000 species. This family includes many important crop species, such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena* L.), and pepper (*Capsicum annuum*).

Brinjal eggplant (*Solanum melongena* L.) is one of the most important Solanaceous crops, ranking third in the total production and economic value in the genus *Solanum*, and fourth in most widely produced vegetable crop. The world production of eggplants was approximately 52.3 million tons in 2017, with China being the main producer (Li et al., 2019). It is one of a dozen or so species of the Solanaceae, or nightshade family, that have been selected and developed as human food plants (Weese and Bohs, 2010).

Eggplants are a rich source of several secondary metabolites among which phenylpropanoids are a notable mention. Chlorogenic acid comprises of 70-90% of total phenolics in the flesh tissues while anthocyanin comprises the fruit skin (La Camera et al., 2004).Phenylpropanoids contribute to all aspects of plant responses towards biotic and abiotic stimuli. They are not only indicators of plant stress responses upon variation of light or mineral treatment, but are also key mediators of the plant's resistance towards pests (La Camera et al., 2004). A wide variety andplasticity occur in the phenylpropanoid profile stemming from a set of enzymes falling under superfamilies like oxygenases, ligases, oxidoreductases, and various superfamilies of transferases.Some of these enzymes might exhibit overlapping specificities *in vitro*, but their developmentally and spatially controlled expression specifically contributes to tissueand plant-specific chemical phenotypes (Vogt et al.,2010).The plant shikimate pathway is the entry to the biosynthesis of phenylpropanoids.

Objective of the present review:

1. Overview of phenylpropanoid synthesis and its significance in *Solanum* and closely allied species.
2. Study of a gene involved in phenylpropanoid pathwaywith bioinformatics tools.

1. Overview of phenylpropanoid synthesis and its significance in *Solanum melongena*

1.1 Pathways of chlorogenic acid synthesis

Different types of phenylpropanoids are found in plants (Fig. 1). Winter and Herrmann (2003) determined that quinic acid esters of hydroxycinnamic acids are the major class of polyphenols in eggplant fruit, with chlorogenic acid as the predominant compound. Chlorogenic acid (5-O-caffeoyl-quinic acid; CGA) is an important and biologically active dietary polyphenol, playing several important and therapeutic roles such as antioxidant activity, antibacterial,hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective,anti-obesity, antiviral, anti-microbial, anti-hypertension, free radicals' scavenger and a central nervous system (CNS) stimulator. Chlorogenic acid has important anti-obesity (Cho et al. 2010), heart-protective, and DNA-protective functions (Wang et al. 2016). It is formed by the esterification of caffeic acid and the aliphatic alcohol, quinic acid (1(OH)-3,4/5-tetrahydroxycyclo-hexane carboxylic acid) (Fig.2). The biosynthesis of CGA in eggplants is as shown below (Fig.3). It is estimated that human consume upto 1g of CGA per day (Chen et al., 2009).

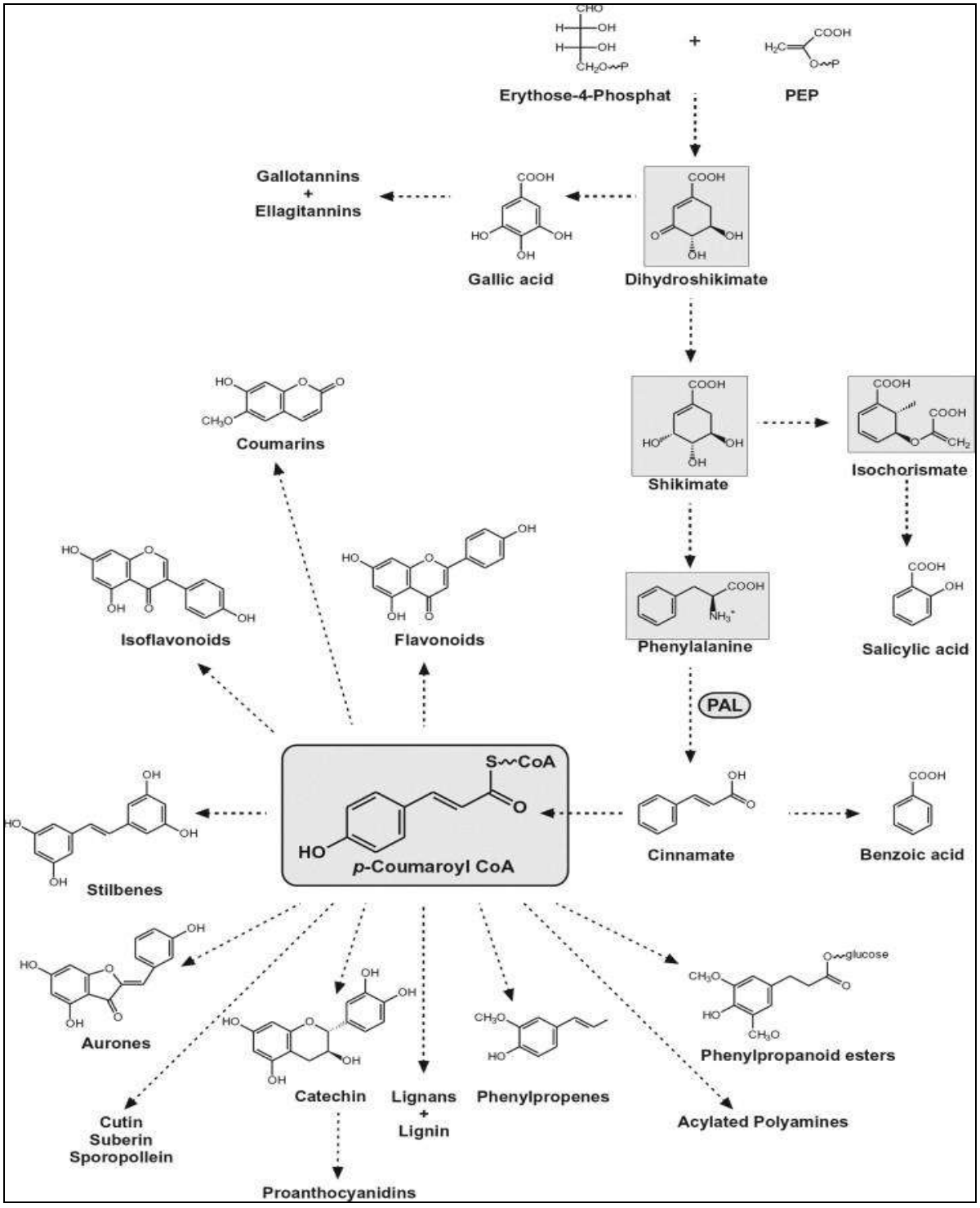


Fig1.Phenylpropanoids in plants (Adopted from Vogt et.al. 2010).

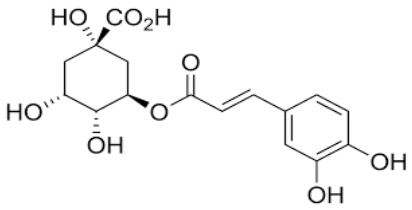


Fig2. Chemical structure of Chlorogenic acid

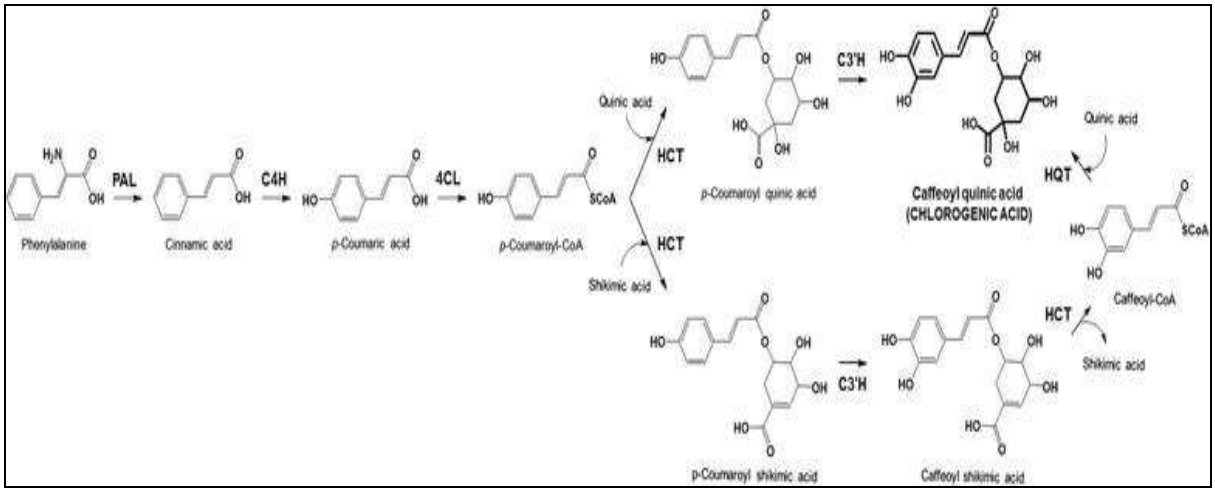


Fig3. Chlorogenic acid (CGA) synthesis pathway in eggplant. (Adopted from Gramazio et. al 2014)

As shown in Fig.3 the enzymes involved in this pathway are: -

1. Phenylalanine ammonia lyase (PAL)
2. Cinnamate 4- hydroxylase (C4H)
3. 4-Coumarate-CoA ligase (4CL)
4. Hydroxycinnamoyl-CoA transferase (HCT)
5. p-coumaroyl ester 3'-hydroxilase (C3'H)
6. Hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT).

The content of potentially health beneficial hydroxycinnamic acid conjugates is substantial in eggplant fruit from commercial cultivars, ranging from about 0.5-1.5% on a dry weight basis. This is in accord with the value of about 600-660 mg/kg fresh weight reported by Winter and Herrmann (2003), and puts eggplant on a par with sweet cherry, kiwi, and several other fruits that are among the highest in phenolic acid content (Whitaker and Stommel, 2003).

1.2. Variation of CGA content in the species of *Solanum*

Hydroxycinnamic acid amides (HCAs), conjugates between hydroxycinnamic acids (e.g., caffeic acid, ferulic acid and p-coumaric acid) and amines (e.g., putrescine, spermidine and spermine) are widely distributed in the plant kingdom. They have been suggested to have possible functions in several plant developmental processes, including tuberization and flowering (Leubner-Metzger et. al. 1992).

Eggplant presents a wide morphological and molecular diversity (Hurtado et al. 2012), as well as a broad variation for composition traits, including total phenolics and CGA content (Arivalagan et al., 2012) due to both genetic and environmental factors. Some wild relatives, like *S. incanum*, present higher CGA contents than those of eggplant. Interspecific hybridization is possible among eggplant and its relatives allowing for desirable traits in related species to be readily introgressed into the domesticated species (Kaushik et al. 2017). The Prohens lab (Valencia, Spain) argued for the value of such introgression (Plazas et al. 2013; Prohens et al. 2007, 2017) and has used relatives, such as *S. incanum* L., to increase chlorogenic acid levels. They have focused on increasing chlorogenic acid synthesis

(Prohenset al. 2013) and on decreasing browning that degrades chlorogenic acid and other phenolic compounds (Gramazio et al. 2014; Kaushik et al. 2017). Another example is introgression from wilt resistant *S. aethiopicum* lines imparting tolerance in *S. melongena*. Remarkably, high-density genetic maps led to the discovery of an orthologous wilt resistance locus in *S. melongena* with exploitable allelic variation (Gramazio et al. 2018). Improved resistance through introgression can help to establish food security.

Chlorogenic acid (CGA) is one of the most abundant phenolic compounds in tomato (*Solanum lycopersicum*). Hydroxycinnamoyl CoA quinate transferase (HQT) is the key enzyme catalysing CGA biosynthesis in tomato (Carlaclé et. al, 2008). Increased CGA accumulation was associated with increased UV-protection in transgenics with altered HQT activity. However, manipulation of HQT activity reaults in more complex alterations in the profiles of phenolics (Carlaclé et. al, 2008).

Chlorogenic acid, caffeic acid, p-coumaric acid and ferulic acid were detected in small quantities in resting whole tubers of irradiated and nonirradiated potatoes. During wound healing their content increases many folds, and in addition, neo and crypto isomers of chlorogenic acid accumulates in the wound healing tissue (Ramamurthy et. al. 1992). The increased formation of phenolics is accompanied by a parallel rise in phenylalanine ammonia-lyase activity. Chlorogenic acid contributes to about 56% (involving the neo and crypto isomers upto 88%) of the phenolics formed during healing in *Solanum tuberosum* (Ramamurthy et. al, 1992).

2. Study of a gene involved in phenylpropanoid pathway with bioinformatics tools.

2.1.1An introduction to BLAST

One of the most widely used tools of the NCBI (National Center for Biotechnology Information), BLAST stands for Basic Local Alignment Search Tool.

Key features of BLAST:

- Widely used sequence similarity search tool
- Finds highly similar local alignment between two sequences (protein and DNA)
- Includes a model of score distributions for random local alignments
- Provides statistical significance for the alignments
- Expect value= the number of hits with the same or better score that could be expected by chance
- BLAST informs about non chance similarities between biological sequences.
- Non chance similarities may point towards homology or evolutionary lines

All BLAST searches begin with a sequence of protein or nucleotide.

BLAST can be used to understand or find out:

- What/ who a particular nucleotide or protein sequence is related to (homology/ conserved domains).
- If a particular nucleotide or protein sequence is already in the database. (Identification, involving finding matching organism in the database or organism of origin)

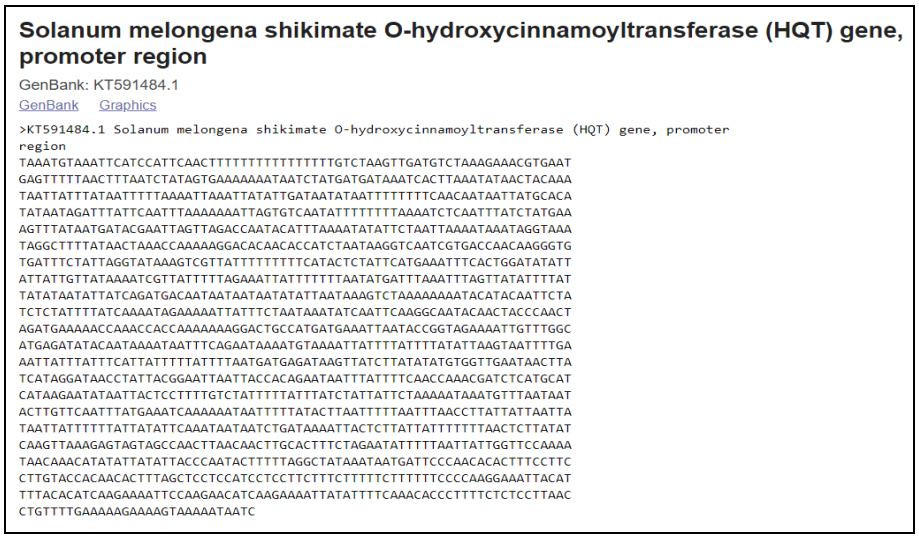
- Where a particular nucleotide or protein sequence is located and how it is organised (annotation and assembly in a genome)
- Comparison of sequences and frame shifts across organisms.

BLAST is most commonly used in the following formats:

- Nucleotide search programs
  - BLASTn
    - traditional BLAST algorithm,
    - most sensitive nucleotide search
  - megaBLAST
    - larger sequence size than BLASTn
    - different search models
      - contiguous megaBLAST (similar sequences)
      - discontiguousmegaBLAST (cross species comparison)
- Protein search programs
  - BLASTp
  - translating searches – useful for unannoted protein coding regions.
    - BLASTx - translated query
    - tBLASTn - translated database
    - tBLASTx – translated query and database

2.1.2Applications of NCBI and BLAST in HQT gene study

The HQT gene sequence has been retrieved from NBCI Nucleotide databse and has been analysed for homology and other aspects. The extracted snapshots of current bioinformatics study of HQT gene have been given in the following sections.



Solanum melongena shikimate O-hydroxycinnamoyltransferase (HQT) gene, promoter region

GenBank: KT591484.1

GenBank Graphics

>KT591484.1 Solanum melongena shikimate O-hydroxycinnamoyltransferase (HQT) gene, promoter region

TAAATGTAAATTCATCCATTCAACTTTTTTTTTTTTTTTTGTCTAAGTTGATGTCTAAAGAAACGTGAAT
GAGTTTTTAACTTTAATCTATAGTGAAAAAAATAATCTATGATGATAAATCACTTAAATATAACTACAAA
TAATTATTTATAATTTTTAAAATTAAATTATATTGATAATATAATTTTTTTTCAACAATAATTATGCACA
TATAATAGATTTATTCAATTTAAAAAAATTAGTGTCAATATTTTTTTTAAAATCTCAATTTATCTATGAA
AGTTTATAATGATACGAATTAGTTAGACCAATACATTTAAAATATATTCTAATTAAAATAAATAGGTAAA
TAGGCTTTTATAACTAAACCAAAAAGGACACAACACCATCTAATAAGGTCAATCGTGACCAACAAGGGTG
TGATTTCTATTAGGTATAAAGTCGTTATTTTTTTTTCATACTCTATTCATGAAATTTCACTGGATATATT
ATTATTGTTATAAAATCGTTATTTTTAGAAATTATTTTTTTAATATGATTTAAATTTAGTTATATTTTAT
TATATAATATTATCAGATGACAATAATAATAATATATTAATAAAGTCTAAAAAAAAATACATACAATTCTA
TCTCTATTTTATCAAAATAGAAAAATTATTTCTAATAAATATCAATTCAAGGCAATACAACTACCCAACT
AGATGAAAAACCAAACCACCAAAAAAAGGACTGCCATGATGAAATTAATACCGGTAGAAAATTGTTTGGC
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AATTATTTATTTCATTATTTTTATTTTAATGATGAGATAAGTTATCTTATATATGTGGTTGAATAACTTA
TCATAGGATAACCTATTACGGAATTAATTACCACAGAATAATTTATTTTCAACCAAACGATCTCATGCAT
CATAAGAATATAATTACTCCTTTTGTCTATTTTTATTTATCTATTATTCTAAAAATAAATGTTTAATAAT
ACTTGTTCAATTTATGAAATCAAAAAATAATTTTTATACTTAATTTTTAATTTAACCTTATTATTAATTA
TAATTATTTTTTATTATATTCAAATAATAATCTGATAAAATTACTCTTATTATTTTTTTAACTCTTATAT
CAAGTTAAAGAGTAGTAGCCAACTTAACAACTTGCACTTTCTAGAATATTTTTAATTATTGGTTCCAAAA
TAACAAACATATATTATATTACCCAATACTTTTTAGGCTATAAATAATGATTCCCAACACACTTTCCTTC
CTTGTACCACAACACTTTAGCTCCTCCATCCTCCTTCTTTCTTTTTCTTTTTTCCCCAAGGAAATTACAT
TTTACACATCAAGAAAATTCCAAGAACATCAAGAAAATTATATTTTCAAACACCCTTTTCTCTCCTTAAC
CTGTTTTGAAAAAGAAAAGTAAAAATAATC

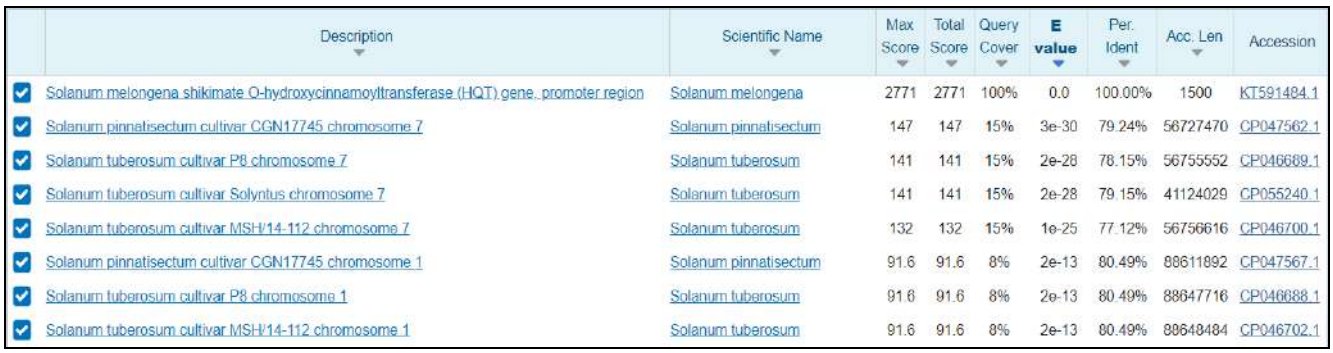
Fig. 4. The nucleotide sequence of HQT gene (>KT591484.1) in *S. melongena* (source: NCBI).

Job Title	ref|NM_001247921.2|
RID	ABUFAFFG114 *Search expires on 05-21 12:47 pm* Download All
Program	Blast 2 sequences Citation
Query ID	NM_001247921.2 (nucleic acid)
Query Descr	Solanum lycopersicum hydroxycinnamoyl CoA quinate tra ...
Query Length	1456
Subject ID	KT591484.1 (nucleic acid)
Subject Descr	Solanum melongena shikimate O-hydroxycinnamoyltransf ...
Subject Length	1500

Filter Results

Percent Identity to | E value to | Query Coverage to | Filter | Reset

No significant similarity found. For reasons why click here



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Solanum melongena shikimate O-hydroxycinnamoyltransferase (HQT) gene, promoter region	Solanum melongena	2771	2771	100%	0.0	100.00%	1500	KT591484.1
Solanum pinnatisectum cultivar CGN17745 chromosome 7	Solanum pinnatisectum	147	147	15%	3e-30	79.24%	56727470	CP047562.1
Solanum tuberosum cultivar P8 chromosome 7	Solanum tuberosum	141	141	15%	2e-28	78.15%	56755552	CP046689.1
Solanum tuberosum cultivar Solyntus chromosome 7	Solanum tuberosum	141	141	15%	2e-28	79.15%	41124029	CP055240.1
Solanum tuberosum cultivar MSH/14-112 chromosome 7	Solanum tuberosum	132	132	15%	1e-25	77.12%	56756616	CP046700.1
Solanum pinnatisectum cultivar CGN17745 chromosome 1	Solanum pinnatisectum	91.6	91.6	8%	2e-13	80.49%	88611892	CP047567.1
Solanum tuberosum cultivar P8 chromosome 1	Solanum tuberosum	91.6	91.6	8%	2e-13	80.49%	88647716	CP046688.1
Solanum tuberosum cultivar MSH/14-112 chromosome 1	Solanum tuberosum	91.6	91.6	8%	2e-13	80.49%	88648484	CP046702.1

Fig5. Nucleotide BLAST study of the HQT gene from eggplant shows similarity in nucleotide sequence with these other species of *Solanum* that include several wild species (distant to *S. melongena*) indicating sequence similarity (source: NCBI)

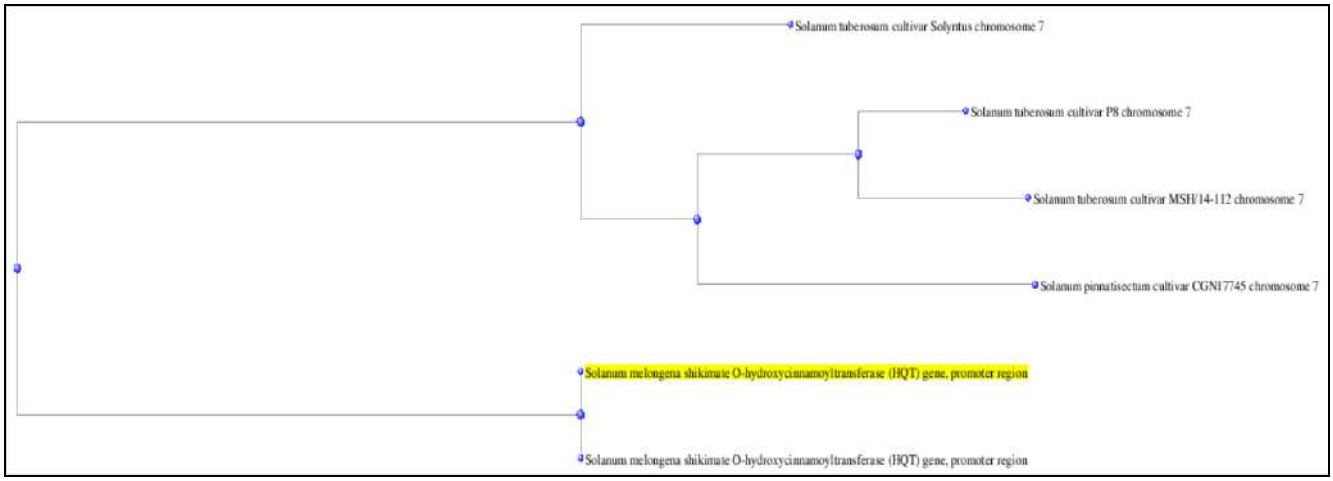


Fig6. Graphical representation of the HQT gene similarity in the species of *Solanum*(*Solanum melongena* highlighted in yellow). (Source: NCBI BLAST)

The above data shows sequence similarity between HQT gene of *Solanum melongena* and other related species. Though we have seen high CGA content in wild species *S. incanum*, the data for the same is not available in the NCBI database as of yet. BLAST with HQT gene sequence of *S. melongena* and shows no significant similarity withother relatively distant species of the family like*S.pinnatisectum*and *S. tuberosum.*

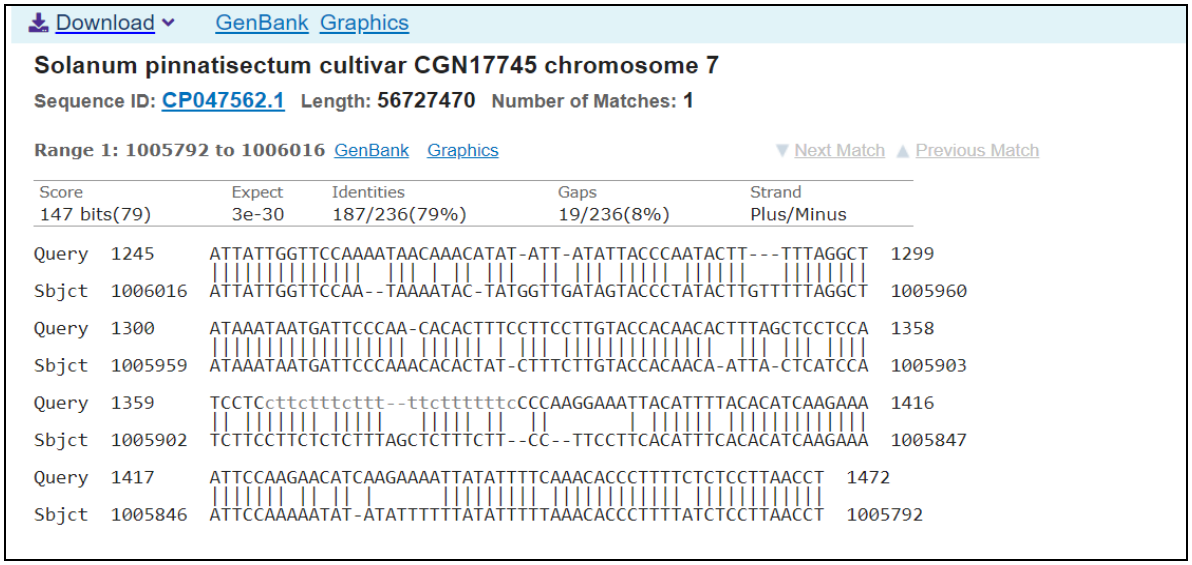


Fig.7 Alignment of nucleotide sequence of *S. melongena* with *S. pinnatisectum* in HQT promoter region of DNA. (Source: NCBI BLAST).

This image (Fig.7) shows that *S. pinnatisectum*has about 79% similarity with *S. melongena* HQT gene and produces CGA in low amounts.This result points towards a degeneracy in the nucleotide sequences of the closely related *Solanum* species like *S.lycopersicum.*Study of the enzymatic pathway of all possible biosynthetic mechanisms of CGA synthesis in plants might givea better view of HQT gene sequences and their homology across the Solanaceae group.

2.2.1A look into the HQT protein sequence

Chlorogenic acid synthesis can involve different pathways (Tuan et. al, 2014). However, in case of *Solanum* sp. CGA synthesis happens through the HQT gene mediated pathway leading to the end product being Caffeoyl quinic acid or Chlorogenic acid (Gramazio et.al 2014) as is evident from the pathway (Fig.8).

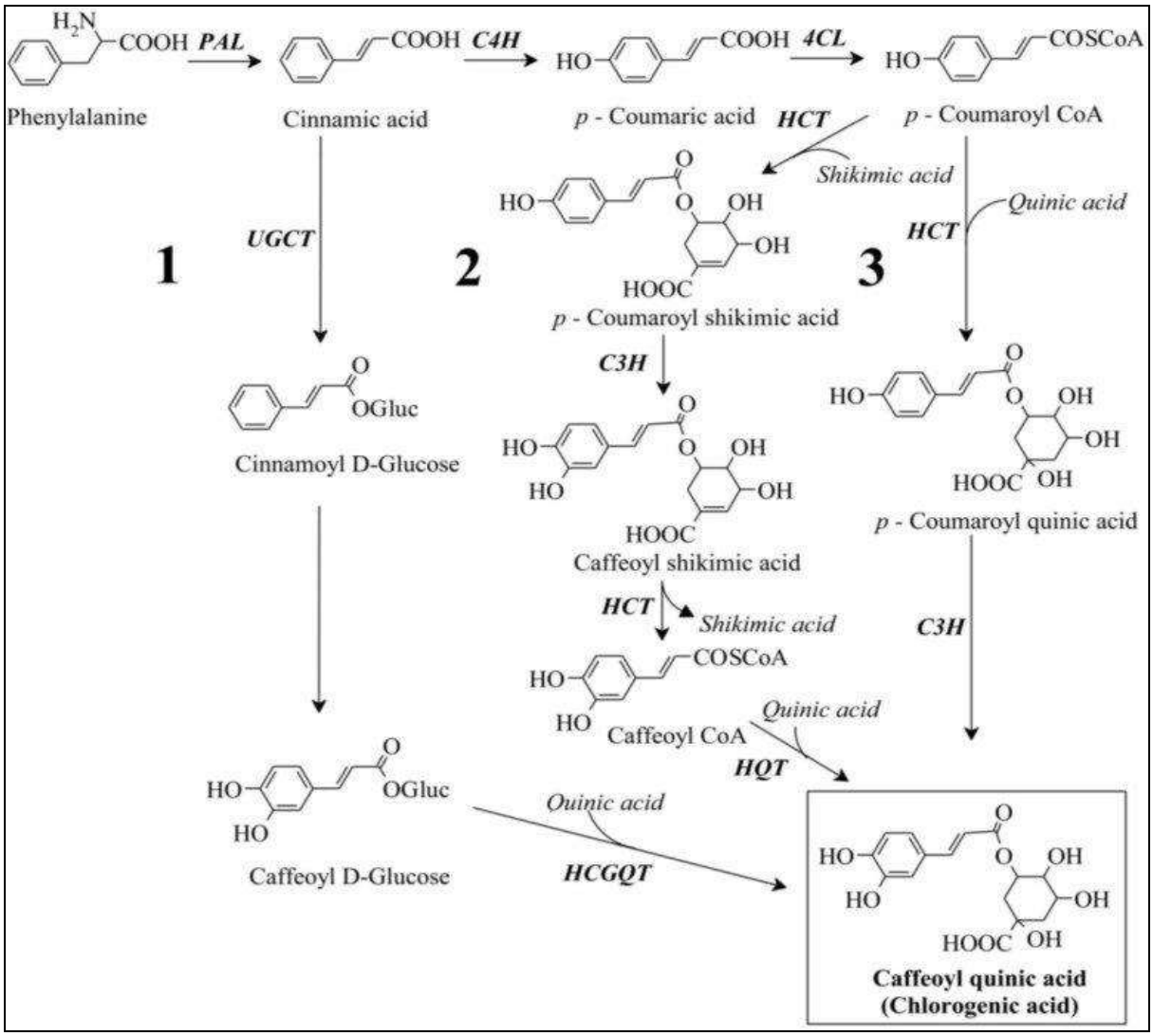


Fig8. Different pathways leading to chlorogenic acid synthesis in plants. Enzymes mentioned here are: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; HCT, hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase; C3H, p-coumarate 3'-hydroxylase; HQT, hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase; UGCT, UDP glucose: cinnamate glucosyl transferase; HCGQT, hydroxycinnamoyl d-glucose: quinate hydroxycinnamoyl transferase. (Adopted from Tuan et. al,2014)

2.2.2HQT protein BLAST:

BLAST gives us an exact idea about protein homology and rules out the codon degeneracy problems. We also get a complete idea about protein evolution and conservation.

HQT is the key enzyme that completes the Phenylpropanoid pathway and CGA synthesis. We can examine the nature of any enzyme and its domain that involves functional evolution. Following sections describe the analysis of the HQT gene across Solanaceae and also in coffee, where highest accumulation of CGA is reported (Jeon et. al. 2019).

a) HQT protein sequence and BLAST result within Solanaceae

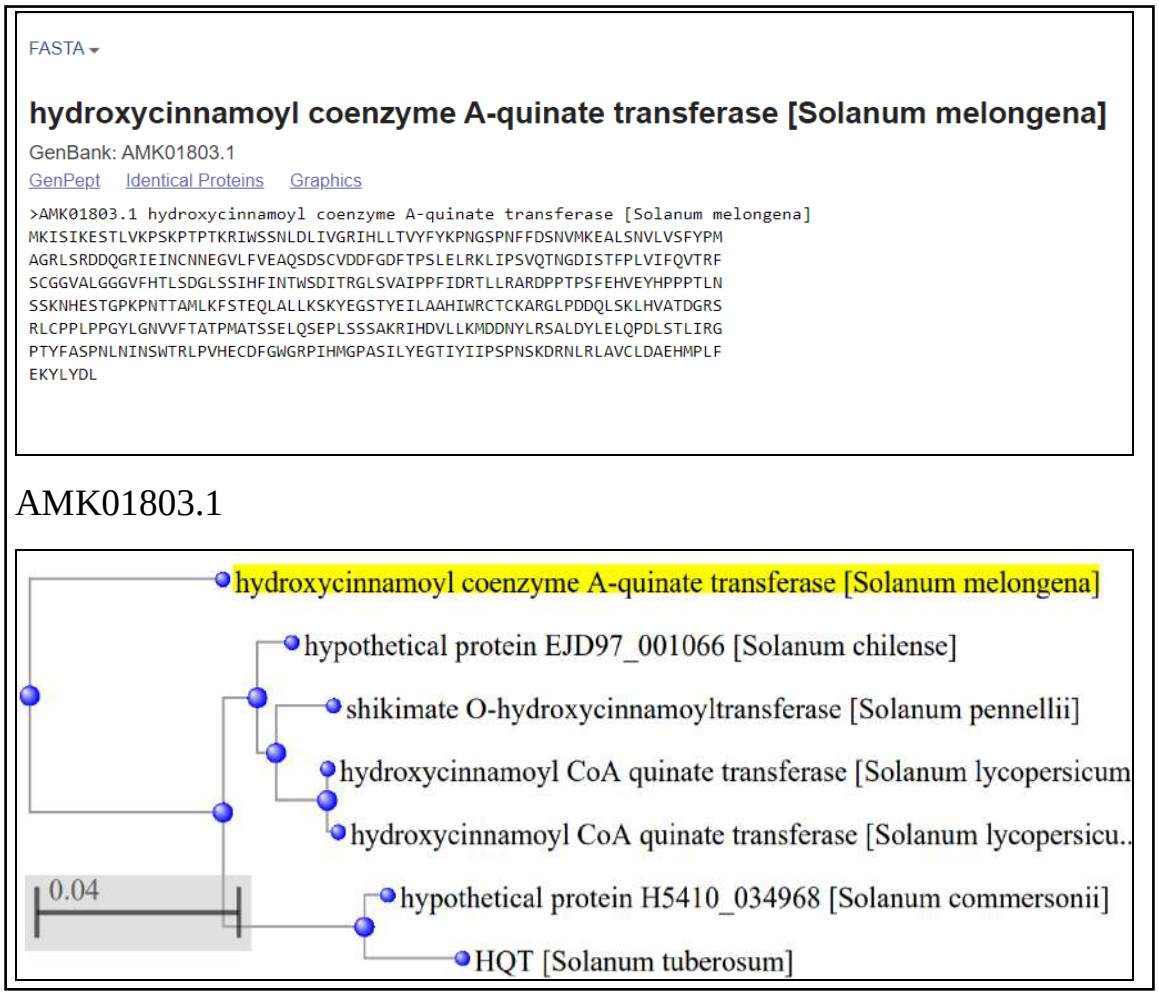


Fig9.HQT gene of *Solanum melongena*: amino acid sequence in FASTA format and BLAST of the same within Solanaceous plants (Source: NCBI).

A close relationship with several related species of *Solanum*, even some wild species like *S. chilense*, *S. pennellii* and others have been observed. The protein BLAST helped overcome the codon degeneracy problem associated with the DNA sequence. However, the DNA sequence remains important to initially screen presence of HQT gene in unexplored *Solanum* species, followed by analysis of the polypeptide sequence.

b)HQT protein sequence and BLAST result in different plant species

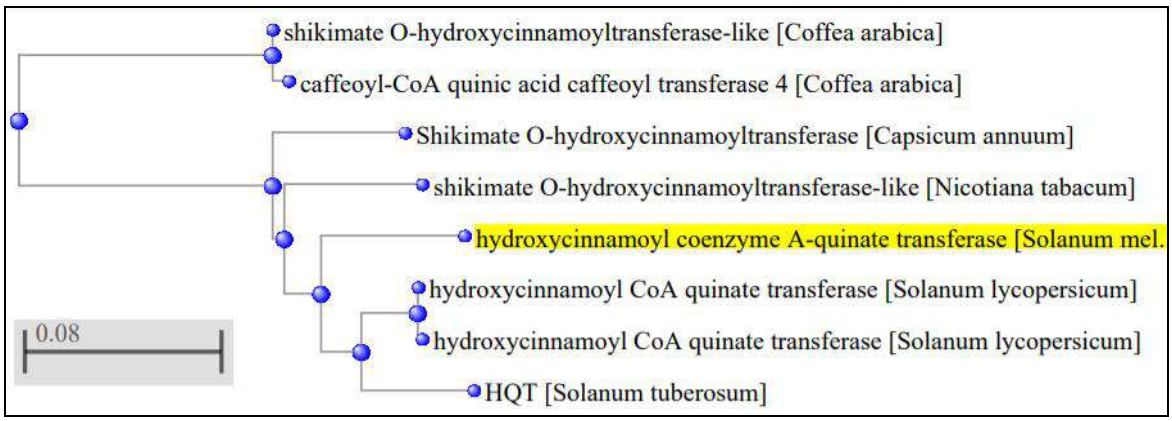


Fig10. Protein BLAST of HQT gene sequence of *Solanum melongena* compared with some other members of the Solanaceae family and *Coffea arabica*. (Source: NCBI BLAST).

The data shows sequence similarity with many other Solanum genera as well as Coffee, indicating the conserved nature of the HQT protein sequence.

2.2.3Implication of Multalinin HQT protein study

Multalin is a multiple-sequence alignment tool for protein and nucleic acid sequences created by Florence Corpet. We can input our sequences into the data entry box using FASTA, EMBL-SwissProt, or Genbank formats and hit “start Multalign” to run the program with the default parameters. Users have the option to tweak parameters related to alignment and data presentation. The default output, which is generated quickly, is an easy-to-read alignment of the sequences. Overall, Multalin is a fast and easy tool for sequence alignment needs.

Multi-alignment of the HQT protein has been carried out using sequences from *Solanum melongena, S. tuberosum, S. lycopersicum, Nicotiana tabacum, Coffeea arabica,* and *Capsicum annuum*(Fig. 11).

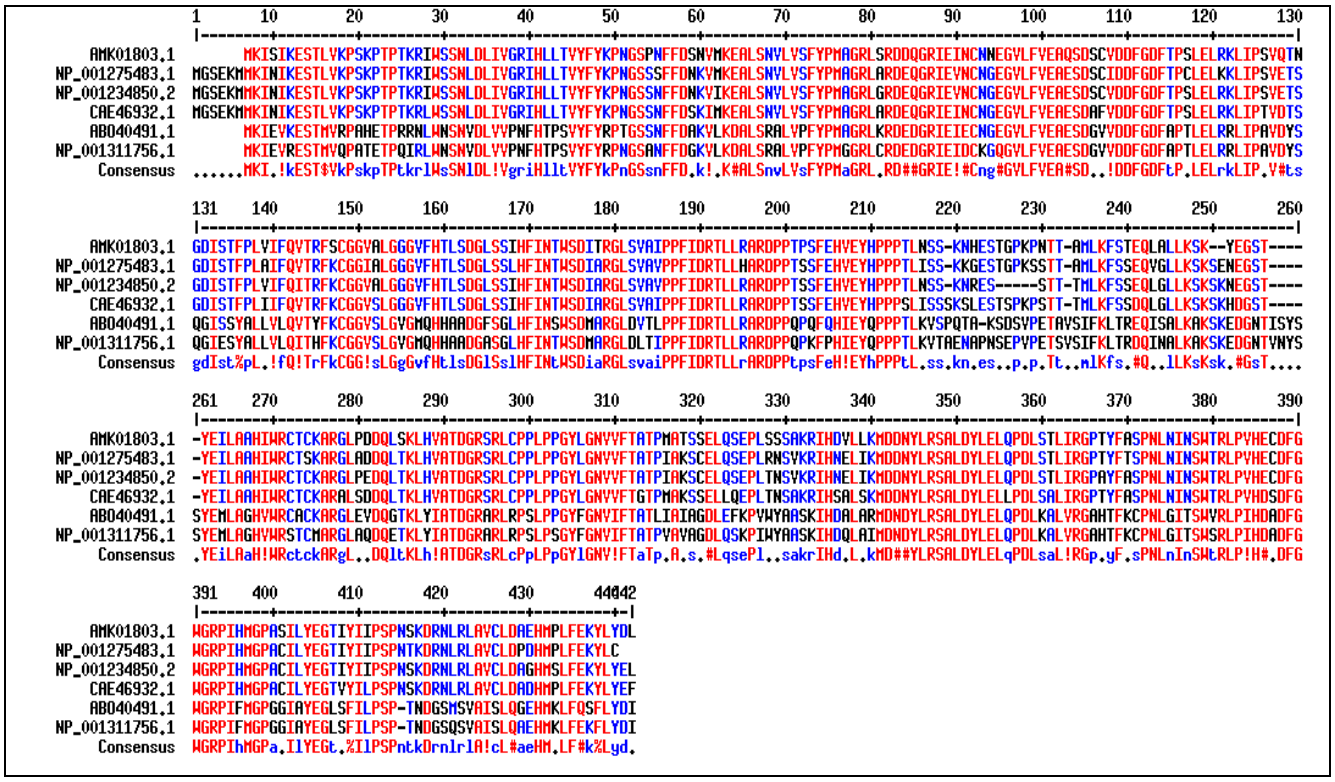


Fig11. Multi-alignment of the HQT protein showing protein homology and conservation. (source: multalin.toulouse.inra).

In the above alignment sequence, consensus symbols used are:
! is anyone of IV
$ is anyone of LM
% is anyone of FY
# is anyone of NDQEBZ
Gap weight: 12 Gap length weight: 2 Consensus levels: high=90% low=50%

The above alignment shows that almost the entire length of the protein isconserved across not only the Solanaceae members (including *Solanummelongena*) but in coffee as well. Hence, the functional constraint on theentire HQT protein shows a transferase domain which is highlyconserved. This data is a prerequisite to study CGA biosynthesis inunstudied wild resources of eggplant and further extend the study toanalysis of expression profiles.

2.2.4 Implication of protein structure databases for HQT study

Pfam is a database of protein families that includes their annotations and multiple sequence alignments generated using hidden Markov models. The Pfam database is a widely used resource for classifying protein sequences into families and domains (Mistry et. al, 2020). Pfam 33.1 (the latest launch) contains 18,259 families and 635 clans. The Pfam website provides different ways to access the database content, providing both graphical representations of and interactive access to the data (Finn et. al, 2014).

Using this bioinformatic tool the HQT protein from *Solanum melongena*has been studied. The search results show that out of the 427 amino acids in the protein sequence, 1-425 fall into the transferase family of enzymes which points to its highly conserved nature (as seen

from the Multalin study, Fig. 12).This further solidifies the fact that the entire HQT protein is a conserved domain.

Sequence search results

Show the detailed description of this results page.

We found 1 Pfam-A match to your search sequence (**all** significant)

Transferase

Show the search options and sequence that you submitted.

Return to the search form to look for Pfam domains on a new sequence.

Significant Pfam-A Matches

Show or hide all alignments.

Family	Description	Entry type	Clan	Envelope		Alignment		HMM		HMM length	Bit score	E-value	Predicted active sites	Show/hide alignment
				Start	End	Start	End	From	To					
Transferase	Transferase family	Domain	CL0149	1	427	1	425	1	432	434	347.8	7.5e-104	n/a	Show

Fig12. Entire protein domain of the HQT enzyme shows transferase activity as seen from the Pfam database. (source: EMBL-EBI,Pfam database.)

The Protein Databank Europe or PDBe is another useful protein database that can help us visualise the protein at its crystal level in 3-D viewing. The HQT enzyme crystal structure when visualised using this tool gave the following structures:

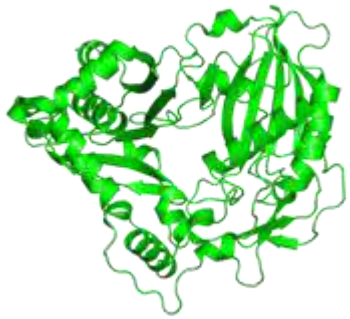


Fig13. 3-D structure of Hydroxycinnamoyl transferase showing secondary and tertiary foldings. (Source: PDBe database).

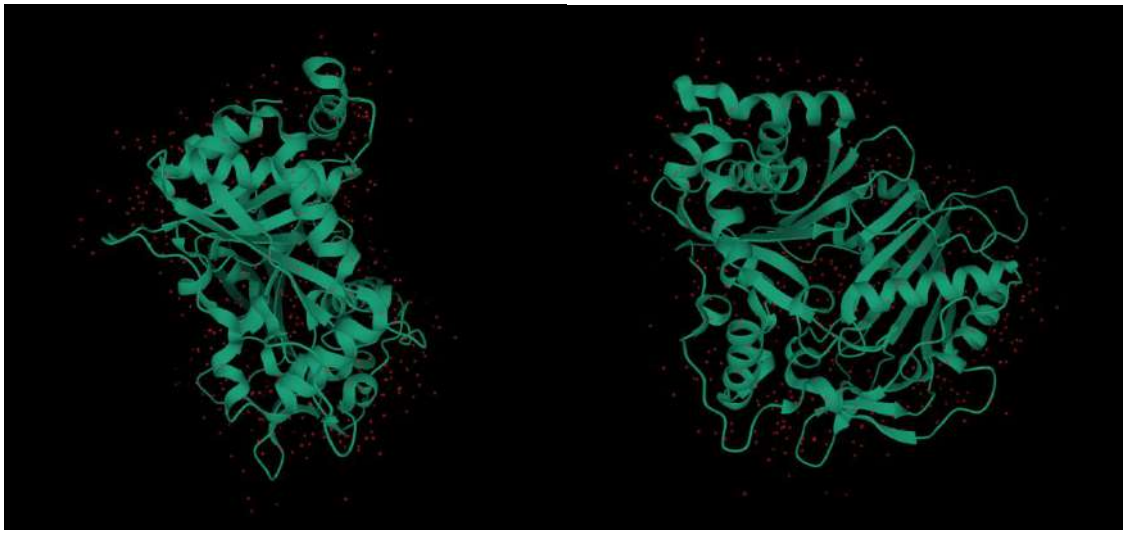


Fig14. Three-dimensional structure of HQT showing the water molecules as red dots all around the protein in different viewpoints. (Source:PDBe database).

Conclusion and future prospects

The biosynthesis pathway leading to the production of chlorogenic acid (CGA) via the Hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase (HQT) protein in *Solanum melongena* is a highly conserved pathway conferring resistance to the plant from various pathogenic attacks including wilt protection. Therefore, the genes involved in this pathway are very important sets of conserved to semi-conserved proteins.They are appropriate proteins to be screened among unexplored wild relatives of *S. melongena*. Current review addresses the in-silico tools that aid in the process of decoding defence mechanism via CGA route and also gives an outline of progress, made in this field. Taking into account the vulnerability of brinjal fruits to pest attack, rotting in unfavourable environments and other severe disease conditions, CGA pathway genes offer excellent scope to unravel crop wild relatives as the reservoir of resistance genes for improvement of the genetic background of eggplant cultivars.

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A. INTRODUCTION:

Haploid refers to those plants having gametophytic chromosome number which are developed through gametic cell division leading to callus formation subsequently to embryogenesis/gametogenesis. Haploid culture is the method where the haploid plants are produced from the haploid microspore cells when the anthers are excised in an aseptic condition at a critical stage and cultured on a medium.

The haploid plants production due to the totipotency of microspores, is the main strategy of anther culture. Different factors affect the anther culture in different crops. There are the remarkable impacts of the factors like genotype, stages of pollen development, physiological status of donor plants, anther wall factors, pretreatment, culture density and growth regulators in the initial anther culture. Various growth regulators like NAA, 2,4-D are responsible for induction of microspore embryogenesis. The culturing of pollens, at different developmental stages (e.g., pollen tetrad, young-uninucleate, mid-uninucleate, late-uninucleate, binucleate, and mature pollen) is found to be great importance for the induction of haploid plants. The response to the anther culture was controlled by genotypic factors. The induction phase of androgenesis depends on the nutrient medium. It was reported (Nitsch *et al*. 1969) that the existence of proper nutrients in the culture medium played an important role for the continued division of the induced microspores to the formation of embryos or callus. Stress pretreatment, cold (Huang & Sunderland, 1982) or osmotic (Roberts-Oehlschlager & Dunwell, 1990; Cistué *et al*., 1994,1999), sucrose substitution by maltose (Raquin,1983; Hunter, 1988), lowering the concentration of ammonium nitrate (Olsen, 1987) and glutamine synthesis (Henry & de Buyser, 1981; Olsen, 1987; Cho & Zapata, 1988) are utilized in the induction medium as they have been considered as the essential factors for enhancing the efficiency of androgenesis in different species. The application of colchicine for the induction of microspore division, to promote gametic embryogenesis in several species, including sugar beet (Levan 1945), maize (Hu *et al*. 1991), *Brassica* (Mollers *et al*. 1994), wheat (Barnabas *et al.* 1991) and rice (Alemano and Guiderdoni 1994), is well reported.

For the anther culture of *Brassica napus* and *Nicotiana*, and isolated microspores culture, liquid medium was proved to be the appropriate medium. There is the beneficial effect of the content of sucrose in the medium which seems to be species-specific. Breeding different crops through haploid culture techniques has the benefits for the efficient production of completely homozygous lines in one generation. Transgenes or stabilization in the genomes are recognized by the homozygous state for the utilization of double haploid plants within a very short time which reduces the amount of work in plant breeding. Androgenesis is the method where double haploid plants are produced by inducing chromosome doubling which plays an important role in the improvement of the plant breeding programmes. Many improved crops including rice varieties and cereals like barley, wheat, maize have been developed. (Kim *et al*., 2020) reported that the regeneration of microspores in *Raphanus sativus* L. were done by analysing QTL (Quantitative trait loci).

B. BRIEF HISTORY:

Anther Culture is the technique of culturing young anthers which is used to produce haploid plants under specific controlled conditions in laboratory. The first anther culture technique was reported by Guha and S.C. Maheswari in *Datura* in 1964. The effect of two new growth-promoting substances, kinetin and gibberellic acid on the growth of excised anthers of *Allium cepa* was shown by Vasil in 1957. It was found that gibberellic acid acts as a regulator of cell elongation and cell division. Tetrads and one-celled microspores were produced from the development of anthers of *Allium cepa* excised at leptotene or leptotene-zygotene stage that took place in media containing kinetin

and gibberellic acid. Therefore, it was concluded that the cell division is induced by kinetin and the number of cell division is increased due to the presence of gibberellic acid.

Guha and Maheswari in 1964 first reported *in-vitro* production of haploid plants derived from immature anthers of Solanaceous species *Datura innoxia*. The cell division occurs and embryos differentiation occurs in the pollen grains of *Datura* also shown by them in 1966. In 1967, Bourgin and Nitsch were the first workers to obtain haploid plants from culturing isolated anthers of *Nicotiana*. The development of haploid plants through *in vitro* culture of anthers and the pollen isolated was successful with many other crop species like rice, wheat, barley.

Niizeki *et al* (1971), found that the rice plants were also obtained by the anther culture. In 1973, Bhojwani *et al*. suggested the degradation of cytoplasmic information concerned in differentiation of gametophytes that takes place after mitosis of the vegetative cell in anthers of *Nicotiana tabacum*. According to Oono K, the production of haploid plants of rice (*Oryza sativa*) was developed by anther-culture and explained their use for breeding in 1975. In the same year, the anther-derived plants of *Hyoscyamus niger* L. were cultured by Corduan G. Two regeneration systems were followed for the anthers of *Hyoscyamus niger* L. In one system, plants are produced by development of embryoids and in the other system, plants are produced by development of callus out of microspores. For the production of haploid plants, anthers were both cultured in the early mononucleate stage of microspore development and in the tetrad stage of microspore development. Regulation of the ploidy level regenerates the anther-derived plants. Dunwell J.M. (1976), investigated the anthers of *Nicotiana tabacum* which was found that the factors like growth environment, age of the parent plant, and the stage of pollen development are highly responsible for the induction of embryoids in *Nicotiana tabacum.* The variation was found among doubled haploid lines obtained from anthers of *Nicotiana tabacum L.* by Burk, Matzinger (1976). Bajaj YPS, Reinert, Heberle (1977) demonstrated the factors required for enhancing *in vitro* production of haploid plants in anther and isolated microspore cultures. The concept of N6 medium and its applications like initiation, growth, and differentiation of callus in anther culture of cereal crops was explained by Chu C in 1978. It was proved that the N6 medium which consists of nutrient mixture of inorganic salts like vitamins, macroelements, microelements and amino acids was proved to be suitable for the anther culture of cereal crop. Baenziger along with his co-workers- Wesenberg, Galun, Feldman in 1983 studied about the variation among doubled haploid lines obtained from anthers of 'Kitt' wheat. The production of haploid plants through anther-culture of cereals and grasses was proposed by Wenzel G, Foroughi-Wehr (1984). In 1987, two regeneration systems involved for the production of wheat haploid plants from microspores was found by Armstrong TA *et al*. The formation of embryoids from microscopes cultured on P1 media was the first step for both systems. The first system involves the germination of embryogenesis occurs by induction of embryoids. The second system involves the production of plants via green centers: organogenesis. Bjørnstad *et al.* (1989) suggested the effects of donor plant environment and light during incubation of anther cultures of some spring wheat (*Triticum aestivum* L.) cultivars. The effect of composition and concentration of carbohydrate on anther-culture response in barley (*Hordeum vulgare* L) was also discovered by Finnie SJ *et al.* in the same year.

According to Barnabas, following Phaler and Kovacs in 1991 stated that the colchicine has direct effects on the microspore embryogenesis to produce di-haploid plants in wheat (*Triticum aestivum* L.). In 1992, the pollen derived plantlets from anther-culture of Ichang papeda hybrids was pointed out by Deng XX *et al*. Ball, Zhou, Shane T *et al*. demonstrated in 1993 that the growth hormone 2,4-D, Indole-3 acetic acid (IAA) is responsible for enhancing the callus induction in anther-culture of spring wheat. In the year 1994, Alemano L and his co-worker Guiderdoni proposed the effect of colchicine supplemented media on both anther culture efficiency and doubled haploid plant recovery frequency, which could be increased the doubled haploid plant production from rice (*Oryza sativa* L.) anther culture. The addition of colchicine had led to significant increase of anther callusing frequency

or ability of callus green plant production. In 1996, Yeung and other scientists Rahman, Thorpe found the comparative development of zygotic and microspore-derived embryos in *Brassica napus* L.

In 2001, Bagni,and Tassoni proposed the Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants which are required for *in vitro* organogenesis and embryogenesis. Castillo AM *et al*. in the same year preferred the efficient production of androgenic doubled-haploid mutants in barley by the application of sodium azide to anther and microspore cultures. Bouvier L, along with his co-workers Guerif, Durel in 2002 finalized the assay of isozyme markers with microsatellite markers for homozygosity assessment and chromosome doubling of pear haploid plants. The homozygosity of doubled haploid clones of pear was confirmed using isozyme markers and microsatellite markers, PCR-amplified with primers. Cistué L *et al.* (2003) proposed the doubled haploid production in barley anther culture. In 2004, Vagera J *et al.* reported that the androgenesis can be induced *in-vitro* in mutated populations of barley, *Hordeum vulgare*. Ashok Kumar HG *et al*. in that year found the outcome of polyamines on androgenesis of *Cucumis sativus* L. In 2005, renowned Indian scientist, Swapan kumar Dutta briefly explained the factors responsible for controlling the androgenic haploids and the applications of androgenesis for double-haploid production in crop improvement. Chiancone B *et al*. in 2006 worked on *Citrus clementina* and found the outcome of polyamines on the growth of plants of *Citrus clementina* by *in vitro* anther culture. The growth regulators like Aliphatic polyamines which includes SPD(Spermidine), SPM(Spermine) were found to be responsible for cell division, flower initiation and growth of pollen tube. Later many workers worked on different plants and concluded the somatic embryogenesis in tissue culture is induced by the Spermidine. In 2008, Broughton reported that the there is the beneficial consequence of the co-cultivation with ovary tissues in the anther culture of many cereal crops. In 2009, Castillo, with other co-workers found the Chromosome doubling in monocots. Moyo M *et al.* (2011) stated that in *Sclerocarya birrea* there is recalcitrant effects associated with the development of basal callus-like tissue on callogenesis and rhizogenesis. In 2014, Behar N *et al*. found the effect of explant type for the *in-vitro* micropropagation of an endangered medicinal plant (*Curcuma caesia* Roxb) production. In the same year 2014, Mohiuddin *et al*. reported the increased production of rice with high yielding varieties development. In the year 2020, (Kim *et al*.,) the regeneration of microspores in *Raphanus sativus* L. were done through genetic maps from maternal and paternal maps and the basis of the regeneration rate helps in analysing QTL (Quantitative trait loci).

C. TECHNIQUES OF HAPLOID CULTURE:

The techniques that are proved to be suitable for haploid culture are:

Liquid medium-

Liquid medium was suitable for the anther culture of *Brassica napus* and also for the culture of isolated protoplasts. It was observed that in the anther culture of *Brassica napus* on agar media, the plants were obtained from microspores (Thomas and Wenzel,1976; Wenzel *et aI*., 1977; Keller and Armstrong., 1978) but the microspores were not able to grow as the exposure of anthers were inappropriate in agar medium. A direct culture of isolated microspores was carried out in a liquid medium and the haploid plants were obtained in the isolated microspore cultures because the microspores were suitable with the all components of the liquid medium. The optimal conditions were provided to the microspores having the ability of morphogenesis. In *Nicotiana tabacum* the pollen grains having the ability of embryogenesis during the formation of early flower reported by Horner and Mott (1979). The application of liquid media for the induction of haploid plants in isolated microspores cultures (Nitsch, 1974).

Lichter (1981) reported that the anthers of *Nicotiana* cultured in a liquid medium opened and burst after few days of incubation and the microspores were released in the liquid medium. Anthers of *Brassica napus* did not open and as the microspores remain closed in the anthers, so the microspores were not released in the liquid medium unless the anther walls were opened due to the rupture of the growing embryoids.

The high sucrose content in the liquid medium was found to be relatively favorable for embryoid formation but unfavorable for plantlet development (Keller *et al*. 1975). The anthers of *Brassica napus* was cultivated on agar media containing 10% sucrose (Keller and Armstrong, 1978). To determine the effects of the sugar content, the anthers were placed in the liquid medium containing different amounts of sucrose. It was observed that the high concentration of sucrose would be optimal for the embryogenic anthers in the liquid medium.

The effect of hormone content in the liquid medium was also found to be of greater importance. The induction of embryogenesis of microspores in the anther culture of *Brassica* was influenced by the concentration of hormones like auxin and cytokinin (Lichter ,1981). It was found that due to the absence of any phytohormones embryoid formation could not occur in the liquid media.

In *Brassica*, the development of embryoids into plants in the liquid media were observed (Lichter,1981). After incubation period of about 1 month of the anthers of *Brassica*, the embryoids developed into a globular shape. Then they were transferred to the hormone-free medium having sucrose and developed into a cotyledon-like structure and a rhizoid pole or even roots. A system of stem embryogenesis developed in many embryoids (Thomas *et al*.,1976). After several transfers new embryoids were developed directly into plantlets.

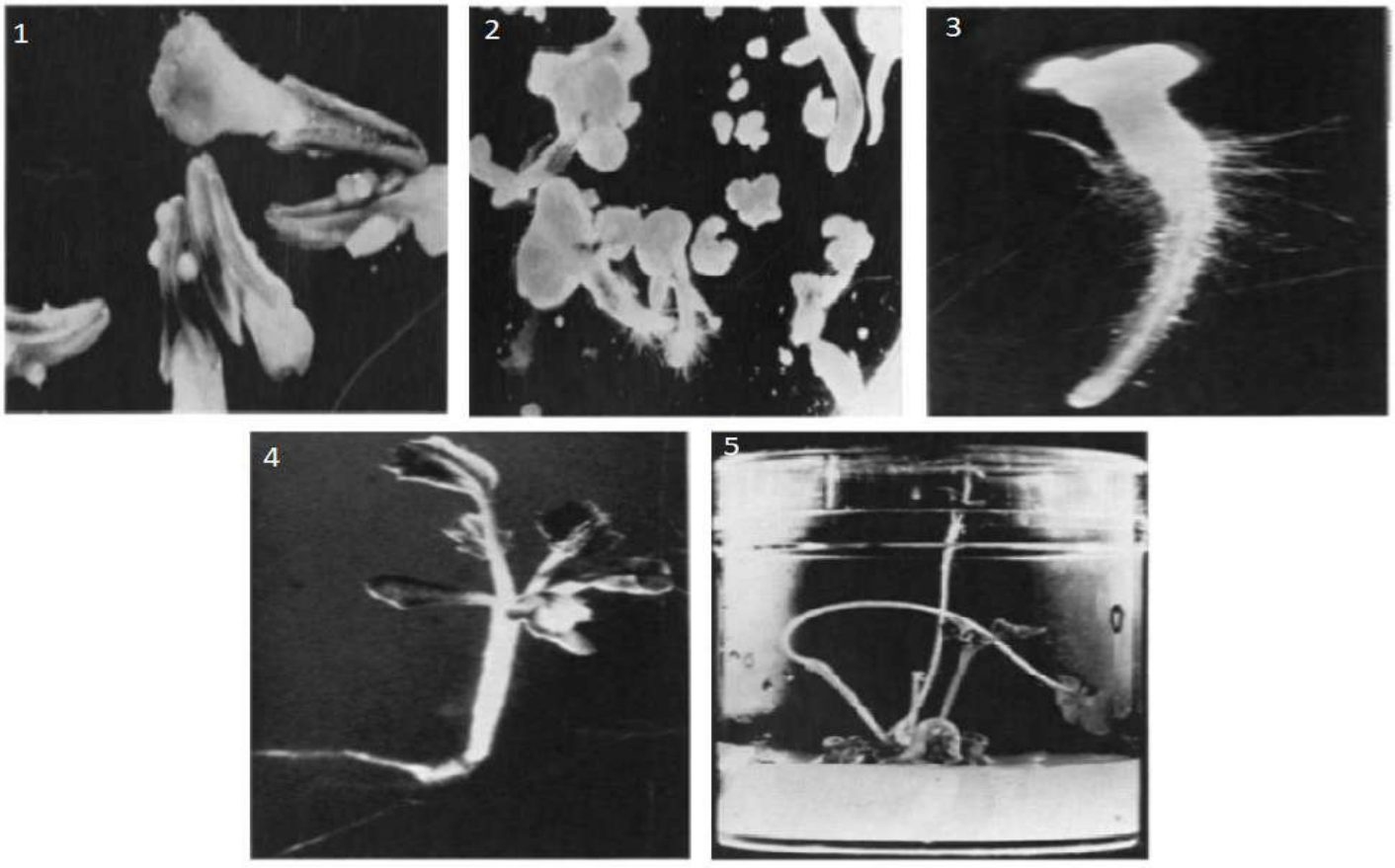
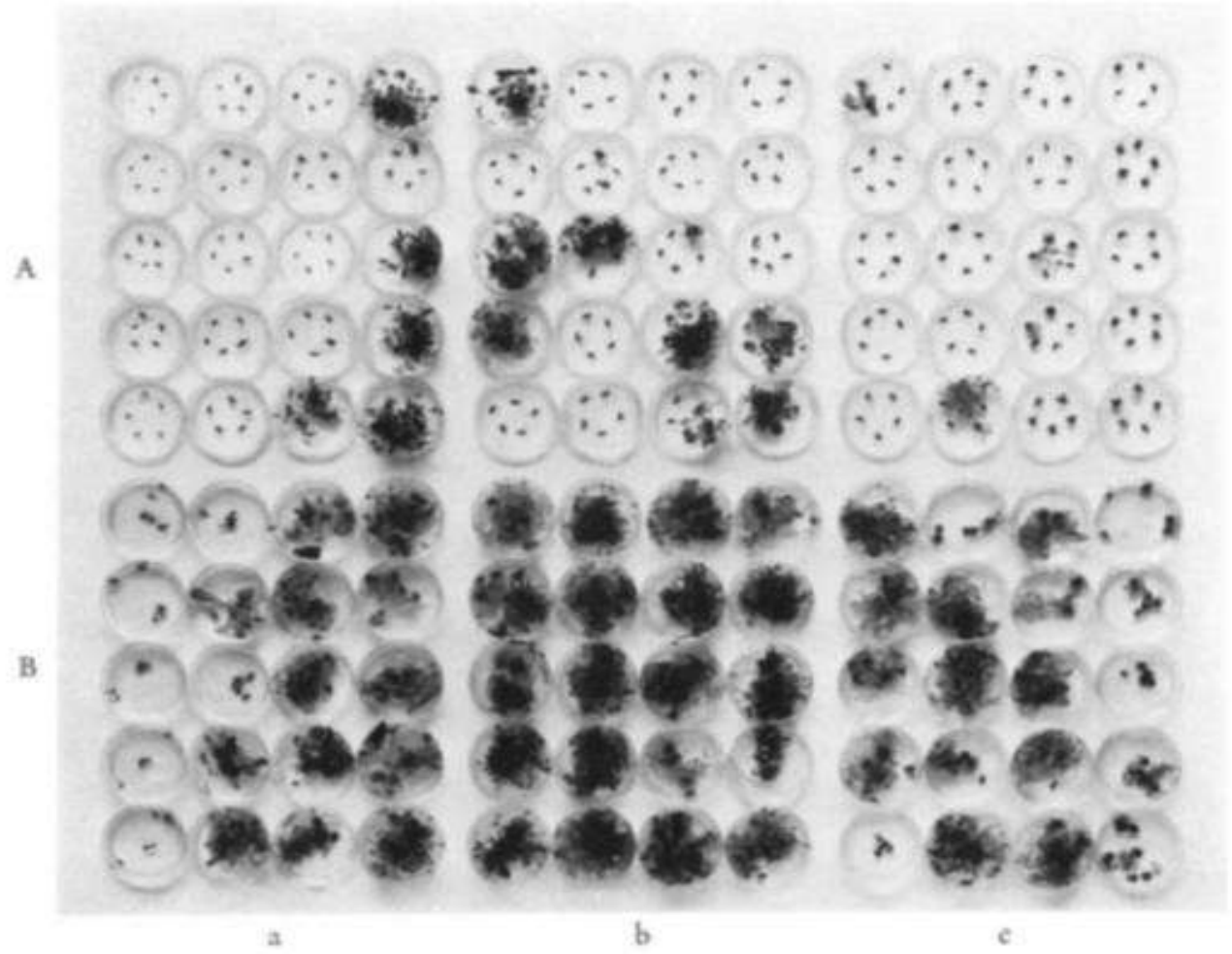


Fig 1-5: Development of embryoids from anther culture in liquid medium (Lichter, 1981) Fig: 1) Anthers with embryoids of globular shape. Fig. 2) Embryoids with cotyledon-like structures. Fig. 3) A seedling like embryoids and rhizoids. Fig. 4) Small plantlet from an embryoid. Fig. 5) Plantlets growing on embryogenic structures.

It was also found that the number of plantlet-producing anthers in *Nicotiana* were increased by the use of **liquid medium.** In 1967, the first successful anther culture of *Nicotiana* was performed on agar media by Bourgin and Nitsch. Subsequently the liquid media were also used and good embryoid formation was obtained. It was reported (Wernicke *et al*.,1976) that anthers of *Nicotiana* were seen floated on the surface of the liquid culture medium when they were kept onto the medium. The anther sacs burst and after about 4 weeks of culture the first embryos could be seen emerging from the bursting anther sacs. They developed into little plantlets within a few weeks. The percentage of plantlet-producing anthers was comparatively higher when culturing onto a liquid medium than culturing on a medium solidified with agar. The embryos were found dropped out of the anthers and formed suspended into the medium.



Comparison between (A) agar and (B) liquid culture medium in *Nicotiana tabacum* (Wernicke *et al*.,1976) a) anthers at the tetrad to uninucleate stage; b) anthers at about the first pollen mitosis; c) anthers at the binucleate stage.

In some instances, the concentration of embryos was found very high and so exhaustion of the medium occurred which caused the delaying of the further development. As the plantlets were so close in contact with the liquid, so an abnormal growth or the hyperhydric appearance was observed. Therefore, this could be stopped when the anthers are transferred to a solid medium after about some weeks of culture.

The liquid culture medium was not only considered as the suitable medium for anthers when cultured in the optimal stage of the first pollen mitosis. (Wernicke *et al*.,1976) reported that the best results found in anthers during the stage of tetrad to uninucleate and at the binucleate stage in the liquid culture medium than the anthers cultivated on an agar medium. Anthers were transferred after 7, 14, and 21 days from an agar medium to a

liquid medium and vice versa for determining the critical period of culture. It was found that the inhibition of the development obtained throughout the whole culture period; mainly during the first two weeks.

D. CYTOLOGICAL STUDIES:

It was found that the haploid plants were appropriate material for detailed cytological studies. (Ecochard *et al*. 1969) from the cytological evidences reported that the meiotic stages were observed in haploid chromosomes showing segmental homology during pachytene and first metaphase. Detailed investigations were studied on the cytology of the haploid in tomato plants (Humprey, I934; Lindstrom Koos, 1931; Newcomer, 1941; Rick, 1945). The cell division rate was found to be slow in the haploid roots. 12 somatic chromosomes were counted and identified in tomato (*Lycopersicon esculentum* L.) on the basis of the karyotypes reported by Ramanna & Prakken (1967).

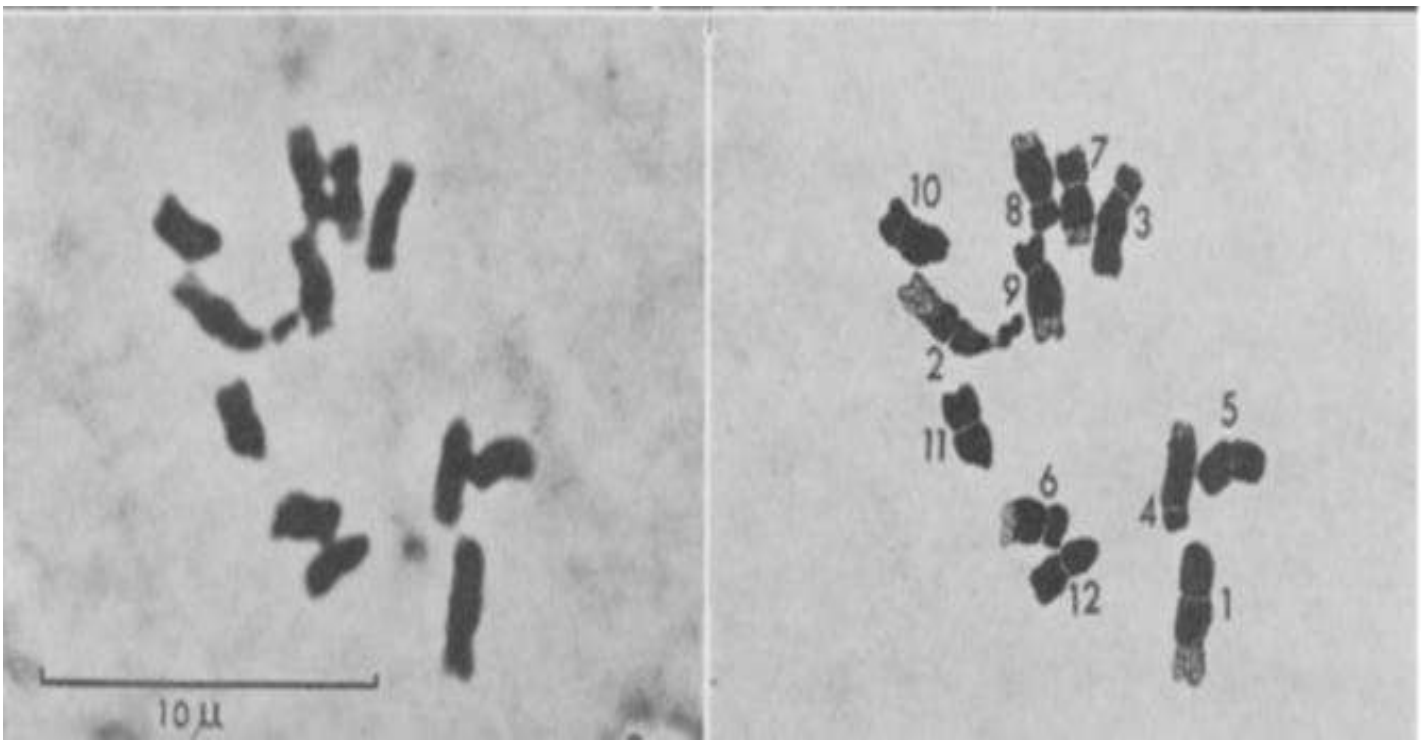


Fig: **Somatic chromosomes and their identification in *Lycopersicon esculentum L.* (Ramanna & Prakken,1967)**

The division stages from first prophase to second anaphase of meiosis was observable within the same anther. Ecochard *et al*. 1969, reported that at pachytene stage, proximal chromatic and distal achromatic parts are clearly visible in the chromosomes. In the pollen mother cells all the stages involved in meiosis could be easily identified and observed. In the tomato, haploid chromosomes were observed forming pairs during meiosis-I. The chromosomes observed in tomato exhibits pairing non-homologously in translocation monosomics (Rick & Khush, 1966). The univalent chromosomes were found in two groups at metaphase and anaphase-I (Ecochard *et al*. 1969). In the univalents, the identification of normal diploid pachytene chromosomes, centromeres, telomeres and each specific chromosome marker (Ramanna & Prakken, 1967) can be done. So, the univalent involved in non-homologous pairing is determined.

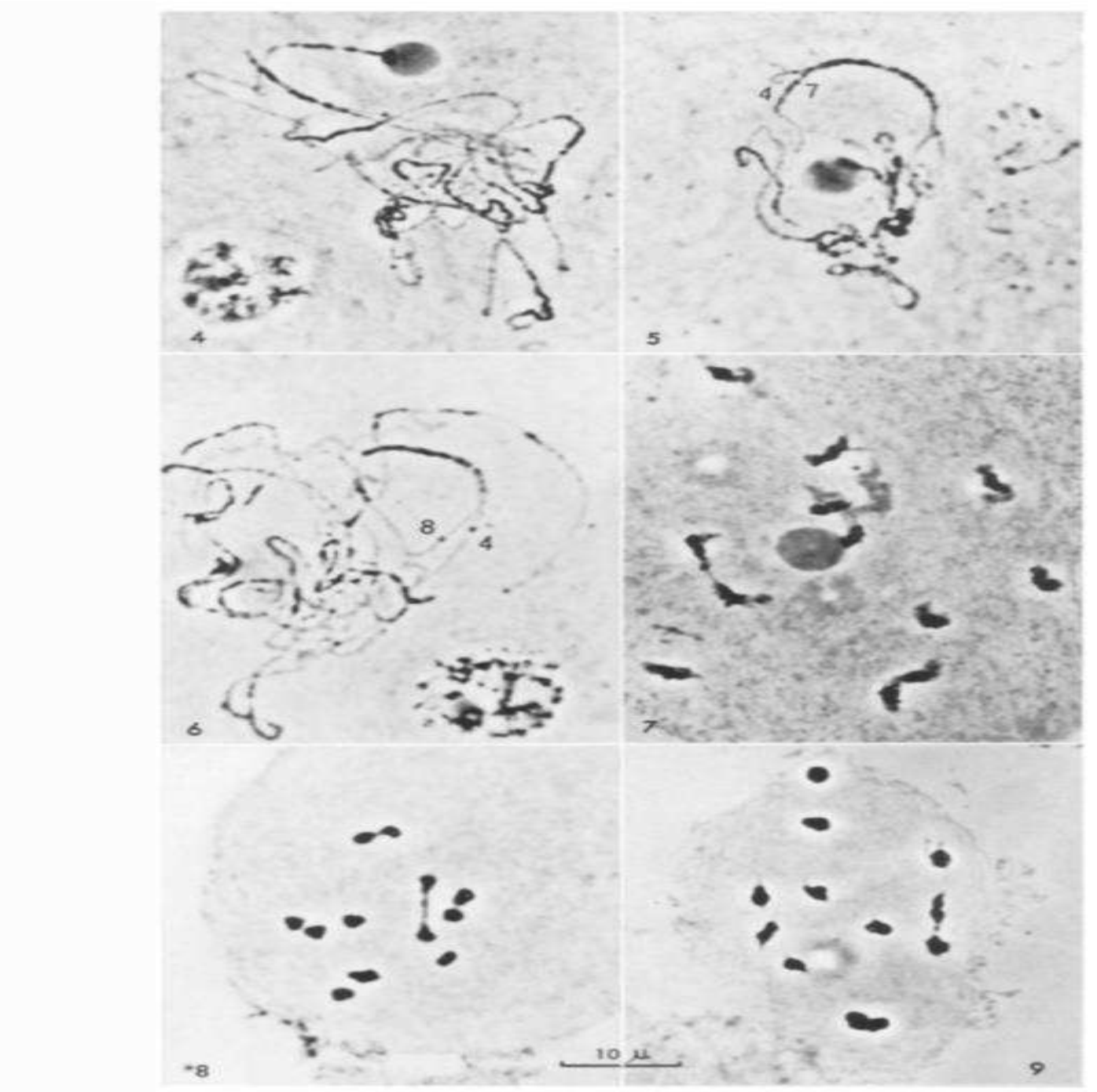


Fig: (4-9) Photographs showing meiotic chromosomes in tomato (Ecochard *et al*., 1969) 4- Pachytene, with 12 univalents. 5- Pachytene with paired chromosomes (4 and 7). 6- Pachytene with paired chromosomes (4 and 8). 7- Diakinesis with bivalents. The achromatic part of the nucleolar chromosome 2 is associated with a long achromatic segment of chromosome 1. 8- Metaphase I with one true bivalent. 9-Metaphase I chromosomes with spindle attachment.

The cytological evidences supporting x=5 in genus *Zea* as the result of the chromosome number and meiotic configuration of *Zea*. The chromosome number in *Zea mays* is 20. (Molina *et al*. in 1986) reported in *Zea mays*(2n=20), the formation of 10 bivalents in meiotic stage was observed and regarded as typical allotetraploid. It was also reported by Molina *et al*. in 1986 that the observation of secondary association and one to five groups of two bivalents in diakinesis-metaphase I stage.

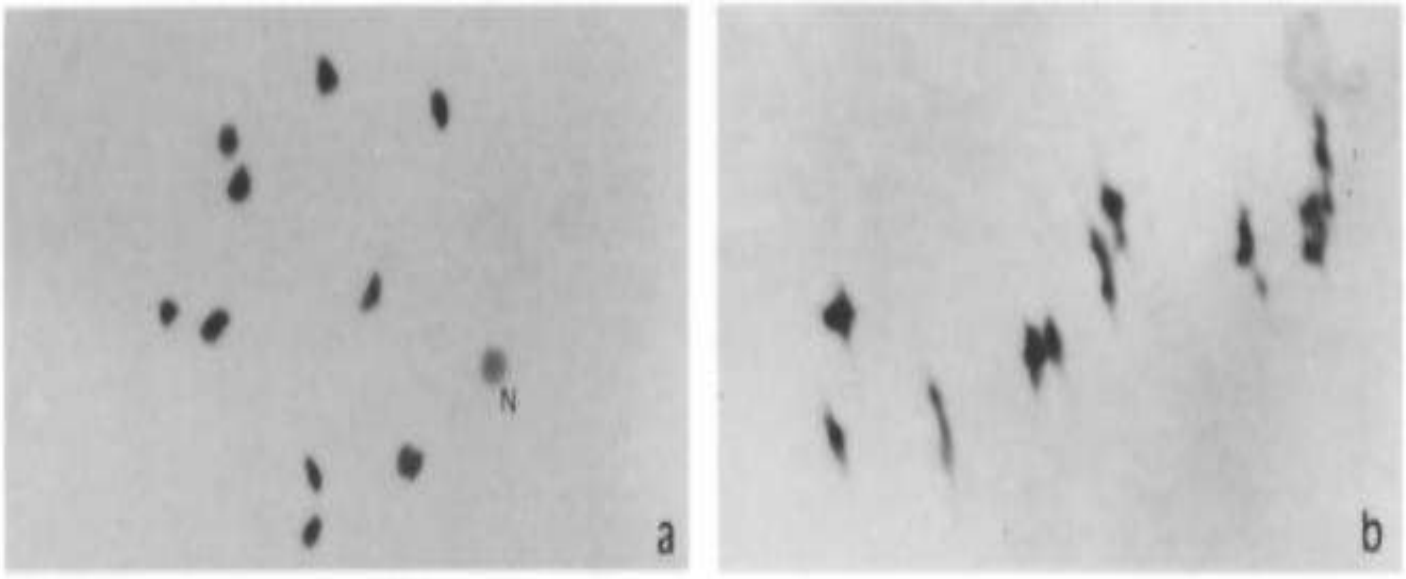


Fig: (a-b) Photographs showing meiotic chromosomes in *Zea mays* (2n =20) with 10 bivalents (Molina *et al*., 1986); a-Diakinesis stage. b- metaphase I stage.

E. FACTORS AFFECTING THE HAPLOID CULTURE:

Anther culture is used for the induction of haploid plant formation via androgenesis from male gametophytes like microspores or pollen grains. Homozygous plants are produced rapidly which express both the dominant and recessive traits when induction of chromosome doubling takes place. Androgenesis via anther culture was first successful in Solanaceae family by Guha and Maheswari.

There are many factors which affect the method of anther culture. They are discussed below:

a) **Physiological status of the donor plants**
b) **Stage of pollen development**
c) **Anther wall factor(s)**
d) **Genotype**
e) **Pre-treatment of cultured anthers/pollen grains**
f) **Culture medium**
g) **Culture density**
h) **Effect of light**

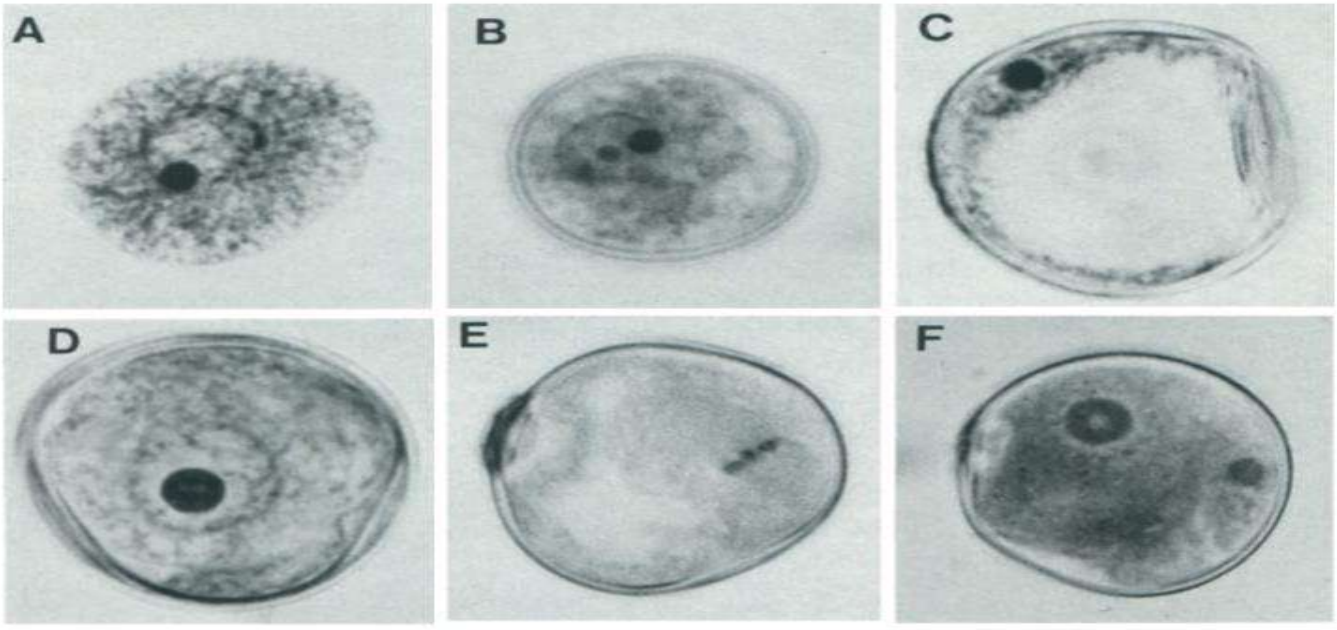
Physiological status of the donor plants

Both callus induction and organogenesis are affected by donor plant growth conditions. The physiological status of the donor plants and the season for the anthers explant which are responsible for the formation of callus was reported by (Dunwell *et al*., 1985). The rate of callus formation is dissimilar in different seasons i.e. in various temperatures. So, it was conformed (Collins 1977; Nitsch *et al*. 1982) that optimum conditions are required for anther culture in different plants species and some particular environmental stresses are required for the production of embryogenesis (Sunderland 1978). Collection of anthers from plants should be supplied with proper nutrients and maintained in a healthy state. At the beginning of the flowering season, flowers taken from

the relatively young plants are considered as more responsive. The anthers that are taken from field-grown plants showed a better response in comparison to those that are taken from greenhouse-grown plants reported by Vasil (1980). Although the optimal growth conditions vary from species to species, but the anther culture response in the temperature under which the growth of donor plants has been determined by studies on barley (Foroughi-Wehr and Mix 1976), oilseed rape (Keller and Stringham 1978; Dunwell and Cornish 1985). In *Brassica napus*, the yielding of pollen embryos was improved when the donor plants grown under lower temperatures reported by Bhojwani and Razdan in 1996. In *Nicotiana,* Dunwell in 1981 examined the outcome of preculture environment and the effect of both photoperiod and light intensity for yielding the microspore embryos. The physiological status of the anthers in the panicles are determined by conditions which are required for the growth of donor plants (Szarejko, 2003). The number of pollen grains are enhanced but unfavourable for the plants development due to the presence of low temperature in short days reported by Heberle-Bors and Reinert, 1981; Heberle-Bors, 1989.

Stages of pollen development

There is an optimal stage of pollen development for each individual species. The stages of pollen development at the time of anther excision and culture are the important factor which affect the induction of androgenesis. The embryos or callus production in response to anther culture generally takes place within the short period of microsporogenesis. The optimal stage for response is different in different species. Chen *et al*. (1991) studied the relationship between pollen stage and plant production in rice and demonstrated the mid-uninucleate microspore stage was the proper pollen stage for the anther culture of rice crops.



A-F: Photographs showing various stages of pollen development in rice (Chen *et al*.,1991). A) Early uninucleate microspore. Microspore just released from callose wall of the microsporocyte; note the absence of vacuole. Nucleus located in the center of the dense cytoplasm, and small nucleolus. B) Early uninucleate microspore. Exine and intine are visible. C) Mid-uninucleate microspore - a large vacuole is formed, nucleus located at one end of the microspore, with small nucleolus. D) Late uninucleate microspore, vacuole has disappeared, nucleus moved away from the end of the microspore, and nucleolus has enlarged. E) First mitosis. F) Bicellular microspore with a large vegetative and a small generative cell.

Calli which were obtained from older microspores exhibited a lower capacity for plant regeneration and calli which were derived from microspores at optimal stage produced less albino plants (C.C. Chen 1977, Genovesi and Magill 1979). The optimal responsive stage of cereals was found to be responsive in the mid -uninucleate stage (Ouyang *et al*. 1973; Sunderland 1980; Miao *et al*., 1978). In *Brassica napus*, the best stages observed was at late-uninucleate and early-bicellular stages of pollen mitosis for the induction of first pollen grains reported by Telmer *et al*. (1992). In *Arabidopsis thaliana*, the microspores exhibit high embryogenic competence was observed at early meiosis (Gresshoff and Doy, 1972). In *Datura innoxia*, pollen at or just after pollen mitosis was considered as an optimal stage. In case of *Lycopersicon esculentum* Mill, according to some researchers, early mitosis was considered as an optimal stage while uninucleate stage was considered by some other researchers (Gresshoff and Doy 1972; Zamir *et al.* 1980; Zagorska *et al*. 1982; Summers *et al*. 1992). However, microspores in anthers from metaphase I to just before the tetrad stage of telophase at meiocyte, had shown better response (Seguí-Simarro and Nuez, 2005) for the induction of androgenesis in tomato (*Lycopersicon esculentum* Mill). Therefore, according to Chen *et al*. (1984), the most reliable method for the identification of the anther stage was the cytological analysis of the microspores. So, developmental stage of microspore during anther excision period is an important factor for the androgenetic induction.

Anther wall factor(s)

Anther wall also plays an important role to the induction of haploid plant in anther culture. According to Pelletier and Ilami (1972), the anther is emitted from the wall and functions in the cultured anther to promote embryogenesis from the pollen grain. Anther tissue attained the condition which favors pollen embryogenesis during the first day(s) that it is necessary that the anthers maintain this state until the embryoids can attain an autonomous function.

However, the low callus-forming ability caused by anther browning at an early stage in culture was probably due to the production of quinones which are toxic to the microspores. Many reports indicated that these results were the result of the wall factor. During the culture of rice anthers some process for minimizing the oxidation of phenolics may significantly increase the callus production ability. High temperature inside the transfer chamber should also be avoided because it appears to enhance the phenolic oxidation reaction. Cultures are maintained in the dark is also helpful because illumination is stimulatory to the production of phenolics. Kohlenbach *et al*. (1978) demonstrated that in liquid anther culture of tobacco, the tapetum material is responsible for the androgenetic development of isolated microspores. The tapetum layer has been thought to have a nutritive function in in vivo microsporegenesis. Therefore, the nourishment which is provided in the development of isolated pollen is provided by anther wall. Moss and Heslop-Harrison, 1967 found that the tapetum releases more DNA which are required for the development of the spores. Tapetum cells are beneficial because the synthesis of DNA is faster in polyploid than in diploid cells, and therefore they can be used as reservoirs of DNA when a sudden demand occurs. The reproducing cells with DNA is provided in the anther wall and other substances for the rapid multiplication of essential cells.

Genotype

There is the strong effect of the genotype of the donor plants on pollen plant formation. The genotype response to androgenesis (Anderson *et al*.,1987) in the development of callus from microspores is the most important factor. There are some species of plants which showed a better result to pollen embryo development. Anther culture is genetically controlled. Anthers of the japonica rice shows a better response than indica rice

subspecies. In anther culture of *Hordeum*, each genotype is different with respect to androgenic response. So, success of anther culture is predominantly dependent on genotype of donor of anther. It was observed by Jacobsen and Sopory in 1978 that high responding genotypes of potato have been obtained from crosses of low responding genotypes. In rice it was observed that there was the response of the genotypic differences occurred in anther culture (Guha-Mukherjee 1973; Lin *et al*. 1974; Oono 1975) and in different crops like in tomato (Gresshoff and Doy 1972), wheat (Bajaj *et al*. 1977), and maize (Miao *et al*. 1978; Genovesi and Collins 1982).

Pre-treatment of cultured anthers/pollen grains

The application of stress-pretreatment, cold (Huang & Suderland, 1982) or osmotic (Roberts-Oehlschlager & Dunwell, 1990; Cistue *et al*., 1994, 1999) was an important factor for the induction of androgenesis in different species. Certain physical treatments like temperature shock and chemical treatments like auxins, kinetin are required to cultured pollen grains or anthers prior to standard culture room conditions which has proved that they play an important role in the *in vitro* androgenesis. The application of appropriate treatments is essential for the good haploid production. In *Nicotiana tabacum*, the bud is pretreated at a temperature of 5^0C for 72 hours.

Temperature shock- Keller and Armstrong (1979) found in their work with *Brassica* species that the high temperature shock i.e. temperature treatment about 30 to 35°C when applied to fresh anthers, prior to incubation greatly induced anther culture response. Touraev *et al*. (1997) showed that it was possible to use the heat shock treatment for replacement of the starvation pretreatment. The best anther culture response levels in *Brassica* species were obtained when cultures were treated at 30°C for 14 days. When pretreatments such as chilling, high temperature, high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, ethanol, c-irradiation, high medium pH, heavy metal treatment are done in the anther culture, the induction of anther and in microspore culture are improved reviewed by Shariatpanahi *et al*. (2006). The dissolution of microtubules and dislodging of the spindle occurs causing abnormal division of the microspore nucleus by the temperature shock. It was proved that initial high temperature shock is essential in plants like oats, capsicum. The optimum temperature required for *Nicotiana tabacum* is 25^0C for the formation of embryoids. Androgenesis is stimulated when pretreatment of anthers is done at higher temperature. There was an increase in anther response with an increase in temperature resulted in the formation of number of albino plants (Wang *et al*. 1978). For the regeneration process in rice anther culture (Chen *et al*. 1982), it was found that slightly lower temperatures (20 to 25°C) were favorable.

In cold treatment, weak or non-viable anthers and microspores are killed and the material gets enriched in vigorous anthers. The anther culture of *Cyclamen persicum* Mill is pretreated at 5°C for first few days(Ishizaka and Uematsu, 1993) for the induction of androgenesis. It is possible that aging of the anther wall is retarded by cold pretreatment, allowing a higher proportion of microspores to change their developmental pattern from gametophytic to sporophytic. The frequency of symmetric divisions of the microspores leading to the formation of embryo is increased when cold treatment imposed earlier to the first pollen mitosis.

Culture medium

The composition of medium is one of the most important factors for determining the success of anther culture and the mode of development. The requirements of culture medium differs with respect to age and genotype of

the anther. The basal media that are commonly used for anther culture are N6 medium (Chu 1978), MS medium (Murashige and Skoog), Nitsch and Nitsch (1969) medium and B5 medium (Gamborg *et al*. 1968).

Sucrose is essential for androgenesis of many plant species, especially for the Gramineae. It was first demonstrated by Nitsch in 1969 for *Nicotiana* and by Sunderland in 1974 for *Datura innoxia*. Sugars are the source of carbon essential in the basal medium and also involved in osmo-regulation. In rice anther culture indicate that the osmotic pressure of the nutrient medium is at least partly responsible. The usual level of sucrose is 2–4% but higher concentration of sucrose favors androgenesis in cereals.

Chelated iron has been shown to play an important role in the induction of androgenesis. Globular embryos are differentiated into heart-shaped embryos and further into complete plants. Activated charcoal removes the inhibitory substances like both endogenous and exogenous growth hormones from the culture medium.

The presence of nitrate, ammonium salts as well as amino acids appear to play a very special role at different stages of the developmental process. However, glutamine is important for most of the plant species as an aid to achieving the *in vitro* differentiation of a cell into a complete plant. Pollen embryogenesis can be induced on a simple mineral-sucrose medium in plants like tobacco, yet for androgenesis to be completed, addition of certain growth regulators is required. For example, in cereal anthers, both growth hormones like auxins and cytokinins are required and optimal growth response depends on the endogenous level of these growth regulators.

Culture density

Culture density is one of the factors in isolated pollen culture and microspore culture response. The amount of plant regeneration can be enhanced when the culture density of microspores is influenced and there is the importance of the optimal density required for plant regeneration and the microspore development (Hoekstra *et al*., 1993). The cell density plays an important role in the differentiation of plantlets from calli. The effect of culture density in barley plants was studied by Davies and Morton(1998). According to the report, the maximum production of regenerant barley plants was found by isolated microspore culture (IMC) than anther culture at optimal cell densities.

Effect of light

The role of light in the induction of androgenesis has been studied by Maheshwari *et al*. in 1980. The action of light during the first stage of culture in liquid and solid medium was studied. The callus formation inhibits but the shoot formation is stimulated when the light act differentially on the two stages of androgenesis. For rice anther culture the light conditions that were used were assorted from complete darkness (Niizeki and Oono 1968; Harn 1969; Woo and Tung 1972; Guha-Mukherjee 1973) to continuous illumination (Nishi and Mitsuoka 1969; Niizeki and Oono 1971). Complete darkness and 16hr light period were compared, and it was obtained that for the initiation of callus, darkness was slightly more advantageous (Cornejo-Martin and Primo-Millo 1981). The light intensity was found not favorable for formation of the callus, plant regeneration has to be conducted under light, and higher light intensity was generally recommended for plant regeneration. In maize, the effect of two wavelengths, the blue (475 nm) and the red (630 nm) were obtained (Nitsch *et al*. 1982).

F. MEDIA COMPOSITION:

Plant tissue culture media must generally consist of the following components-

Basal media:

Clapham (1973) first discovered from barley microspores that the presence of higher content of ammonium ion in the LS medium (Linsmaier and Skoog 1965) was restrictive for the formation of callus. The N6 medium containing a low concentration of $(NH_4)_2S0_4$ and a high concentration of KNO_3 was developed by Chu *et al.* (1975). The N6 medium was later proved by (Chu 1978; Genovesi and Magill 1979; Chen *et al*. 1982; Tsay *et al*. 1982) to be the important medium for anther culture of rice and other cereals (Chu 1978; Miaoetal. 1978; Nitsch *et al*. 1982). It was obtained by Lin and Tsay (1974) that the formation of callus from the anthers of rice was four times higher cultured on N6 inorganic salts and MS inorganic substances supplemented with NAA and kinetin than those cultured on MS medium with the same plant growth regulators. Yeh and Tsay (1988) reported that more effective results of inorganic salts with N6 medium in delaying the browning and simultaneously the growth of callus was found to be increased compared to those of MS medium.

- **Macronutrients:**
  The nitrogen has effects on the pH of the medium and thus has indirect effects on the tissue culture (Dougall,1980; Congard *et al*.,1986). Six major macronutrients- nitrogen(N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S) are required for plant cell or tissue growth. They are essential for the morphogenesis. Calcium is the important constituent of the cell wall. The callus deposition is promoted and thus the extension of cell is inhibited due to the presence of high concentrations of calcium (Eklund and Eliasson,1990; Atkinson 1991). The presence of sulphur is considered as the important purity in agar (Pochet *et al*., 1991). The optimum concentrations of P, Mg, S, and Ca range from 1-3mM. Potassium is essential for cell growth of most plant species.

- **Micronutrients:**
  Iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo) in culture media are the essential micronutrients required for tissue culture. Chelated forms of iron and zinc are commonly utilized for the preparation of culture media. Iron is the most critical of all the micronutrients for the induction of androgenesis. Dalton *et al*. (1983) reported that the precipitation can cause due to the imbalance between iron and EDTA. Iron citrate and tartrate may be used in culture media, but these compounds are inconvenient to dissolve and often precipitate after the preparation of media.

- **Carbon source:**
  The sucrose is the preferred carbohydrate or carbon source required for plant cell and tissue culture media. The optimal concentration of sucrose is directed by the components of the solidifying agent (Chaleff and Stolarz in 1981) and the mineral salts in the culture medium. It may change with the developmental stage of the microspores. Chen in 1978 reported that there were the promotive effects of the high concentrations of sucrose on anthers at different developmental stages. High sucrose concentration favors better survival of pollen grains which increases the frequency of androgenesis especially in Gramineae family (Clapham 1973; Ouyang *et al*. 1973; Ono and Larter 1976; Miao *et al*. 1978). In the cultured tissues or cells, as the inhibition of the photosynthesis occurs, so carbohydrates

are needed for tissue growth in the medium. The sucrose in the medium is rapidly converted into glucose and fructose. Glucose is then utilized first, followed by fructose. Sucrose concentration of culture media is generally used at a concentration of 2–5%. of pollen grains, thus improving the frequency of androgenesis.

Growth hormones

The growth hormones like auxins, cytokinins, gibberellins and abscisic acid play an important role for pollen embryogenesis. In *Brassica* sp., both auxins and cytokinins are required for direct pollen embryogenesis. It was demonstrated by Skoog and Miller (1957) that the ration of auxin to cytokinin controlled the type and extent of organogenesis in anther culture. The addition of these ration of hormones is due to the production of morphogenesis. The application of auxins promotes the microspore development (Ball *et al*. 1993) with 2,4-D (2,4-dichlorophenoxy acetic acid) for the induction of callus formation and IAA (1H-indole-3-acetic acid), (alpha-naphthaleneacetic acid) NAA for the development of direct embryogenesis (Armstrong *et al*. 1987; Liang *et al*. 1987). They are used for the induction of rapid cell proliferation. The cytokinins are used in the culture media are BAP or 6-benzylaminopurine, kinetins for the stimulation of cell division, shoot formation and the inhibition of root formation. It was possible to induce the multinucleated pollen for the formation of embryos when cultured. Huang *et al*. in 1985 reported the effects of 2,4-D, NAA, and kinetin on formation of callus and plant regeneration in rice anther culture. Auxins 2,4-D and NAA were equally efficient in promoting microspore callus formation, but callus formed in the presence of 2,4-D is less capable of plant regeneration compared to that formed on medium supplemented with NAA. The optimal concentration of NAA is required in the callus formation medium. The callus formation and plant regeneration increase with the increase in the concentration of kinetin. The effect of two new growth-promoting substances, kinetin and gibberellic acid on the growth of excised anthers of *Allium cepa* was shown by Vasil in 1957. It was found that gibberellic acid acts as a regulator of cell elongation and cell division. Another growth hormone, abscisic acid added in the culture medium for the enhancement of callus growth, shoot or bud proliferation.

Undefined organic supplements:

The tissue culture is favorable in the presence of organic supplements like ground banana, coconut milk, yeast extracts, protein hydrolysates, malt extracts in the culture media was reported (Guha *et al*. 1970; Guha-Mukherjee 1973; Wang *et al*. 1974; Oono 1975; Liang 1978). There is the beneficial effect in the microspore embryogenesis of different species if activated charcoal is added in the culture media (Bajaj 1990). The effect of activated charcoal is generally attributed to the absorption or removal of inhibitory compounds or toxic media substances from the culture medium excreted by the anther wall and the level of growth regulators are regulated (Reinert and Bajaj 1977; Vasil 1980; Heberle-Bros 1985). Example in soyabean, when activated charcoal is added to culture medium, the cell growth is inhibited.

Amino acids:

The results for the supplementation of exogenous aliphatic polyamines in the culture medium was reported for the increase in induction of embryos in potato (Tiainen, 1992), in Indian wheat cultivars (Rajyalakshmi *et al*. 1995), in cucumber (Ashok kumar *et al*. 2004) and in clementine (Chiancone *et al*. 2006). The effects of

glutamine, proline for the tissue culture media was reported by (Reinert and Bajaj, 1977; Powell 1990; Achar 2002). The plant cells are provided with amino acids which is the source of nitrogen for the tissue culture induction. The most common sources of organic nitrogen used in culture media are the amino acid mixtures (e.g., casein hydrolysate), L-glutamine, L-asparagine, and adenine. Examples of amino acids included in culture media are glycine, glutamine, asparagine, L-arginine and cysteine, and L-tyrosine which are all used to enhance cell growth. For the stimulation of morphogenesis in cell cultures.

Vitamins:

Plants require vitamins as catalysts for various metabolic processes. In tissue culture media, vitamins like thiamin(B_1). Nicotinic acid, pyridoxine(B_6) are used. Thiamin(B_1) and Nicotinic acid are required for the induction of good levels of response of anther culture. The cooperative interaction of the thiamin with the cytokinin was reported by (Digby and Skoog, 1966). Drew and Smith (1986) suggested that the vitamin, riboflavin is required for the inhibition of the callus formation and thus the quality of the shoot growth is improved.

Solidifying agents:

The most commonly used gelling agent used for the plant tissue culture media is Agar. The higher the agar concentration, the stronger is the water binding. The effects of gelling agents were studied and it was reported that the gelrite is the gelling agent which is found to be the essential one in anther culture of *Oryza sativa*. The reaction of Agar gel with the media constituents do not occur and are not digested by the enzymes present in the plant which remains stable at all feasible incubation temperatures. Dunwell (2010) reported that the anthers which floated on liquid medium covered with agar-solidified medium. The agar concentrations commonly used in the media are 0.5%- 1.0%. In maize, anthers when placed on the agarose-based medium responded significantly better. Gelrite, another gelling agent which is synthetic aids in detecting contamination.

pH:

Another important factor for the induction of gametic embryogenesis is pH (Stuart *et al*. 1987). The pH of the media varies in the anther culture and it was established that before autoclaving process, the pH of the culture media is in the acid range about 5.7-5.8.

G. HAPLOID CULTURE IN FEW CROPS:

Anther culture in monocot plants (*Oryza sativa*)

Niizeki and Oono in 1968 first reported the production of haploid plants of rice (*Oryza sativa L*.) through anther culture. Chu *et al*. (1975) suggested the N6 medium containing the low concentration of $(NH_4)_2S0_4$ and a high concentration of $KN0_3$ was efficient for production of anther culture in rice (Chu 1978; Genovesi and Magill 1979; L.J. Chen *et al*. 1982; Tsay *et al*. 1982). The effects of B5 (Gamborg *et al*. 1968) and a modified LS medium (R3, Chaleff and Stolarz 1981) was found to give good results for having the higher concentration of

nitrate-nitrogen and lower concentration of ammonium-nitrogen (Chaleff and Stolarz 1981; Zapata *et al*. 1982). Glycine, ammonium nitrate, Inositol, Nicotinic acid was the basal media that was commonly used in rice anther culture reported by Chen *et al*. Lin and Tsay (1974) reported that the first pretreatment of the inflorescence of rice was done at the temperature of 8^0C for 1 week, and then anthers were dissected and inoculated on N6 inorganic salts and MS organic substances with the addition of NAA and kinetin. It was obtained that the formation of callus from the anthers of rice was four times higher cultured on N6 inorganic salts and MS inorganic substances added with NAA and kinetin than those cultured on MS medium with the same plant growth regulators. According to Chen *et al.* (1991) the technique of anther culture for the japonica subspecies of rice has been found. Many haploids and spontaneously doubled haploids are readily attained for practical uses. The effects of the addition of organic supplements like yeast extracts, casein hydrolysate and coconut water in the N6 media enhanced induction of androgenic callus in indica rice varieties (Roy and Mandal, 2005).

It was observed by Chen and Lin (1976) that collection and culturing of anthers were more productive at the beginning of the flowering period than those harvested at the end of the flowering period. The success of the anther culture in *Oryza sativa* depends on genotype and physiological state of the donor plants. Chen *et al*., 1991 reported the reproductive growth especially the meiosis of pollen mother cells in *Oryza sativa* plants, was found more sensitive to extreme temperature than vegetative growth. It was shown by histological studies that microspore formation was inhibited by low temperature and abnormal multiplication of tapetum layer cells was obtained.

The stages of pollen development at the time of anther excision and culture are the important factor for the induction of androgenesis in *Oryza sativa* (Cha-um *et al*, 2009; Silva, 2010) and the best response occurs in *Oryza sativa* only at the mid-uninucleate microspore stage (Chen *at al*., 1991).

The anther culture in rice involves the two-step process- the initial development of calli and green plants regeneration from embryogenic calli. The basal medium containing high content of nitrate-nitrogen and low concentration of ammonium-nitrogen, about 4-6% sucrose, 2 mg/I NAA, and 1 mg/l kinetin is considered as the most suitable medium for callus formation. The collection of young rice panicles done when the stages of microspore are at mid- to late-uninucleate stage as the early or mid- to late-uninucleate stage is considered as the best responsive stage in anther culture of rice (Datta and Wenzel, 1998). The detached panicles are then wiped and are pretreated with a cold temperature shock (8 to 1O°C) for 8 to 14 days has proved to be beneficial for the culture. Incubation of the inoculated anther cultures are preferably done in complete darkness at 28°C. The MS basal medium containing sucrose supplemented with 0.5 mg/l NAA and 2 mg/I kinetin has put forward for plant regeneration. The transfer of callus onto the regeneration medium usually done 10 days after emergence and kept at the temperature of 20 to 25°C under artificial light for callus regeneration. If failure of callus formation occurs at the induction phase, then various pretreatments are tried. If failure of callus formation occurs due to the inability of the pollen to support division and growth, then improvement in culture medium and culture methods should be attempted. The transfer of the green plantlets to the rooting medium and then the transfer of the rooted plants to the pots (Mishra *et al*, 2013).

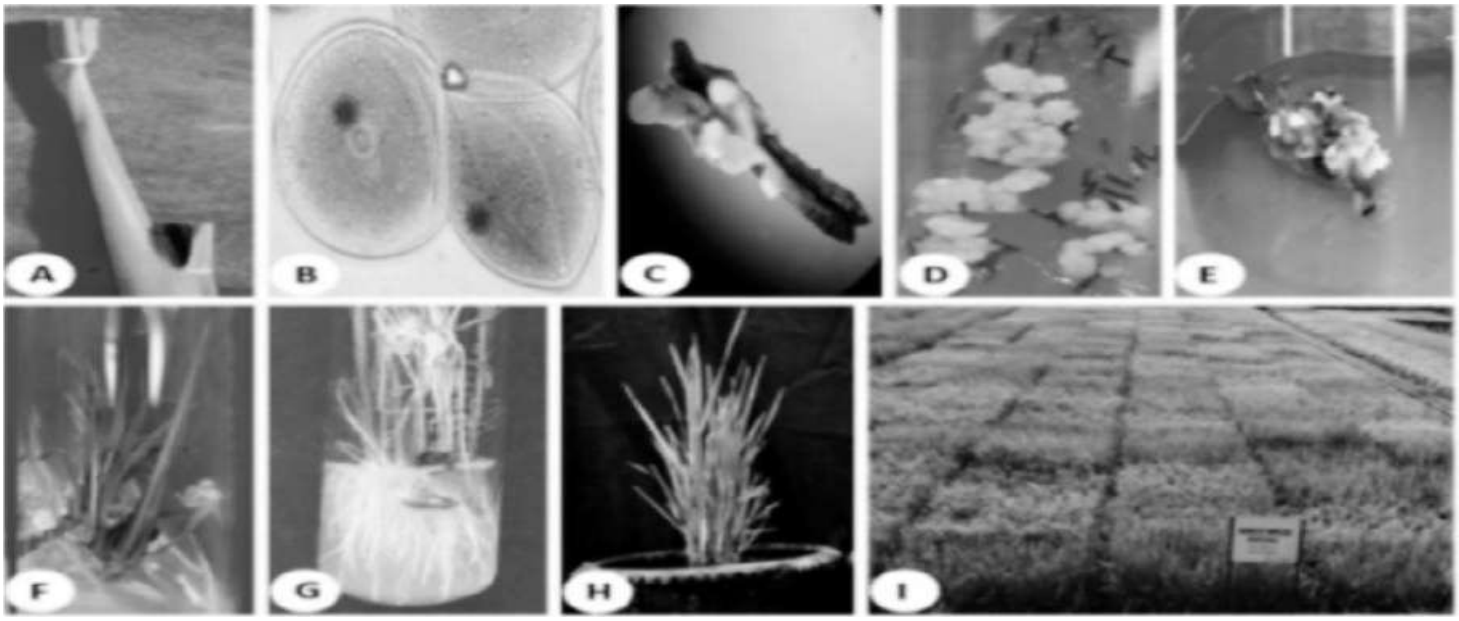


FIG: Photographs showing *In vitro* androgenesis and plant regeneration in rice (Mishra R., 2014)A. Young detached panicles collected having microspores at mid- to late-uninucleate stage; B. Cytological confirmation of mid- to late-uninucleate stage; C. Microscopic view of anther with multiple calli; D. Callus induction; E. Callus regeneration; F. Green plant regeneration; G. Rooting; H. Anther derived plants in net-house condition; I. Anther derived plants in field for agronomic evaluation.

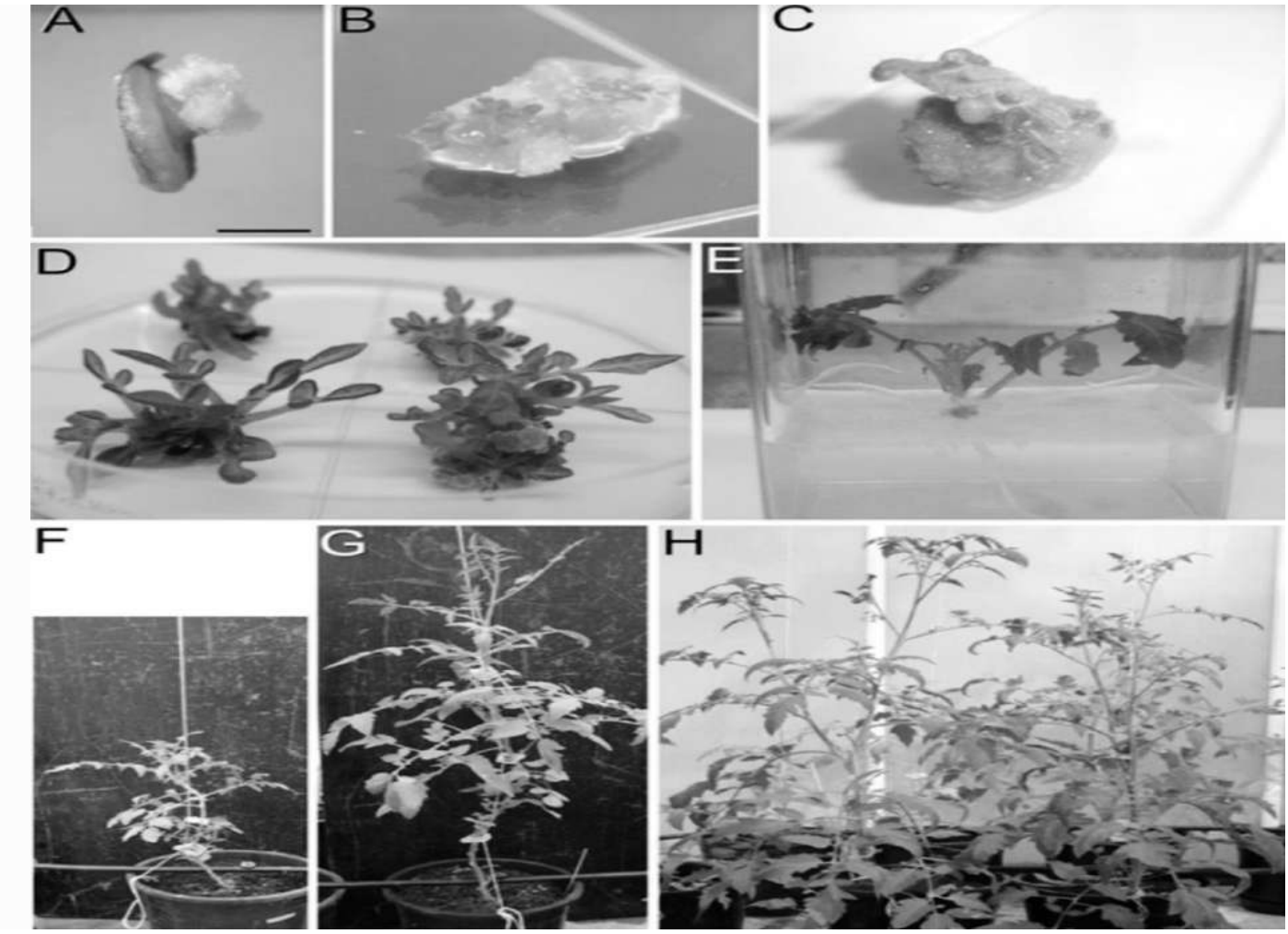
Anther culture in vegetable:(*Lycopersicon esculentum*)

In vegetables like tomato, spontaneous haploid plants were found by Morrison in 1932. The anther culture of tomato (*Lycopersicon esculentum* L.) was first reported by Sharp *et al*. in 1971. It was found that the improvement of tomato androgenesis can occur by controlling the microspore developmental stage, the growth conditions of the donor plants and improving the culture medium, especially both growth regulators, and the cultivation conditions (Sharp *et al*. 1971; Gresshoff and Doy 1972; Debergh and Nitsch 1973; Zagorska *et al*. 1982; Summers *et al*. 1992). It was found that the microspore stages, temperature, medium and the genotype were the important factors for the induction of haploid plants. The pH changes in the medium also responsible for the induction of androgenesis in tomato. The meiotic metaphase I through telophase II is represented through the excision stages of tomato plants (Seguı-Simarro and Nuez 2005). The responsive stage obtained was the first pollen mitosis (Touraev *et al*. 2001), however, in tomato anther culture meiocyte was found as the inducible stage (Gresshoff and Doy 1972; Seguı́-Simarro and Nuez 2005, 2007; Shtereva *et al*. 1998; Summers *et al*. 1992; Zamir *et al*. 1980). Another important factor is the genotype for the induction of androgenesis. In different tomato cultivars, it was obtained that some male- sterile mutant lines especially sensitive to the induction of androgenic calli (Seguı́-Simarro and Nuez 2005,2007; Shtereva *et al*. 1998; Zagorska *et al*. 1998, 2004; Zamir *et al*. 1980).

The chromosome number was found haploid in low content of regenerated plants. However, according to Chlyah and Taarji (1984), the haploid callus was obtained from the low amount of calli from anthers and once many shoots were enhanced by successive subcultures. The haploid callus but no organogenesis was obtained when the culture of pollen grains of tomato was segregated from the nurse culture with the help of a small filter paper. Nitsch (1973) reported that the liquid medium was suitable for the development of first stages of embryo formation in anthers of *Lycopersicon esculentum.* The isolation of anthers from plants at the beginning stages

of flowering produced the higher content of callus formation and organogenesis (Shtereva *et al*. 1998). The two different stages were found as optimal stages in tomato. The shoots which were obtained was the best result for anthers in meiosis stage (Gresshoff and Doy (1972). Gao *et al*. (1980) reported the uninucleate microspore stage as the optimal stage in tomato. The prophase stage in meiosis as it was a long process therefore the size of the flower bud and the anther in early prophase I or late telophase II was distinguishable. It was also found that the correlations between the flower length, anther length and the stage of microsporogenesis was poor and they were determined by many factors like temperature of donor plants, stages of microspore development. When the callus-inducing medium was used in initial stage, this stage was carried out either in darkness (Gresshoff and Doy 1972) or in 10hr photoperiod (Gao *et al*. 1980). The single step of the callus-inducing medium was also carried out in 16hr photoperiod.

The role of light in two stages of androgenesis inhibits callus and shoot formation is stimulated (Chlyah *et al.*,1990). The temperature about 25^0C -27^0C was found favorable in tomato culture (Gao *et al*. 1980). Cold pretreatment about 4^0C was applied to excised tomato flower buds in early stages of meiosis for about the time of 24, 48 or 72 hr before anther culture or to the beginning of the culture for about 24hr or 48hr. It was obtained that the callus formation was stimulated in all the treatments (Chlyah *et al*.,1990).



REFERENCE: Photographs showing Anther culture and plant regeneration in tomato (Patricia *et al*., 2011). a Anther with a young callus emerging from the anther locule. b Callus with shoot initials at its surface. c Regenerating shoot over the surface of the callus. d Developing shoots and leaves over old, necrosing calli. e *In vitro*, regenerated and rooted tomato plantlet. f–g Tomato plants regenerated from anther cultures, acclimated

and grown at the greenhouse. f Haploid regenerant. g DH regenerant, h normal tomato plants, used as donors of anthers.

Anther culture in cash crop:(*Hordeum vulgare*)

The haploid plants are obtained from the anther culture in barley (*Hordeum vulgare*) was first reported by Clapham D in 1973. The regeneration of plants was done through *in vitro* from anthers or isolated microspores in barley (Bednarek *et al*. 2007). For the production of many embryos in barley, the use of defined medium played an important role in an isolated microspore culture system, such as donor plant growth (Hunter, 1988), nitrogen source and balance (Olsen, 1987; Mordhorst and Lörz, 1993),sugar type (Hunter, 1988; Scott and Lyne, 1994), high glutamine (Olsen 1987) hormones (Ziauddin *et al*., 1992), density and osmolality (Hoekstra *et al*., 1993) and pretreatments (Oehlschlager and Dunwell, 1990).The ability of androgenesis in barley is determined by the developmental stages of microspores (Jacquard *et al*.,2003). The anthers of *Hordeum vulgare* were cultured at uninucleate stage. Kao 1981; Olsen 1987 reported the effect of ficoll in anther culture of barley. It was confirmed that the regeneration of plants and the quality and amount of embryoids can be improved and increased by ficoll. Hou *et al*. 1993, reported the beneficial effect of gelrite in anther culture of barley. Further it was found the beneficial effect of filcoll is more in comparison to gelrite in barley anther culture.

The two distinct mechanisms-embryogenesis (induction and development of a microspore embryo) and plant regeneration results the androgenic response. Ohnoutkova *et al*. ,2019 studied the process of anther culture in barley. It was found that the induction of pollen embryogenesis occurs after few days of keeping the cultured anthers in dark at the temperature of 26^0C which was observed with acetocarmine staining. When the pollen embryos or calli were found to be grown after some weeks of cultivation of anther, they were transferred onto the regeneration media. Well developed rootlets are found to be grown in the plantlets and then these plantlets with well-developed roots are transferred to Jiffy peat and kept in a plastic bag until the plants from barley anthers are tested for their ploidy level for several days so that the humidity is maintained until.

In anthers of barley (*Hordeum vulgare*), male meiosis takes place in specialized cells called meiocytes. Flow-cytometric estimation of DNA content regulates the ploidy of regenerated plants from barley anthers. It was obtained that when anthers are collected from triplets, early prophase I (leptotene and zygotene) was observed. Based on cytological evidences in leptotene and zygotene stage the frequency of actual recombination process is likely occurs(Fig-1C) (Barakate *et al*., 2014; Colas *et al.*, 2016).

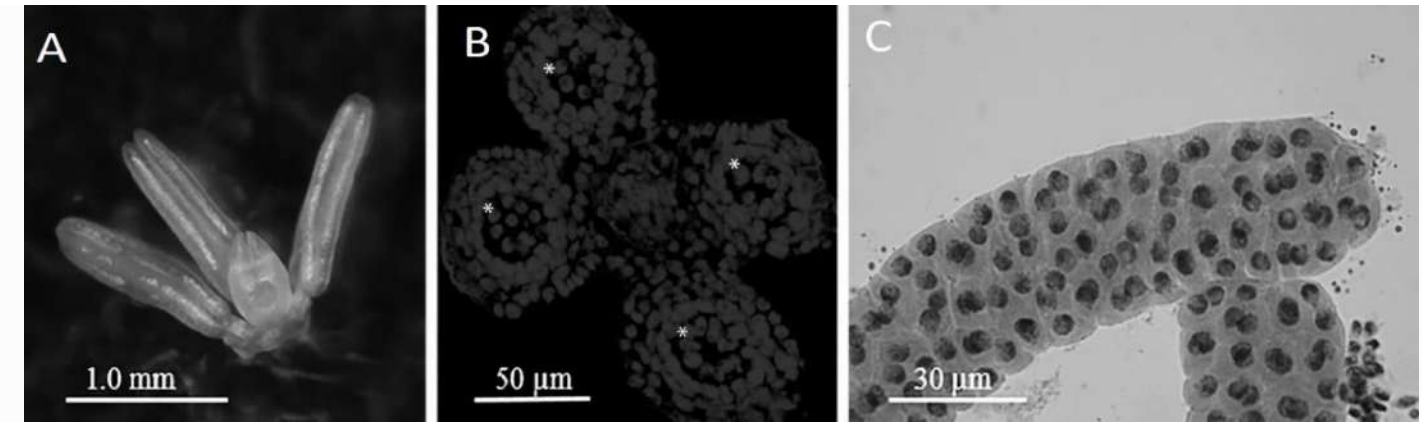


FIG (A-C) Photographs showing Barley anthers and meiocytes by Lewandowska *et al*. (2019) (A) Mature barley anthers at late meiosis stage. (B) Anther's Cross-section showing 4 pollen sacs (asterisk). (C) Meiocytes at zygotene stage within pollen sac after an anther squash (Barakate *et al*., 2014; Colas *et al*., 2016)

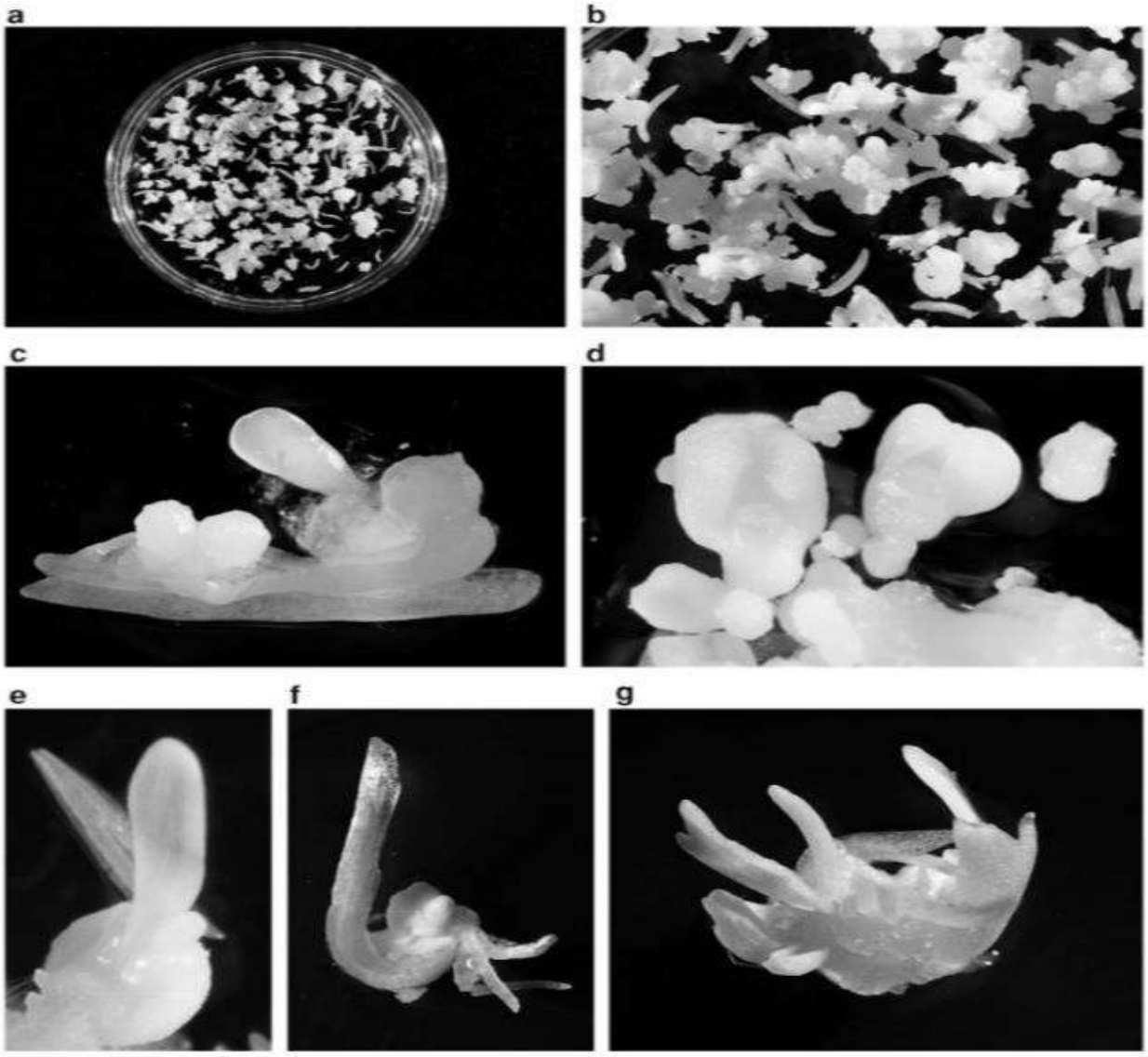


FIG (a-g) Photographs showing induction and regeneration processes in barley anther culture by Ohnoutkova *et al*.,2019 (a-d) Formation of pollen embryos or calli on induction medium after 4–5 weeks. (e–g) Regenerated structures were transferred onto the regeneration medium.

H. APPLICATIONS:

For the first time the application of anther culture in intersubspecific heterosis breeding was reported by Li *et al*. (1992). The anther culture is considered as the successful technique as the unusual segregation is controlled between crosses of two varieties of rice- indica and japonica when uses in intersubspecific heterosis breeding programmes. In crop improvement, the haploids are used in the 'gametoclonal variation' which mainly defines the differences in morphological and biochemical characteristics both in chromosome number and structures which are found among regenerated plants from gametic cells which are cultured (Evans *et al*. 1984; Morrison and Evans 1987). When only one clonal member is capable of doubling the chromosome to give rise to viable pollen which further can be used in other sibling crosses with other clonal members. The development of double-haploid lines for the purposes of the crop improvement mainly for the achievement of homozygosity in diploid or alloppolyploid species and thus initiatons in a breeding programme are preserved. Besides the improvement in crop-breeding programmes, the haploids and double-haploids both are used in many research programmes

like in studies of mutation, gene mapping and genomics. Haploids provide the reliable information on the location of major genes and Quantitative trait loci -QTLs (Khush and Virmani, 1996). Double haploids DHs also provide the information for genetic analysis such as QTL (Datta, 2005).

The production of double haploid plants is important for the improvement of breeding programmes of self-pollinating cereal crops like barley. Less time is consumed for the development of completely pure homozygous lines. In plant breeding programmes isolated microspore culture is effective in production of haploid or double haploid plants so that new varieties of plants are developed in very short time (Ferrie *et al.* 2011, Dwivedi *et al*. 2015 and Germana *et al.*2006). The development of advanced genomic, transcriptomic, proteomic and imaging tools which helps in identification of many genes which are related in microspore reprogramming and embryogenesis. Induction of embryo formation and androgenic plants regeneration results due to stress treatment or pre-treatment which helps in development of microspore into androgenesis. Using microspore cultures transformation protocols like particle bombardment has been used for the regeneration of fertile barley plants (Datta, 2005).

On the identification of QTL associated with androgenesis, the production of many double haploid lines takes place for barley breeding programs. The applications of doubled haploids, such as breeding cycle is shortened by immediate fixation of homozygosity, selection productivity is high, gametoclonal variants production results the increase in genetic variability, and recessive genes are expressed which is appropriate for breeding purposes (Devaux and Pickering, 2005). The selection of transgenic plants from bombarded microspores using isolated mother cells with the help of herbicide resistance gene *Bar* (Jähne *et al*.,1994; Yao *et al*.,1997). The doubled haploid has been accepted as it is important in plant biology and genetics (Suriyan *et al*.,2009), genome mapping (Hussain *et al*., 2012) or in analysis of QTLs (Chauhan and Khurana, 2011).

I. CONCLUSION:

This paper has described about the anther culture and its techniques, the factors responsible for the anther culture, the composition of media in the anther culture and its applications. Anther culture technique has been recognized as an efficient supplement to the conventional inbred line development which is usually achieved through a number of inbreeding cycles. The study provides insights into the application of anther culture which is considered as the successful technique that leads to the improvement in crop breeding programmes. The undesired heterozygous plantlets (double, triploid and tetraploid plants) can be easily produced by the anther or microspore culture. Thus, it has been observed that anther culture technique is successful in many crops through ages.

The effect of mutation is studied by the haploid cultures. The mutations can be easily induced by the haploid and double haploid system (Szarejko,2003) and thus the desired mutant traits can be easily selected. For example, introduction of mutations was successfully done during microspore embryogenesis in rapeseed for herbicide resistance, disease resistance, salt tolerance, and also for seed quality traits (Badiger,2017). The technique of anther culture explains the resolving the problems of cytology. The haploids are very useful in cytological studies as the ploidy of regenerated plants can be determined. The production of haploid plants by the *in vitro* techniques has a major impact in the field of biotechnology as well as in breeding programmes. Less time is consumed for the breeding cycles and also the homozygous plants is completely developed which makes the double haploid technology a suitable technology as it has a significant impact in the field of genetics, QTL mapping, genomics and also in plant breeding programmes. The nutritional value is improving through transgenic breeding and also there is reduction in time, labor and and cost for plant breeding programmes. Conventional cytological techniques such as counting the chromosome number in root tip cells or measuring the amount of DNA content using flow cytometry helps in determination of ploidy level. The importance of colchicine in the medium for chromosome

doubling also reported by Castillo *et al*.,2009. It is one of the most preferred techniques that has been used by many scientists for the development of the double haploid lines in crop improvement.

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INTRODUCTION

Cell culture technique was first introduced by the German scientist, Gottileb Haberlandt, at the beginning of the 20th century. Since then a lot of modifications have been proposed in culture system in order to enhance their performance as well as widen their application. Muir *et al.*, in 1954, designed the first plant cell suspension culture which underwent a lot of modification through closed continuous culture, then open continuous culture and finally into a chemostat culture which is most frequently used. But then the technique of immobilized plant cell culture was introduced further for much more product yield. Considering the same objective but for large scale production of commercially important plant-based products, various bioreactors were introduced such as stirred tank bioreactor, air-lift bioreactor, bubble column bioreactor, rotating-drum bioreactor, membrane bioreactor having several pros and cons, each suitable for culturing a specific type of cell. Also several disposable bioreactors were introduced to make the culture process more eco-friendly. As time passed, several scientists observed that by simply manipulating the bioreactor designs, culture media and several other parameters according to the need of their experiment, the yield can be increased as well as duration of the experiment can be decreased, thus adding to the advantage.

The main principle underlying the plant cell culture is the ability of a plant cell to differentiate into a whole plant provided that all the necessary nutrients and plant hormones along with the necessary environmental conditions must be supplied to the aseptic medium. This ability of a plant cell to regenerate into a whole plant is referred to as totipotency. Sahai *et al*. (1985) observed that plant cells in cultures show some unique features that influence the choice of bioreactors to culture them. They have thin perforated cell wall enclosing the protoplast containing numerous large vacuoles. It has been observed that plants cells are sheer-sensitive and on being agitated mechanically they lyse easily. Thus, plant cells are required to be mixed very gently. This can be achieved using an air lift bioreactor. Using this process, production of secondary metabolites can be enhanced from plant cells. Also, plant cells of dimensions ranging between 20-100µ can rapidly settle in the suspension culture. It has been observed that plant cells in bioreactors have low metabolic processes. Plant cells have a relatively high specific density because of the presence of intracellular starch. Hence, require a low amount of gases and nutrients, leading to simplifying the design of the bioreactor as well as scaling up the process.

The main aim behind introducing these alterations is scaling up the production of a broad spectrum of secondary metabolites which can be further used in pharmaceutical industries for treatment of various diseases, or in the food industries as aromas or dyes. Eibl *et al.* (2018) proposed that a great advancement in the plant cell culture technology has been made for the production of food additives and cosmetic ingredients. The plants producing these metabolites are either season dependent or are rare or produce them in very less quantities. Hence the concept of plant cell culture was essentially introduced to ensure that these problems can be overcome at least upto certain extent. Also large scale production of medicinally important secondary metabolites would ensure their availability to a larger mass at a comparatively cheaper rate. There are several companies that have made notable contribution in commercializing these plant based proteins and metabolites. This finally led to the introduction of “molecular farming” by Satish *et al.* (2019).

BRIEF HISTORY

Plant cell culture and its components under defined physical and chemical conditions *in vitro* are considered as important tools in both basic and applied studies and are also exploited for commercial applications. The early studies led to embryo cultures, root culture, the first tissue culture and cell culture. The period between the 1940 and 1960 marked the development of new techniques accompanied by improvements of the existing ones. The mid-1960s witnessed the application of cell culture under cell behavior, plant modification and improvement and product formation. The late 1990s saw a continuous expansion in the application of the *in vitro* technologies in a broad range of plant species.

Culture of single cells under aseptic conditions was first proposed by Gottileb Haberlandt in the year 1902. However, in the year 1942, Gautheret demonstrated through his experiments that the plant cells in the culture medium gradually became insensitive to the auxin, which is considered to be a significant growth regulator. Muir *et al*. (1954) for the first time reported suspension culture of plant cells. Later in the same year, they modified the cell culture technique during culturing the calluses of *Tagetes erecta* and tobacco by shaking them and thereafter, placing them on filter paper kept on a pre existing and well established callus cultures. This method gave rise to the concept of nurse cultures. This was followed by publishing cultivation of plant cells using bioreactors by Melchers and Engleman (1955). Nobecourt, too in the same year, demonstrated through his experiments that variability of meristems which are obtained from callus changed when the cells were cultured in the suitable medium.

Bergmann (1959) also added a few modifications in the cell culture technique. He proposed the incorporation of single cells in a 1-mm layer of solidified medium leading to the formation of a uniform layer of single cells which is used to obtain uniform cell colonies. Kohlebach (1959) successfully demonstrated the mechanical isolation of mesophyll cells from *Macleaya cordata* forth establishment of culture. The year 1959 was considered essential in the history of plant cell culture because for the first time large-scale culture of plant cells was designed by Tulecke and Nickell from the cell suspensions of *Ginkgo*, *Lolium*, Holly and Rose using simple spurged 20-L carboys. This project was sponsored by NASA to examine if food could be supplied during space flight using plant cell culture.

Hildebrandt along with his coworkers in the year 1960, proposed the idea of using a "conditioned medium" for culturing single cells. "Conditioned medium" is a medium in which cells and tissues have already been grown. Bellincampi (1987) defined conditioning factor as a substance that can easily be recovered from the culture medium and maintained indefinitely after lyophilization. No plant hormone can substitute a conditioning factor. A conditioning factor must be thermostable, water soluble, has a low molecular weight and is species unspecific. However the nature and corresponding chemical entities of conditioning factors are still unknown. In this method a higher concentration of one or more metabolites excreted by the cells present in culture medium is maintained so as to obtain an efficient growth of single cells. . A number of techniques based on production of conditioning factors were developed with the aim to increase the plating efficiency. However, in 1965, along with Vasil, he demonstrated that whole plant of tobacco can also be grown from a single cell by adding coconut water to the fresh medium instead of using the previously demonstrated "conditioned medium".

Butcher (1977) suggested that the interaction between plant and microbes can also be well studied using cell culture. For the first time, in the year 1978, Zenk demonstrated the use of large scale plant cell culture for the production of pharmaceuticals suggesting its industrial implications. Filner (1978) studied the regulation of inorganic nitrogen and sulfur assimilitation; whereas, Fowler in the same year studied carbohydrate metabolism using cell suspension culture. Biochemistry of virus multiplication as well as phytotoxin action was also studied extensively by Rottier and Earl respectively by using cell suspension culture in the year 1978.

Flick (1983) demonstrated plant modification and improvement using cell culture as they provide the advantage of isolation of variants. Neumann and Reinhard (1985) illustrated the study of primary and secondary metabolism using cell suspension culture. Zimmerman (1986) demonstrated the use of stirred tank reactor and a variety of air-driven reactors to commercially row cells on a large-scale.

The beginning of 21st century has seen the use of plant cell cultures for the *in vitro* production of various secondary metabolites used in pharmaceutical, agrochemical, food, flavors and cosmetic industries. Vanishree *et al.* (2004) designed another technique for production of various pharmaceutically active agents such as Shikonin from *Lithospermum erythrorhizon* and Berberine from *Coptis japonica*. Wilson and his coworkers designed various plant cell culture technologies for the production of active cosmetic ingredients or food additives. Satish *et al.* (2019) employed "molecular farming" using genetically modified plants and plants in the *in vitro* system for producing various pharmaceuticals.

CELL CULTURE TECHNIQUES

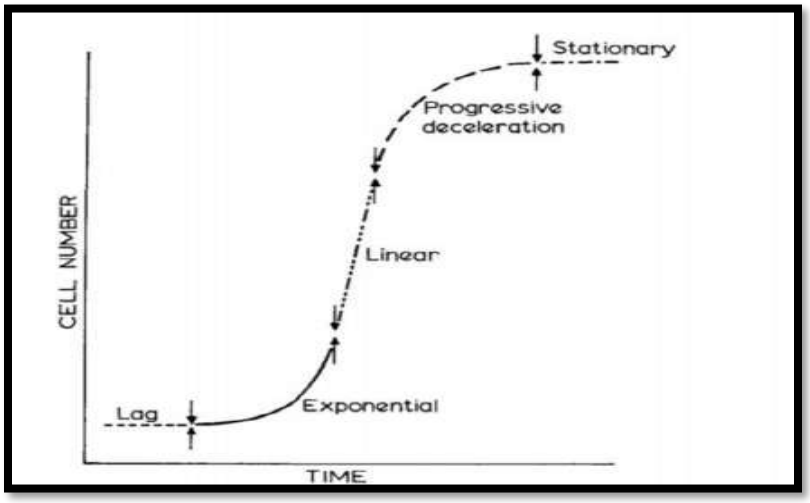
Sahai *et al*. proposed that the selection of appropriate techniques for a plant cell culture relies on the kinetics of product formation as well as cell growth. As suggested by Payne (1992) there are generally two types of product synthesized such as growth associated plant product and non growth associated plant product. Based on the type of product formation, the operating strategies have been determined. It has been demonstrated that for a growth associated plant product, one step batch fermentation or a single-stage continuous culture is beneficial. Delinking of cell growth and product formation in order to achieve non growth associated plant products, can be achieved by two-stage batch or continuous culture or immobilized cell culture. According to Helwig *et al*. (2004), *in vitro* cultivation of plant cells can be achieved by several methods such as cell suspension culture and immobilized culture.

TYPES OF CELL CULTURE TECHNIQUES

1. CELL SUSPENSION CULTURE TECHNIQUE

A. BATCH CULTURE

Batch culture is considered the most common type of cell suspension culture. In this type, a definite volume of nutrient and culture media is used to grow plant cells. The cells in the culture media continue to grow until one of the essential nutrients is depleted from the media and the growth ceases.



A characteristic sigmoid curve growth of the dividing plant cells in the batch culture, has been proposed by King *et al*. (1973), which consisted of the following phases –

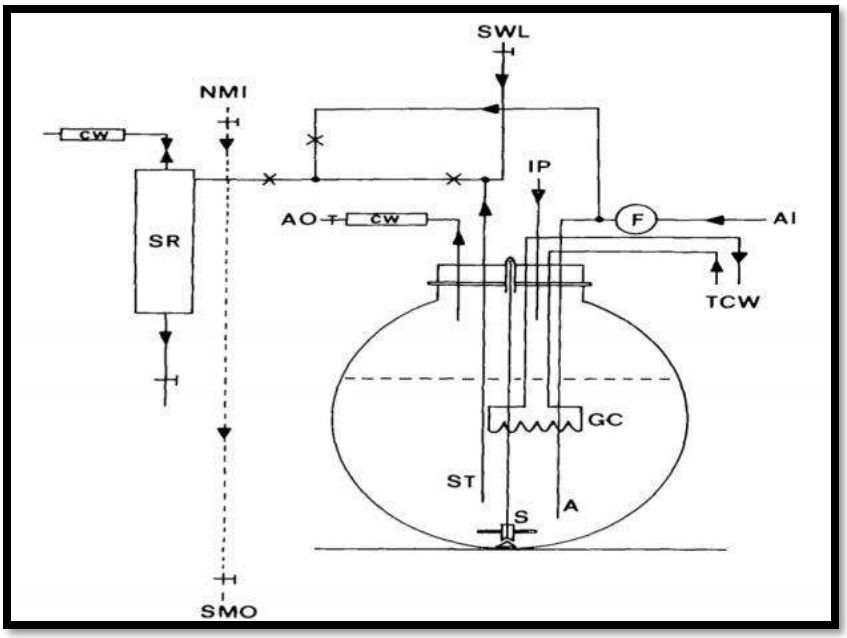
i. **Lag phase** – cells prepare themselves to divide
ii. **Exponential phase** – cell division rate is highest
iii. **Linear phase** – cell expansion increases but cell division decreases
iv. **Progressive deceleration phase** – both cell division and elongation slows down
v. **Stationary phase** – cell size and number becomes constant

While studying the growth curve, Street, in the year 1976, concluded that the overall increase in the number of cells is very less, that is, only 10-15 folds and the exponential phase is also very small, which is only 2-3 generation, as compared to the entire growth cycle.

Campbell (1957) after analyzing the growth of various plant cells in batch culture, concluded that even if a constant exponential growth phase can be attained for a small amount of time, the other cell metabolism parameters do not increase simultaneously and hence, no steady-state is achieved, which leads to the absence of a 'balanced growth'. This results in prominent alterations in the composition of a cell as observed during culturing *Acer* cells, *Rosa* cells and *Galium mollugo* cells. Further, King *et al.* (1973) also proposed that due to rapid uptake of endogenous metabolites in the cells, much earlier than depletion of a primary nutrient from the medium, the cell division slows down and ultimately stops. Scragg (1990) demonstrated that by employing the batch mode of cultivation, the unstable but rapidly growing culture of *Helianthus annus* has been successfully scaled up to a volume of 80 litres in an air-lift bioreactor in three 10-day subcultures. He also demonstrated the growth of *Catharanthus roseus* in suspension culture for the production of alkaloid in air-lift bioreactor using batch culture has resulted in scaling up of the process and thus, leading to lowered accumulation of serpentine. For a long period of time, stirred tank reactors with modified impellar has been used for large scale cultures since it provides enhanced mixing under low sheer

Sahai *et al*., mentioned that there are a few essential factors contributing to the process of batch culture. These include biomass growth rate or doubling time or batch cycle time, product concentration, maximum cell density in the bioreactor (gm dry weight per litre reactor volume), yield coefficients, $Y_{x/s}$ [gm cell mass (x) per gm substrate (s)] and $Y_{p/s}$ [gm product (p) per gm substrate(s)].

Muir (1954),for the first time proposed that agitating the cells of tobacco and *Tagetes erecta* in suspension cultures can prevent its re-aggregation. This is done using an orbital platform shaker. Clips of appropriate size is fitted with the platform of the shaker for holding the flasks. These clips are often interchangeable so that flasks of different sizes can be used. Different impellers of varying size, type and position with respect to air-liquid interface are used to regulate the agitation speed which in turn, affects the rate of sheer inside a bioreactor. Hooker *et al.* (1990) observed that a large flat-bed turbine impellar with a high width-to-diameter ratio is optimal for growth and phenolics production as it offers more gradual velocity gradients as compared to regular flat-bladed disc turbines. This offers better distributed mixing patterns excluding dead zones at low sheer in suspension culture of *N. tabacum.*



BASIC BATCH CULTURE UNIT

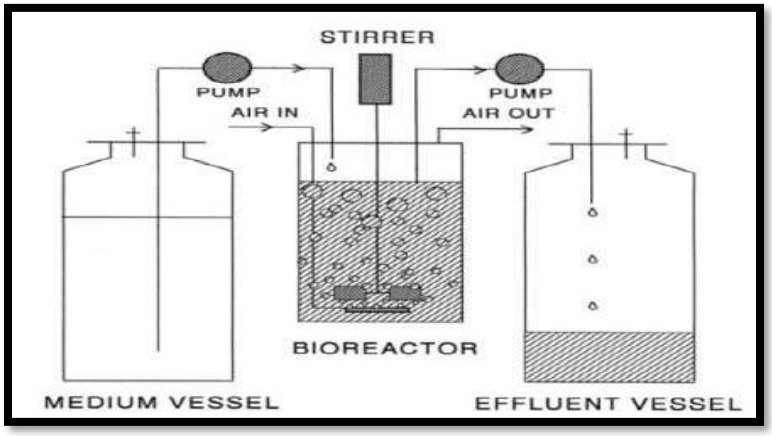
The diagram, proposed by Wilson *et al.* (1971), depicts the direction of flow of batch culture using the solid line and removal of culture using the dotted line. X indicates a screw or spring clip attached to a silicone rubber tubing line. A is the aerator. AI depicts air inlet. AO is air outlet. CW is non absorbent cotton wool filter. F is the miniature air filter. GC is gas coil. IP refers to inoculation port. NMI refers to new medium input. S is the stirrer. SMO depicts stale medium or culture outlet. SR is the sample receiver. ST is the sample tube. SWL refers to sterile water line. TCW refers to temperature-controlling water supply.

Using batch cultures for culturing plant cells has proven to be advantageous because they not only provide a sufficient volume to the culture for facilitating frequent aseptic sampling but also any desired temperature for the culture can be maintained as well as agitated to different extents. The culture can be easily supplied with different levels of oxygen by varying the rate of aeration and break-up of the gas stream using sintered spargers which is an industrial tool specially designed for agitating a liquid by means of compressed air or gas supplied using a pipe. Batch cultures also facilitate determination of carbon dioxide and other volatiles in the exit gas stream as well as continuously monitoring the pH of the culture as it is considered an important factor regulating the production of secondary metabolites.

Although it offers a few advantages, batch cultures have limited usage in culturing plant cells, since a high initial capital investment is required as batch cultures rely heavily on sophisticated technology. Batch culture does not permit for continuous monitoring or controlling of environmental factors which results in limiting growth in cultures as well as change in the composition of the nutrient medium. It offers a limited to more intensive studies of the changes in growth and metabolism and does not aid infrequent sampling of a single population of cells constant. Fowler, (1977) suggested that because of the above mentioned drawbacks, batch cultures are not considered suitable for studies of cell growth and metabolism. To a certain extent, these problems are overcome by continuous cultures.

B. CONTINUOUS CULTURE

The same basic batch culture unit can be modified and used as a continuous or semi-continuous culture system by modifying it to incorporate provisions for regulated medium inflow by monitoring the excess medium or cell suspension (chemostat principle) or continuous monitoring of the cell population density via the optical properties of the cell suspension (turbidostat principle). In this type of culture, there is a constant inflow of fresh medium which is balanced by a constant efflux of an equal amount spent medium, plus cells resulting in development of a steady state at dilution rates smaller than the maximum specific growth rate of the culture. In industrial applications, continuous culture might be important because of the relatively long run times of a production process and the controlled conditions.

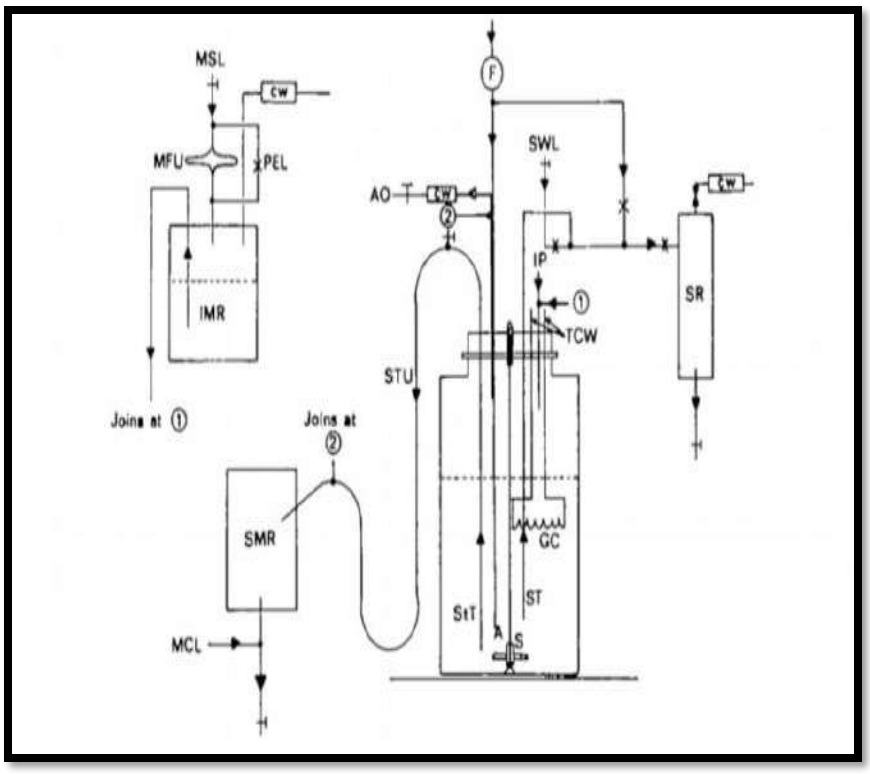


BASIC SET-UP OF CONTINUOUS CULTURE UNIT

In the figure, proposed by Van Gulik (2001), we see that the medium vessel supplies medium continuously to the effluent vessel with the help of a pump via bioreactor containing a stirrer which incorporates air into the bioreactor.

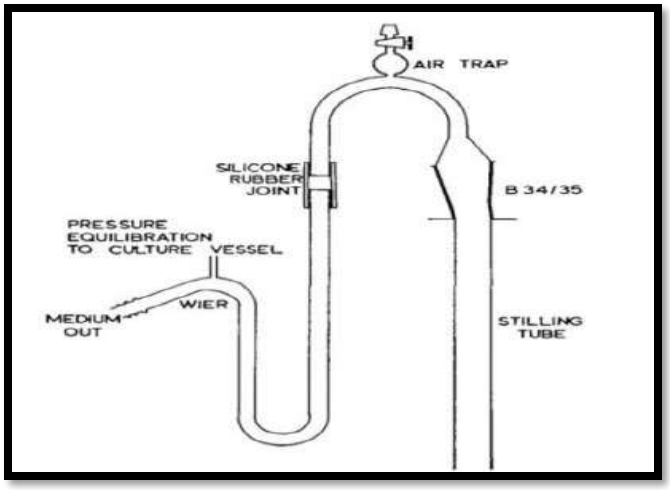
Closed continuous culture system

Experiments led to the development of a closed continuous culture system permitting in which there is a continuous replacement of medium without interrupting the aeration and stirring. A specially designed siphon tube unit and a system for continuous controlled entry of new medium and balancing overflow of spent medium is incorporated in this system. Here the cells are separated mechanically from the media that is flowing out and is added back into the culture. This leads to an increase in the biomass of the plant cells as the growth proceeds.



FLOW DIAGRAM OF CLOSED CONTINUOUS CULTURE UNIT

The diagram, proposed by Wilson *et al.* (1971), depicts the direction of flow of closed continuous culture using the solid line and removal of culture using the dotted line. X indicates a screw or spring clip attached to a silicone rubber tubing line. A is the aerator. AI depicts air inlet. AO is air outlet. CW is non absorbent cotton wool filter. F is the miniature air filter. GC is gas coil. IP refers to inoculation port. NMI refers to new medium input. S is the stirrer. SMO depicts stale medium or culture outlet. SR is the sample receiver. ST is the sample tube. SWL refers to sterile water line. TCW refers to temperature-controlling water supply. IMR depicts intermediate medium reservoir; MCL depicts mercuric chloride solution line; MFU depicts medium filter unit; MSL depicts medium supply line; PEL depicts pressure equalizing line; SMR depicts stale medium reservoir; StT depicts stilling tube; STU depicts siphon tube unit.



SIPHON TUBE UNIT

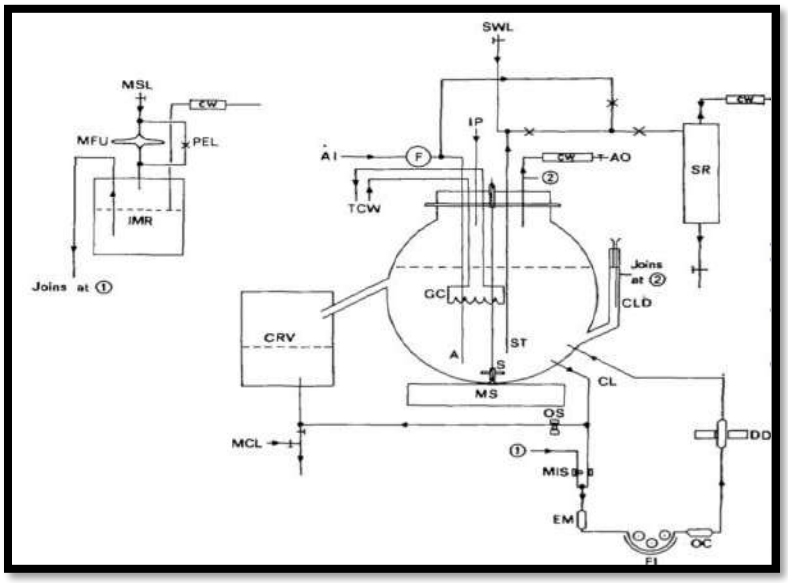
The figure, proposed by Wilson *et al.* (1971), depicts a siphon tube which is specially designed and inserted in a closed continuous vessel which helps to replenish the media and prevent any air bubble to enter and interrupt the flow of the media.

Open continuous culture systems

It is another type of continuous culture system in which the inflow of the fresh medium is balanced by outflow of an equal amount of the same medium. The rates of the inflowing medium is so adjusted that the volume of the culture medium remains constant and a sub-maximal growth rate is maintained over an indefinite period of time. Open continuous culture systems are of two types –the turbidostat and the chemostat. Both turbidostat and chemostat have been developed and operated over considerable length of time. Both of them involve the addition of a loop to the culture vessel through which the culture is circulated external to the main culture vessel. Also in both types of open system, the culture is harvested at a rate which balances the regulated input of new medium to the medium flowing out.

1. Turbidostat

It is a method of monitoring a continuous culture is by regulating the biomass concentration and controlling the rate of flow of the medium to keep the biomass concentration constant. A device known as spectrophotometer or turbidimeter is used to measure the optical density. A major drawback is that the reliability of turbidimetric measurement of the biomass concentration is questionable since, it is sensitive to changes in cell or aggregate size. Phototrophic cells are the ones that use light as their source of energy. Cultures of this type can be grown using continuous culture technique with light as the limiting 'nutrient' and hence, is termed as photostat. It has been observed that at low biomass concentrations part of the 'nutrient' light leaves the culture through the glass wall or is absorbed by the wall of the vessel. This can be considered as another major drawback of this system. Both the theoretical and the practical aspects differ considerably from the chemostat theory

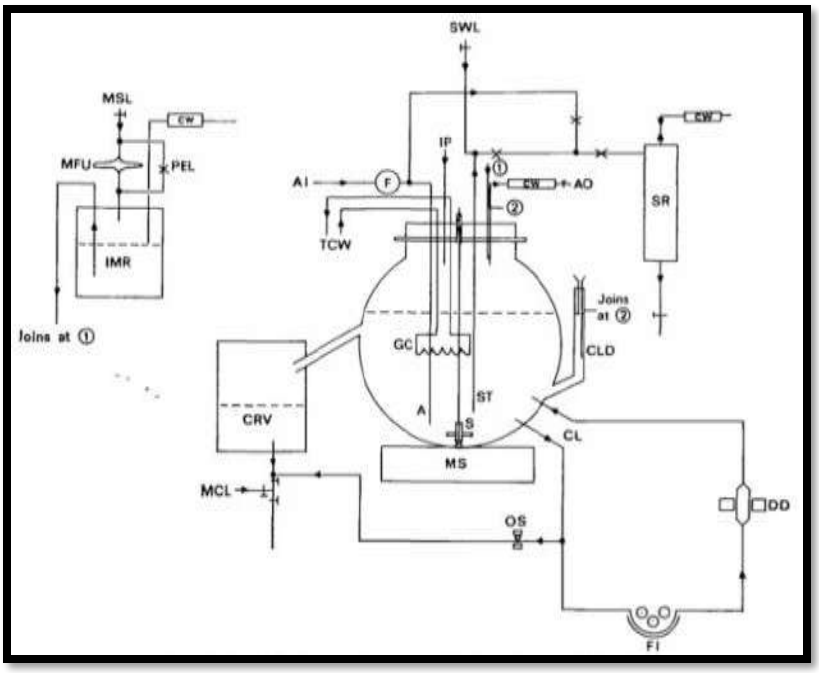


FLOW DIAGRAM OF OPEN CONTINUOUS CULTURE UNIT- A TURBIOSTAT

The diagram, proposed by Wilson *et al.* (1971), depicts the direction of flow of open continuous culture (turbidostat) using the solid line and removal of culture using the dotted line. X indicates a screw or spring clip attached to a silicone rubber tubing line. A is the aerator. AI depicts air inlet. AO is air outlet. CW is non absorbent cotton wool filter. F is the miniature air filter. GC is gas coil. IP refers to inoculation port. NMI refers to new medium input. S is the stirrer. SMO depicts stale medium or culture outlet. SR is the sample receiver. ST is the sample tube. SWL refers to sterile water line. TCW refers to temperature-controlling water supply. EM depicts electrode module. MIS depicts medium input solenoid valve. OC depicts observation chamber.

2. Chemostat

It is considered to be the most common type of continuous culture. The system is controlled by the flow and composition of the influent. It has been observed that a steady-state of cell growth and metabolism can be maintained using this system. It is also useful in determining the effect of individual growth-limiting nutrients on cell growth. Chemostat offer several advantages over batch culture. The average specific growth rate in the culture is identical to the dilution rate provided that the steady state conditions are maintained. All culture parameters (biomass, biomass composition, and nutrients) remain constant for prolonged periods of time in a steady state. This "balanced growth" is a very important tool in studying the growth and production kinetics or various parameters of plant physiology. It is possible to study cell metabolism with various growth limiting substrates (e.g. glucose, nitrate, or phosphate)



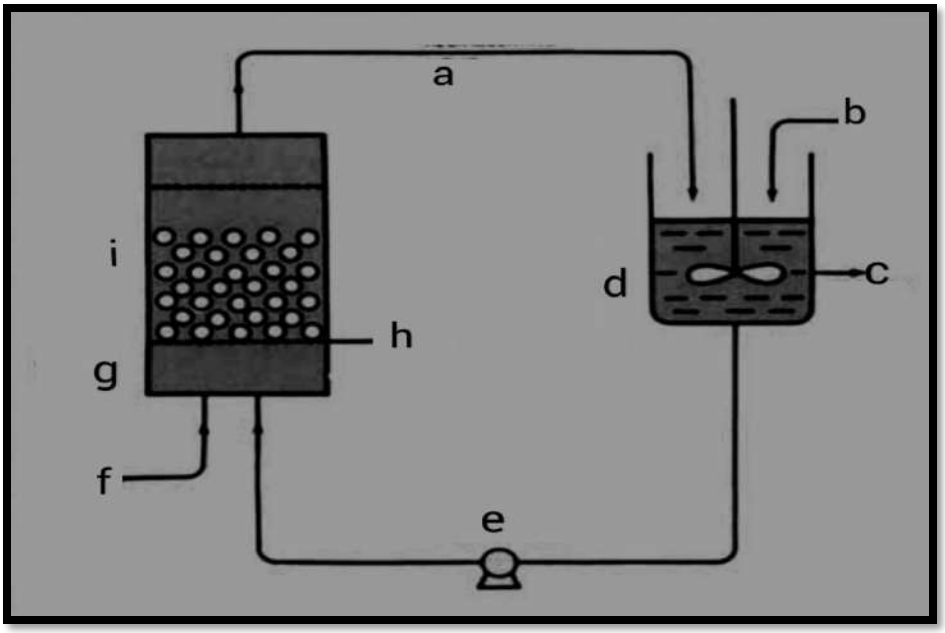
FLOW DIAGRAM OF OPEN CONTINUOUS CULTURE UNIT- A CHEMOSTAT

The diagram, proposed by Wilson *et al.* (1971), depicts the direction of flow of open continuous culture (chemostat) using the solid line and removal of culture using the dotted line. X indicates a screw or spring clip attached to a silicone rubber tubing line. A is the aerator. AI depicts air inlet. AO is air outlet. CW is non absorbent cotton wool filter. F is the miniature air filter. GC is gas coil. IP refers to inoculation port. NMI refers to new medium input. S is the stirrer. SMO depicts stale medium or culture outlet. SR is the sample receiver. ST is the sample tube. SWL refers to sterile water line. TCW refers to temperature-controlling water supply. EM depicts electrode module. MIS depicts medium input solenoid valve. OC depicts observation chamber. CL depicts circulation line; CLD depicts constant level device; CRV depicts culture receiving vessel. DD depicts density detector; FI depicts flow inducer; MS depicts magnetic stirrer motor. OS depicts outlet solenoid valve.

2. IMMOBILIZED PLANT CELL CULTURE TECHNIQUE

This method was first introduced in the plant cells by Brodelius *et al*. (1979) where the plant cells present in late stationary phase of batch cultivation are entrapped using appropriate membrane or gel. It is widely applied for *in vitro* production of metabolites in a continuous fashion. More efficient control over cell density is provided by membrane reactors; thus, allowing a more homogeneous environment inside the reactor. It has been observed that pressure drop and fluid dynamics can be controlled very easily and are more or less independent of the scale of operation. Brodelius *et al*. also proved that alginate gel-entrapped immobilized systems enhanced the production of metabolite in many cases. The use of elicitors for stimulation of formation of secondary metabolites and simultaneously absorbing the product *in situ* using a resin has been considered to be a significant advancement in the application of gel trapped systems.

Archambault *et al.* (1989) designed a technique known as surface immobilized plant cell (SIPC) in which the hydrodynamics can be controlled easily. Moreover, a well separated two-phase system is provided where foaming is reduced to a minimum. It has been noted that using this system for culturing cells from *C. roseus* and *N. tabacum* provides an efficient long-term operation of the culture system. This in turn, enhances the production of secondary metabolites from them. Shuler *et al.*, in 1986, demonstrated that multi-membrane systems have an added advantage of relieving product inhibition by allowing *in situ* separation of the product. A slightly different approach was developed by Yoon *et al.*, three years later, where the cells were contained in a compact tissue-like layer in a membrane bottom boat. This modification allowed the intra-cellular products to be produced.

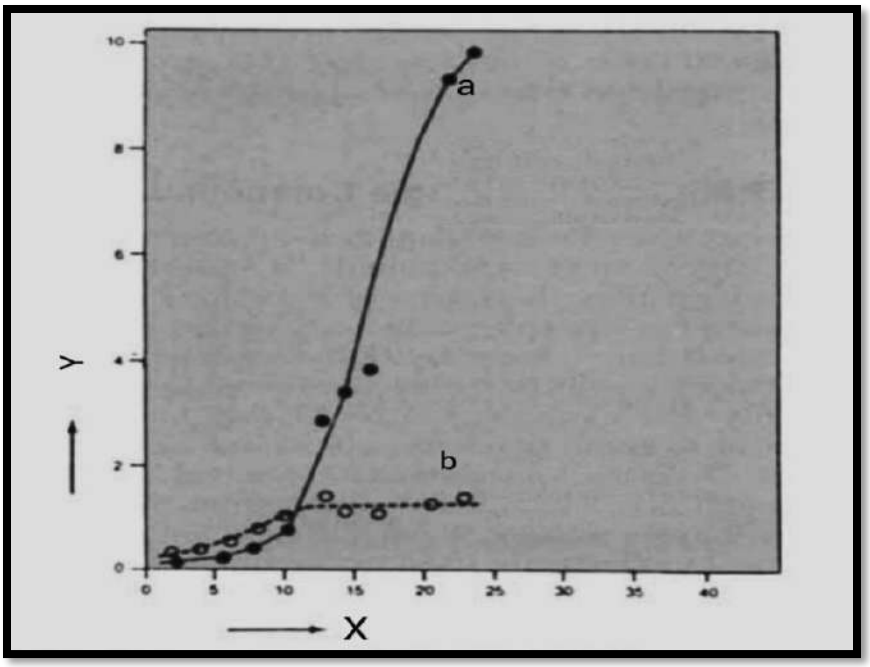


EXPERIMENTAL SET-UP FOR SECONDARY METABOLITE PRODUCTION FROM POLYER ENTRAPPED CELLS IN A COLUMN REACTOR

The figure, proposed by Sahai *et al.* (1985), depicts that the polymer entrapped beads are kept inside the column reactor with the help of a support mesh and these cells are transported through re circulating liquid to a recycle vessel where in fresh medium is added and circulated back to the column reactor via a recycle pump.

a= re circulating liquid, b= fresh medium stream, c= product stream, d= recycle vessel, e= recycle pump, f= fresh air, g= column reactor, h= support mesh, i= polymer entrapped beads.

Using immobilized plant cell cultures instead of batch culture has proven to be advantageous as they permit optimization of product without compromising growth by delinking growth and product formation as well as eliminate the non-productive phases of a batch cycle which at times might occupy up to 75% of the total fermentation time. They also allow separation of product from the biomass and separation procedures within the reactor itself, as the product is extracellular; thus, eliminating costly extraction as well as facilitates continuous processing thus, contributing to large increase in volumetric productivities and continuous removal of metabolic inhibitors opposed to traditional batch culture techniques. They maintain a stable, metabolically active population of slowly dividing or non-dividing cells which is crucial for catalyzing multi-step, multi-enzymatic conversions as well as support viability of plant cells for longer periods of time without cell division. Immobilized cultures also impart protection to cells from high sheer stresses of the liquid as well as provides an enhanced cell-cell contact as well as enhances the possibility of accelerated reaction rates by increasing cell densities and possible channeling of inexpensive substrate into desired product rather than to cell mass and improves product yield by varying the cell aggregation which is a design parameter in an immobilized cell process. Immobilized plant cell cultures are also associated with several disadvantages including low product yield and genetic instability.



PRODUCTION OF ANTHRAQUINONES IN SUSPENSION CULTURE AND IMMOBILIZED CELL CULTURES OF *Morinda citrifolia*

Brodelius *et al.* (1979) proposed the figure to demonstrate using immobilized cultures can be used to enhance the production of secondary metabolites from Anthraquinone as compared to batch culture and that too in lesser number of days. This is because immobilized cultures eliminate up to 75% of non productive phase of batch culture.

X= incubation time (days), Y= Anthraquinones (pmol/cell), a= immobilized cells, b= suspension culture.

PLANT CELL BIOREACTORS

SELECTION OF A BIOREACTOR

As mentioned by Huang *et al.* (2009), the bioreactor for culturing a particular type of cell is selected based on the following criteria –

- Oxygen supplying capacity and air bubble dispersion intensity in broth;
- Hydrodynamic stresses generation intensity inside the reactor and their effect on the plant cell system;
- Mixing adequacy of culture broth at high cell concentration;
- Temperature, pH, and nutrient concentration regulation ability inside the reactor;
- Aggregate size controlling ability;
- Scale-up process is easier
- Simple aseptic operation for long durations.

BIOREACTOR DESIGNS

Mechlers and Engleman, in 1955, first reported the use of bioreactors for cultivation of plant cell suspension. NASA (1959) sponsored a research conducted by Tulecke and Nickell, 1959 and 1960 on the possibility of using the cultures to supply food during space flight which marked the beginning of the large scale cultivation of plant cell suspension. Large carboys and bottles were used during the experiments which were either rolled or bubbled to give good mixing. Stainless steel bioreactors fitted with a motor and agitator soon replaced these make-shift bioreactors. Gradually, air-lift bioreactors were introduced for plant cell culture. Later, a number of different bioreactor designs were proposed to meet a varied range of requirements of different plant cells. The most frequently used bioreactors for commercial plant cell culture as proposed by Ruffoni *et al*. (2010), Georgiev *et al.* (2013) and Lehmann *et al*.(2014) includes –

(i) Stirred-tank reactor

These reactors are reported by Piehl *et al*. (1988) and are commonly used for all cell types. They can be easily scaled-up and can offer good fluid mixing. These are more suitable for high-viscosity cell culture as they are able to achieve high oxygen transfer. They have alternative impellers and offer ease of compliance with cGMP requirements. However, there occurs high shear stress around the impeller which may lead to destruction of the plant cells. Also, heat generated from mechanical mixing may interfere with the maintenance of optimum conditions inside the culture vessel. Requirement of high capital and operational costs along with high energy cost owing to mechanical agitation has been considered as a major disadvantage. Additionally, there is a risk of contamination due to mechanical seal. These problems can be somehow overcome using a bubble column reactor. *N. tabacum* cells have been cultured using this bioreactor since 1971 as reported by Scragg (1991).

(ii) Bubble-column reactor

They are designed by Tanaka (2000) and are suitable for culture of both plant and animal cells. These bioreactors are easy to construct as well as scale up and require a low operational cost. Also, it offers low contamination risk, low shear stress generation and no heat generation owing to lack of mechanical agitation. But the main problems highlighted in this case are poor oxygen transfer capabilities and poor fluid mixing in highly viscous cultures. High levels of foaming under high-aeration conditions are also observed that leads to various complications. *N. tabacum* cells have also been cultured using this bioreactor since 1971 as reported by Scragg (1991).

(iii)Air-lift reactor

These are designed by Smart and Fowler (1984) and are also suitable for both plant and animal cells. These bioreactors are easy to construct and scale up and also offer low operational cost, low contamination risk and low shear stress. Also, there is no heat generation due to lack of mechanical agitation. There are multiple choices of internal draft tubes

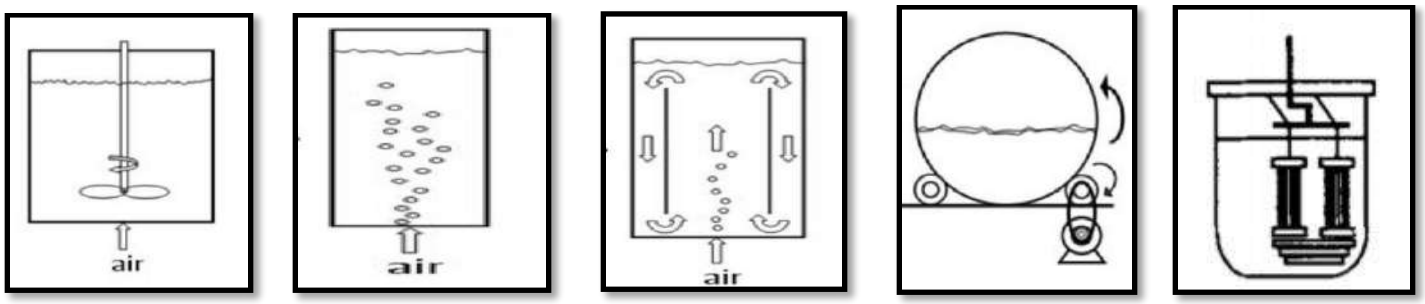
and better oxygen transfer as compared to bubble column. Patterns in which the air circulates also facilitate gas and nutrient transfer. Air-lift bioreactor offers a relatively poor oxygen transfer capabilities and poor fluid mixing for highly viscous cultures. Moreover, high levels of foaming, under high-aeration conditions is often observed. *C. roseus* cells have been cultured using this bioreactor since 1977 as reported by Scragg (1991).

(iv) Rotating-drum reactor

These bioreactors designed by Tanaka (1983) and offer good mixing and aeration, increased oxygen supply and promote high oxygen transfer to cells at high density. But scaling up is difficult as well as there is non-uniform mixing at a very large scale. *C. roseus* cells have been cultured using this bioreactor since 1977 as reported by Scragg (1991).

(v) Membrane bioreactor

These are also a type of bioreactor having the ability to concentrate biomass and protein product in membrane compartment. The extracellular products can be easily withdrawn using these bioreactors. They offer low shear stress and low operational cost. As a backlash, they are difficult to scale up and necessarily, oxygenation is required. They have a low heat transfer rate and are difficult for online monitoring of culture conditions.



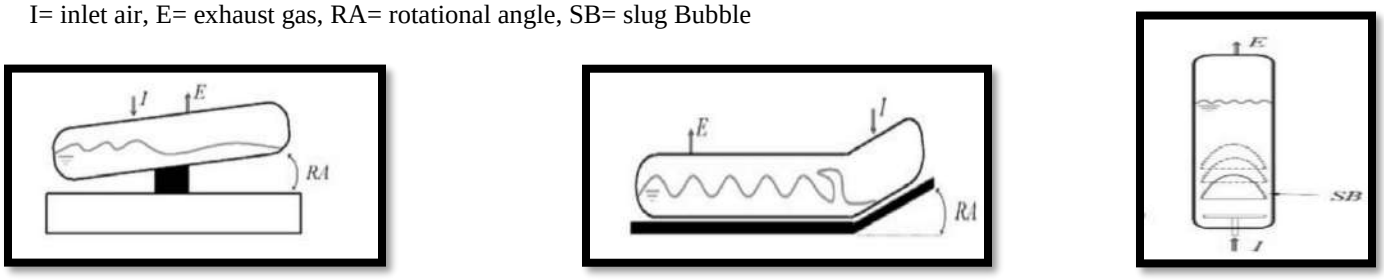
Stirred-tank bioreactor Bubble-column bioreactor Air-lift bioreactor Rotating-Drum bioreactor Membrane bioreactor

Configuration of frequently used bioreactors for plant cell culture as proposed by Weathers *et al.* (2010)

Disposable reactor

Eibl and Eibl (2009) and Ducos *et al.* (2008) proposed the use of disposable and scalable reactors, usually plastic bags as one of the more recent developments in cell culture. The capital costs of disposable culture systems are far less than for the usual stainless steel tanks. There are a number of disposable bioreactors developed by certain companies such as slug bioreactor, wave bioreactor wave and undertow manufactured by Nestle etc.

I= inlet air, E= exhaust gas, RA= rotational angle, SB= slug Bubble



Wave bioreactor Slug bioreactor Wave and undertow

Configuration of disposable bioreactors as proposed by Georgiev *et al.* (2009)

Wave bioreactors are a type of disposable equipment that offers low shear stress, high oxygen mass transfer, low operational cost and increased operational flexibility in batch cultures. These are light weight and useful for high cell density culture. They also reduce cleaning and fulfill in-house sterilization requirements. But they are difficult to scale up and to be applied in advanced cell culture operational strategies. These reactions also offer low heat transfer rate. The wave undertow reactor is a modified version of wave bioreactors, where only one portion of the platform moves up

and down. Thus, it offers involvement of minimal mechanical force. But the slug bioreactors functions quite differently as it involves the formation of a gas bubble at its base, the gradually move upwards, offering a good aeration and mixing inside the reactor.

Scaling up

In order to commercially set up plant cell culture technologies, scale up is considered a crucial step. Successful scaling up of a variety of reactors has been achieved for large-scale production of secondary metabolites. Similar or excess growth obtained as compared to that obtained in shake flasks is considered a benchmark for successful scale-up of most cultures. Zhang and Zhong (2004) demonstrated the scale up from 3 L to 30 L for growth of *Panaxnoto ginseng* cells using a centrifugal impeller bioreactor. They determined the initial $k_L\alpha$ value of the 3 L-reactor at which the highest productivity of biomass and secondary metabolites was obtained. Terrier *et al.* (2007) too successfully demonstrated cultivation in scaled-up slug bubble and wave and undertow reactors using suspension cultures of tobacco and soybean cells with working volumes as high as 50 L and 100 L respectively.

FACTORS AFFECTING CELL CULTURE

1. BIOREACTOR CONSIDERATIONS –

A number of advanced bioreactors have proven to be effective in scaling up of several plant cell systems like a bubble free loop fluidized bed reactor, an external loop air-lift bioreactor, a centrifugal impellar bioreactor. The main goal of designing a bioreactor is to provide an environment that is able to optimize the growth and productivity of the genetically modified, shear sensitive and fragile plant cells. As mentioned by Bisaria and Panda (1991), culturing of plant cells large scale is often met with technological barriers including oxygen transfer, cell aggregation wall growth caused by the adhesion of cells etc.

I. Plant cell growth –

It has been observed that that plant cells grown using suspension culture displays a long doubling time (20–100 h) whereas, Kieran (2001) using Tobacco BY-2 cells, demonstrated that they had a higher growth rate and shorter doubling time (about 12 h) with respect to other plant host species. Gao and Lee (1992) concluded that it was due to the effect of oxygen supply on genetically modified tobacco cells that specific growth rate, maximum cell concentration, consumption rate of glucose and fructose and GUS protein production yield in Tobacco BY-2 cells enhanced.

II. Oxygen demand –

Oxygen is considered to be the most important gaseous substrate that is needed for cellular growth and aerobic metabolism of suspended plant cells in the *in vitro* culture. Trexler (2002) proposed that the physiology and oxygen demand of plant cells during suspension culture in a bioreactor can be monitored using Oxygen uptake rate (OUR) as an indicator. A typical OUR value for plant cells is considered to be about 5–10 mmol-O_2/ (L-h). Although for cell growth, plant cell has a relatively low oxygen demand resulting from their slow metabolism, it has been demonstrated that the volumetric productivity for a high cell density plant cell culture is limited by insufficient oxygen mass transfer due to high apparent viscosity of the cell culture broth and dissolved oxygen. In order to maintain cell growth and viability, concentration of dissolved oxygen very critical. A typical volumetric oxygen mass transfer coefficient ($k_L\alpha$) value required in a bioreactor operation for plant cell cultures is between 10 and 50 h^{-1} is needed for fulfilling the OUR requirements. Mirjalili (1995) concluded from various experiments that high rates of aeration may result in severe foaming problems and GA stripping effects (CO_2, ethylene or other volatile metabolites) which can lead to inhibition of plant cell growth. Hence it is ensured that the design of the aeration system generate as many small bubbles as possible. Although, lower $k_L\alpha$ often results in poor cell growth, Kato (1975) demonstrated that a higher $k_L\alpha$ cannot guarantee

good growth of plant cells. Thus it has been suggested that restricted range of $k_L\alpha$ is beneficial for growth of plant cells in suspension cultures.

III.Aggregation –

Chattopadhay *et al*. (2002) demonstrated that plant cells in suspension culture have a tendency to aggregate into large clumps due to the inability of the daughter cells to separate from the parent cells after division based on the conclusions of Taticek *et al.* (1995) that the secretion of extracellular polysaccharides also contributes to increased cell adhesion. It has been demonstrated by Kieran (2001) that formation of cell aggregates generally promotes cellular organization and differentiation, thus resulting in improvement of production of production of secondary metabolite, as well impacted the mass transfer leading to oxygen, nutrient or chemical inducer in homogeneities inside large cell aggregates. But a major drawback observed by Kieran was that the cells present in the interior portion of the aggregates were unable to get access to nutrient and oxygen, leading to adverse effects on plant cell growth as well as affect yield and quality of recombinant protein. It has experimentally proven by Kieran that moderate cell aggregation (200–500 m) are advantageous over large cell aggregates(∼1–2 mm) since the former enhances sedimentation rates, facilitating media exchange as well as in situ recovery of culture broth during downstream processing whereas the later is undesirable since they complicate the bioreactor operation, enhances mass transfer limitations and makes cell aggregates more susceptible to hydrodynamic stress, resulting in cell damage, attributed to aggregate surface attrition and aggregate shattering

IV.Rheological properties of plant cells in cultures –

Crossgrove (1997) observed that plant cells become spherical to elongated shapes from spherical when cell division is terminated. Curtis and Emery (1993) performed a study on the rheological properties involving 10 different plant cells in shake flasks and concluded that elongated plant cell morphology in tobacco (*Nicotiana tabacum*) batch culture exhibited a power-law type fluid rheological property. This resulted in higher apparent viscosity, compared to spherical cells. It has also been observed that, semi-continuous tobacco cell culture displayed Newtonian rheological behavior and thus, the tobacco cells did not elongate when grown in semi-continuous culture. This confirmed that rheology was dependent on plant cell morphology. Whereas Kato *et al.* (1978) demonstrated that non-Newtonian fluid character was associated with the plant cell morphology. It was observed that elongated plant cell morphology led to a higher packed cell volume (PCV) at a given dried cell weight (DCW) concentration, thus leading to a more loose cellular network under packed conditions. Wagner and Fogelman (1977) pointed that the importance of the actual culture environment was indicated by a morphology change from pellet culture to suspension culture containing mostly single cells in a scale up study from a shake flask to a bioreactor.

V. Shear sensitivity of suspended plant cell cultures –

Dunlop *et al.* (1994) observed that plant cells in suspension culture experience shear stress due to their large volume of intracellular vacuoles and a rigid, inflexible cellulose-based cell wall. It was observed that plant cells are more susceptible to shear stress during the late exponential growth and early stationary phases during which they are of relatively large size and contain large vacuoles. Meijer *et al*. (1993) observed that cellular response of plant cells to hydrodynamic stress includes changes in cell viability due to cell growth rate or membrane integrity, release of intracellular components such as proteins or secondary metabolites, changes in metabolism like OUR, mitochondrial activity, ATP concentration, cell wall composition, increase of calcium ions in cytoplasm and changes in cell morphology and aggregation patterns, are influenced by the intensity and the exposure duration of the cells to shear force.

VI. Foaming and wall growth –

Su (1995) and Abdullah *et al*. (2000) demonstrated that foaming primarily takes place during the exponential growth phase due to the secretion of extracellular proteinaceous compounds, polysaccharides and fatty acids and may become tremendous as a result of cell lysis during the stationary phase. The plant cells in suspension culture and proteinaceous compounds released by them get entrapped in the foam layer. The cells on getting entrapped in the foam layer do not receive enough nutrient and oxygen, resulting in the reduction of suspended biomass and productivity. Generation of a

thick layer due to the release of proteases and secondary metabolites by the plant cells in suspension leads to their adhesion to the reactor wall, impeller shaft and the sensors, thus hindering and disturbing the flow pattern of culture fluid. Under extreme foaming conditions, there lies a possibility that the foam layer might migrate up the gas outlet port and clog the air venting filters, restricting gas flow and making the culture susceptible to contamination. Approaches to reduce the foaming as proposed by Li *et al*. (1995) include reduction of speed of agitation and rate of aeration in a manner that mixing intensity and mass transfer rates are not affected, addition of antifoam reagents that result in a reduction of the surface tension of the culture broth as well as reduce the $k_L\alpha$ value in the bioreactor, application of surface aeration or bubble free-aeration, using a mechanical foam breaker or installing an impeller above the culture broth to serve as a mechanical foam breaker.

2. <u>OPTIMIZATION OF CULTURE ENVIRONMENT</u> –

It was observed that an alteration in the gas phase composition is very effective for inducing the desired change in cell metabolism. Culture environmental conditions such as light, temperature, medium pH and oxygen have been examined for their effect in many types of culture.

I.Temperature –

Normally a temperature range of 17–25ºC is used for the inducing formation of callus tissues and growth of cultured cell. But, it has been observed that different plant species prefer different temperature to optimal growth. Toivonen *et al.* (1992) demonstrated that total fatty acid content per cell in dry weight can be increased by lowering the cultivation temperature. In the same year, Kreis and Reinhard too observed that biotransformation of digitoxin to digoxin is favoured at 19º C whereas at 32º C biotransformation of digitoxin purpureaglycoside is favored by the plant *Digitalis purpurea*.

II. Illumination –

Illumination is considered to be one of the important factors affecting the culture media. Seitz and Hinderer (1988) demonstrated that in cell cultures of *D. carota* and *Vitis hybrids* accumulation of anthocyanin was strongly stimulated by light. Mulder-Krieger *et al.* also, in the same year, demonstrated that illumination of callus cultures of *Marticaria chamomilla* affected the composition of sesquiterpenes in the cultures and if light is not supplied to callus cultures of *Citrus limon*, there was accumulation of monoterpenes in the culture.

III. Medium pH –

The pH of the medium plays an important role in formation of product in cell culture. The pH is generally regulated between 5 and 6 before autoclaving in order to avoid extremes of pH. It has been observed that the concentration of hydrogen ions in the medium changes as the culture develops. Husemann *et al.* (1992) experimentally proved that there was an increase in the external pH from 4.5 to 6.3 and the cytosolic pH by 3.0 units and the vacuolar pH by about 1.3 units during photoautotrophic cell suspension cultures of *Cheno rubrum*. McDonald and Jackman, 1989, demonstrated that during ammonia assimilation pH of the medium decreases whereas during nitrate uptake pH of the medium increases.

IV. Agitation and aeration –

Aeration and agitation is considered to be crucial for large-scale production. Muir *et al.* (1954) first introduced in callus culture of tobacco (*Nicotiana tabacum*), to enhance the productivity of the culture. Kreis and Reinhard (1989) demonstrated that maintaining levels of dissolved oxygen at 50% after 20 days of growth in an airlift bioreactor influenced alkaloid yield of around 3-g/l culture. Also increasing the aeration rates was found to produce a marked decrease in alkaloid productivity. Kreis and Reinhard (1989) demonstrated that airlift and stirred tank bioreactors can allow similar secondary product levels in cultured plant cells, but the characteristics of the stirrer is important for stirred tank vessels.

V.Composition of the gases in medium –

Ambid and Fallot (1981), using fruit suspension cultures studied the effect of the composition of the gaseous environment on production of volatiles. They reported that the synthesis of monoterpenes by Muscat grape suspensions was stimulated by the addition of carbon dioxide in the medium and subsequently induced the formation of linalool. Kobayashi *et al.* (1991) reported that to prevent cell browning and to sustain berberine production in suspension cultures of Thalictrum minus in bubble column reactors the use of carbon dioxide at the 2% level was considered critical.

3. MANIPULATION OF NUTRIENTS –

Stafford *et al*. (1986) proposed that to increase the product accumulation effectively the culture environment must be manipulated by altering several external factors such as nutrient levels, stress factors, light and growth regulators, which would thus lead to the increased expression of many secondary metabolite pathways. Many of the constituents of plant cell culture media are important determinants of growth and accumulation of secondary metabolites.

I. Sugar levels

Plant cell in suspension cultures are generally grown heterotrophically, where simple sugars are mainly used as a source of carbon as well as inorganic supply of other nutrients. Berlin *et al.* (1983) demonstrated that the level of sucrose, affected the productivity of secondary metabolite-accumulating cultures using suspension cultures of *Eschscholtzia californica* where yields of benzophenanthridine alkaloids increased 10-fold to around 150 mg/l by increasing the sucrose concentration to 8% (w/v). They also drew the same conclusion using cell culture of *C. roseus*, where an optimal sucrose concentration of 8% (w/v) was required for the accumulation of indole alkaloid. Do and Cormier (1990) demonstrated regulation of anthocyanin production in *Vitis vinifera* cell suspension cultures due to the osmotic stress created by sucrose alone and with other osmotic agents.

II. Nitrate levels

The level of proteinaceous or amino acid products was observed to be affected by nitrogen concentration of cell suspension cultures. Both nitrate and ammonium are present as sources of nitrogen in plant tissue culture medium such as MS, LS or B5. There is a marked effect of the ratio of the ammonium/nitrate–nitrogen and overall levels of total nitrogen on the production of secondary plant products in suspension cultures. Ikeda *et al*. (1977) demonstrated that reduced levels of NH_4^+ and increased levels of NO_3 promoted the shikonin and betacyanins production, whereas higher ratios of NH_4^+ /NO_3 increased the production of berberine and ubiquinone. Zenk *et al.* (1975), Yeoman *et al.* (1980) and Yamakava *et al.* (1983) demonstrated that a decrease in the nitrogen level in suspension culture enhanced the formation of anthocyanin in *Vitis sp*, anthraquinones in *Moringa citrifolia* and capsaicin in *Capsicum fruitescenes* respectively. Rajasekaran *et al.* (1991) demonstrated that for cultures of *Chrysanthemum cinerariaefolium*, removal of nitrate in the culture increased pyrethrin accumulation in the second phase of culture by two fold.

III. Phosphate levels

The phosphate concentration in the medium was found to have a marked effect on the production of secondary metabolites in plant cell cultures. Sasse *et al.* (1982), proposed that increased levels of phosphate enhanced the growth of cells, whereas it had a negative influence on secondary product accumulation. He observed that when phosphate levels in the culture are reduced, the production of ajmalicine and phenolics in *Catheranthus roseus* was induced, as well as caffeoyl putrescines in *Nicotiana tabacum* and of harman alkaloids in *Peganum harmala* were also induced.

IV. Growth regulators

Dues and Zenk (1982) proposed that concentration of growth regulators such as auxin and cytokinin play an important role in growth and secondary product accumulation in suspension cultures. Zenk *et al.* (1975), Sahai and Shuler (1984), Tabata (1988), Bohm and Rink (1988), Ranjendran *et al* (1992) observed that upon addition of 2, 4-D or NAA or IAA to the suspension culture, there was an increase in the production of anthraquinones in *M. citrifolia*, nicotine in *N.*

tabacum, shikonin in *L. erythrorhizon*, betacyanins in *Portulacca* and anthocyanins in *Populus* and *D. carota.* Cytokinins too have various effects depending on the type of metabolite and species concerned. Seitz and Hinderer (1988) and Mok *et al.* (1976) demonstrated that on adding kinetin, there is an enhanced production of anthocyanins in *Haplopappus gracilus* suspension culture whereas inhibition of production of anthocyanins in *Populus* suspension culture. Bohm and Rink (1988) and Seitz and Hinderer (1988) also reported that Gibberellic acid and abscisic acid are suppressed production of anthocyanins in a number of cultures.

4. SYNERGISM OF ENHANCEMENT STRATEGIES –

It has been reported by Choi *et al.* (1995) that a combination of various enhancement strategies results in many fold times increase stimulation of secondary metabolite is as compared to their individual approach which has been recognized valuable in large scale cultures. Sajc *et al.*, in the same year, also demonstrated that during production of anthraquinones from *Frangula alnus*, using an external-loop air-lift bioreactor in combination with immobilization using calcium alginate and silicone oil for in situ extraction of product increased the productivity almost 10-30 fold times whereas when they were employed separately the productivity increased only up to five times.

5. ELICITATION –

As proposed by Zhao *et al*. (2005), eliciators are compounds of pathogenic origin that induce of expression of variety of genes often associated with enzymes responsible for the synthesis of secondary metabolites by plants as a defense mechanism to attack by pathogens. Adding eliciators such as Jasmonic acid in the culture medium triggers the formation of secondary metabolites in plant cells. Elicitor dosage was found to be dependent on both the tissue density and the free elicitor concentration in the medium. Both biotic and abiotic elicitors were used to stimulate secondary metabolite product formation in plant cell cultures, thus reducing the time required to achieve high product concentrations and increased culture volumes. Various secondary pathways are activated by the plant in response to stress. Boller (1983) and Kohle *et al.* (1985) suggested the use of calcium ions and ethylene as secondary messengers that transmit signals from plasma membrane thus triggering the transcription and translation process during the process of elicitation. Yukimune *et al*. (1996) demonstrated that by adding methyl jasmonate to *Taxus media* culture, 110 mgl^{-1} paclitaxel can be produced in two weeks. Manivanan (2016) demonstrated that Salicylic acid can also stimulate production of various flavanoids in plant cell cultures. Rebecca *et al.* (2021) experimentally demonstrated that heavy metals can be used as elicitor in *Murraya koenigii* for enhanced production of flavanoids.

6. BIOSYNTHETIC PATHWAY ANALYSIS AND CONTROL –

Several approaches have been made for the effective monitoring of the rate-limiting step in the biosynthetic pathway of the natural products by Monitoring the enzymes present in the pathways just after introducing the elicitor, measuring the quantity of enzymes in the cell lines of different biosynthetic pathways, adding the precursor molecules of each biosynthetic pathway, over expressing and transforming the genes involved in the pathway. This method has been undertaken by Bohlmann and Eilert (1994) at the branch point of shikimate pathway for controlling the production of furoquinoline, furanocumarins and epoxides in *Ruta graveolens* and Dagnino *et al*. (1995) while working with two cell lines with different biosynthetic capabilities to produce terpenoid indole alkaloid in *Tabernaemontana divaricata*. Andrey *et al.* (2020) proposed that in order to successfully manipulate the biosynthetic pathway a combination of information derived from genomics, proteomics, metabolomics and transcriptomics is necessary.

7. SCREENING AND SELECTION OF HIGHLY PRODUCTIVE CELL LINES –

Choosing a parent plant having high contents of the desired products for callus induction to obtain high-producing cell lines is an important factor enhancing the production of secondary metabolites. Thus to achieve this, screening of the heterogeneous population for variant cell clones containing the highest levels of desired product was carried out. In this method the heterogeneity in the pre-existing biochemical activity in a population of cells was exploited in order to

obtain highly productive cell lines. Rhodes *et al.*, 1988, employed an alternative strategy involving introduction of mutation of the plant cells used in culture medium in order to obtain overproducing cell lines. The desired mutant cell from a large population of cells were then selected using appropriate selective agents by exposing them to a toxic inhibitor or environmental stress and only cells that are able to resist the selection procedures are then cultured to generate high-yielding cell lines.

8. PERMEABILIZATION –

Cell permeabilization is performed by creating pores in the membranes of the plant cell or its organelles, thus allowing easy passage of various molecules in and out of the cell. Brodelius (1988b) proposed that the measurement of enzyme activity of the primary metabolism, viz. hexokinase, glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase, malic and citrate synthetase allows easy monitoring of the permeability of the cells. Several attempts have been made by Brodelius *et al.* (1983) to permeabilize the plant cells, so that the cell viability and be maintained as well as increased mass transfer of substrate and metabolites to and from the cell can be achieved in a short time period. Permeabilizing agents such as organic solvents including isopropanol, dimethylsulfoxide (DMSO) and polysaccharides like chitosan have been put into use. Also methods including ultrasonication, electroporation and ionophoretic release, high electric field pulses and ultrahigh pressure can be used to increase permeability of the cell.

9. CYCLODEXTRINS –

Many plant cell cultures often due to dramatic decrease of cell viability, hardly convert precursors in the presence of organic phases leading to a reduction in the enzymatic activities of these systems. Thus, bioconversions in the presence of clathering agents such as cyclodextrins was designed to solve the problem of bioconversion of water-insoluble precursors by combining the advantages of apolar systems and aqueous. Haggin (1992) proposed that cyclodextrins are able to form stable inclusion complexes with natural spices and flavoring substances, thus increasing their cyclodextrin cavity. They can also be modified by substituting various functional compounds on the primary or secondary phase of the molecule. The chemically modified cyclodextrins are considered to be more water soluble than native cyclodextrins**.** Cardillo *et al.* (2021) proposed that cyclodextrins triggers accumulation of phytoalexin in *Vitis vinifera* and *Nicotiana tabacum* cell culture as a defense response.

APPLICATION OF CELL CULTURE

Fowler (1986) designed a viable alternative for the production of vast arrays of secondary metabolites, including pharmaceuticals, flavors and fragrances, foods, cosmetics, agrochemicals and fine chemicals is provided by *in vitro* large scale cultivation of plant cells. Curtin (1983) demonstrated the production of various pharmaceutically active agents such as Shikonin from *Lithospermum erythrorhizon* and Berlin (1986) demonstrated Berberine production from *Coptis japonica* commercially using large scale plant cell cultures. Biotransformation of organic compounds, as well as production of novel metabolites that are not normally found in nature can also be accomplished using plant enzymatic systems.

PRODUCTION OF SECONDARY METABOLITES

As proposed by Buyel (2018), plants harbor a varied range of secondary metabolites. Secondary metabolites are referred to those plant derived compounds, specifically small molecules, which do not directly take part in growth and development of an organism, but are intermediates and products of metabolism. Metabolites play an important role in plant defense against pests and pathogens. Ajayi *et al.* (2019) proposed that the secondary metabolites have complex aromatic rings and chiral centres, which have proven to be advantageous as a precursor for synthesis of novel drugs. These plant derived metabolites are considered as an important source of various pharmaceuticals, recombinant proteins, active ingredient in cosmetics and various food additives.

1. **In pharmaceuticals**

A great progress has been made in production of medicinal compounds using plant cell cultures. A broad spectrum of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavanoids, and amino acids has been produced till date. Epinosa-Leel *et al.* (2018) and Davoodi *et al*. (2019) proposed plant has been widely exploited for active plant derived metabolites that are frequently used in pharmacy.

Taxol (Paclitaxel)

It is a complex diterpene alkaloid derived from the bark of the *Taxus* tree. Jordan and Wilson (1995) suggested it to be one of the most promising anticancer agents known due to its unique mode of action on the micro tubular cell system. Srinivasan *et al.* (1995) performed a study on the kinetics of biomass accumulation and observed that paclitaxel production by *T. baccata* cell suspension cultures accumulated is the second growth phase.

Morphine and Codeine

Analgesics such as morphine and codeine have been derived commercially from latex extracted from *Papaver somniferum*. Tam *et al.* (1980) reported that morphine and codeine can be derived from morphologically undifferentiated cultures.

Ginsenosides

Tang and Eisenbrand (1992) reported the production of ginseng from the root of *Panax ginseng*. Since ancient times it has been widely used as a tonic and highly prized medicine for being a fantastic promoter of health and longevity. Proctor (1996) identified the primary bioactive constituents of ginseng to be ginsenosides, which is a group of triterpenoid.

L-DOPA (L-3, 4-dihydroxyphenylalanine)

Guggenheim (1913) isolated it from *Vinca faba* and is known to be a precursor of alkaloids, betalain, and melanine and also catecholamines in animals. It has proven to be potent drug for treatment of Parkinson's disease which is a progressive disabling disorder associated with a deficiency of dopamine in the brain. Since there is a huge demand for large quantities of L-DOPA due to the widespread application of this therapy, introduction of cell cultures as an alternative means for enhanced production is necessary to that they are commercially available at an affordable price.

Berberine

It is an alkaloid, derived from the roots of *Coptis japonica* and cortex of *Phellondendron amurense*. It has been obtained from a number of cell cultures of *Coptis japonica* reported by Sato and Yamada, (1984), *Thalictrum spp*. reported by Nakagawa *et al.*, (1984) and *Berberis spp.* reported by Breuling *et al.* (1985) and shows antibacterial properties.

Diosgenin

Tal *et al.* (1983) reported production of diosgenin from cell cultures of *Dioscorea deltoidea*. Zenk (1978) proposed it to be a precursor for the chemical synthesis of steroidal. They found that diosgenin accumulation in one cell line was greatly influenced by carbon and nitrogen levels.

Capsaicin

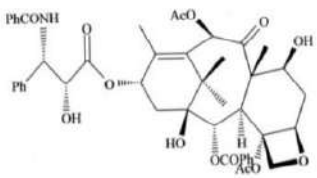
It is an alkaloid obtained from fruits of green pepper, *Capsicum spp*. Sooch *et al.* (1977) it's used in pharmaceutical preparations as a digestive stimulant and for rheumatic disorders and also as a pungent food additive in formulated foods.

Vinblastine and Vincristine

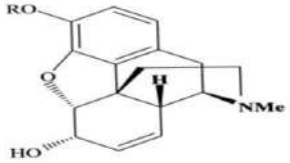
These compounds are dimeric indole alkaloids and are extracted commercially from large quantities of *Catharanthus roseus* since the intact plant are low in their concentrations. Vinblastine is consists of catharanthine and vindoline. They are considered to be valuable drugs in cancer chemotherapy due to their potent antitumor activity against various leukemias and solid tumors.

Podophyllotoxin

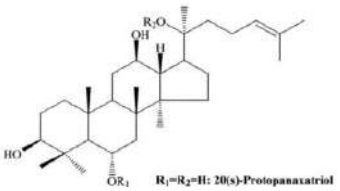
It is extracted from *Podophyllum peltatum* and *Podophyllum hexandrum*. Issell *et al.*, (1984) reported that it a lignan and a precursor for the preparation of its semisynthetic derivatives such as etoposide and teniposide, which are widely used in anti-tumor therapy.



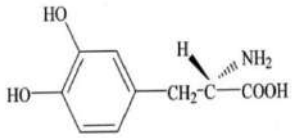
Taxol



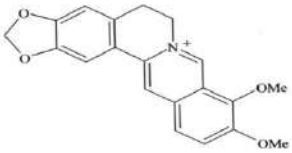
R = H – Morphine, R=Me – Codeine



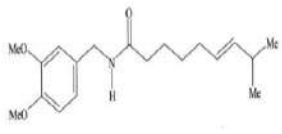
Ginsenosides



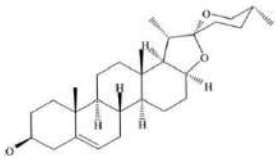
L- DOPA



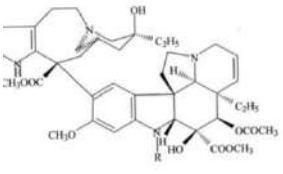
Berberine



Capsaicin

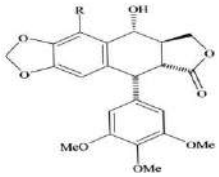


Diosgenin



R=CH_3 – Vinblastin

R =CHO - Vincristine



Podophyllotoxin

Structure of few secondary metabolites used in the pharmaceutical industry

As proposed by Xu and Zhang (2014) the therapeutic proteins produced by modern biotechnological techniques were referred to as 'biopharmaceuticals'. Biopharmaceuticals are considered to be revolutionized modern medicine and represent the fastest growing sector within the pharmaceutical industry. They include protein therapeutics, mainly antibodies and antibody derivatives, vaccines and some serum-derived proteins, like cytokines, growth hormones, interleukins and interferon. These are used for the treatment of diabetes, anemia, hepatitis, cancer and cardiovascular diseases.

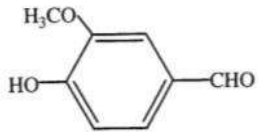
2. In food biotechnology

Davies and Deroles (2014) proposed that production of commercial high-value food additives present in very low quantity in plant or extraction from very rare plant species are primarily targeted using cell culture technique. Very few food related products have had ongoing commercial success, specifically those producing ginsenosides, paclitaxel, berberine alkaloid-based health supplements, and the dye shikonin. Krasteva *et al*. (2020) proposed that a lot of plant-derived natural products have been used as food in the last few years. Vancauwenberghe (2019) and Park *et al*. (2020) proposed that production of flesh-like printed cellular products using food inks containing living plant cells and pectin or alginate matrix have been made possible due to advancements in 3D printing technology.

Aromas and Fragrances

Natural aromas are considered to be the outcome of mixing a variety of compounds and are susceptible to the conservation processes of foodstuffs, such as sterilization, pasteurization, freezing, etc. Enzymatic or chemical

reactions can alter some aromas resulting in their disappearance if stored over long period. Extracting aromas from the cell cultures offer a significant advantage of constant composition and independence of the season. Vanillin, obtained from culture of *Vanilla planifolia* plants is considered to be one of the universally used aromas as proposed by Prince and Gunson (1994). Burri *et al*. (1989) proposed vanillin to be an antioxidant having antimicrobial activity as proposed by Lopez-Malo *et al*. (1995) and anti mutagenic activity as proposed by Kometani *et al*. (1993a). Also, Kurata (1998) reported that from cell cultures of *Tlaeobroma cacao* and *Coffea arabica*, the aromas of cocoa and coffee respectively have been derived.



Structure of Vanillin

Pigments

The use of additional pigments was strongly criticized by the associations of consumers in the 1970s, because most of the colors are produced by chemical synthesis and they are unrelated to any naturally occurring material. The biotechnological methods used for producing natural food colorants consist of growing higher plant cells. Kim (1990) obtained shikonin compounds**,** such as shikonin and its derivatives acetyl and isobutyl shikonin, from the roots of *Lithospermum erythrorhizon*. Since these plants are very rare, the mass cultivation of *Lithospermum erythrorhizon* cells to produce shikonin compounds has been successfully established. Curtin *et al*. (2003) derived anthocyanins from *Vitis vinifera* culture, which are water-soluble pigments, providing coloration to fruits and flowers. They also act as a natural pH indicator and turn red at low pH and blue at pH over six and are commercially used in acidic solutions such as soft drinks, sugar confectionary, jams, and bakery toppings in order to impart them a red coloration. Chen *et al*. (2003) produced crocin from culture of *Crocus sativus* stigma, which is commercially used as a yellow food colorant.

3. In the cosmetics

Zappelli *et al.* (2016) proposed that product approval in the pharmaceutical industry differs from that in the cosmetics industry as no official approval is required and the developments and innovations introduced in the cosmetics industry by manufacturing variety of new cosmetics products every year are strongly based on the demand of the consumer. Schmidt (2012), Fonseca-Santos *et al.* (2015) proposed that the consumer primarily demands effective, safe, and natural cosmetics which are time at the same sustainable and whose manufacture does not harm the environment. Thus, there is an immense interest for production of multiple specific activities for skin care, make-up, and hair care as supplement ingredients in plant cell culture extracts with respect to the cosmetics industry. Also rare or endangered plant species extracts can be obtained using plant cell culture technology. Krasteva *et al*. (2020) proposed that using plant cell culture technology a large number of cosmetics products have been manufactured explaining the renaissance in plant cell culture technology. In 2008, Mibelle Biochemistry laid foundation of usage of plant stem cell culture extracts in the cosmetics industry.

PRODUCTION OF RECOMBINANT PROTEINS

The first report of recombinant protein was production using plant cell culture was in 1990. Since then, different recombinant proteins have been produced in plant cell cultures, including antibodies, enzymes, hormones, vaccines, growth factors and cytokines. Fischer *et al.* (1999) proposed that Tobacco cell suspension culture offers several advantages and hence can be used as model systems for production of recombinant proteins. Helwig *et al.* (2004) added that tobacco suspension-cell lines such as Bright Yellow 2 (BY-2) and *Nicotiana tabacum* 1 (NT-1) have been used as

model systems for production of recombinant-protein. As proposed by Kwon (2003) recombinant proteins have also been produced in rice, soybean and tomato using plant suspension cultures. The advantages of studying such cell lines included the possibility of being more favorable than tobacco in terms of by-product levels since they are derived from food crops and also that plants with higher protein content like soybean and lupin, might more readily facilitate higher expression levels. Other benefits included faster growth, higher expression, levels, more efficient secretion and other advantages concerning, process compatibility. Mason *et al.* (1992) proposed the concept of edible vaccine by integrating genes encoding anitigens of various pathogens with the gene of preferably less expensive plants. As mentioned by Ramachandra Rao *et al.* (2002), costly and low volume products like anti-HIV and anti-cancer can be produced at a large scale using plant cell culture technique, thus paving the way to commercialize a few selected pharmaceuticals.

COMPANIES COMMERCIALIZING PLANT CELL CULTURE TECHNOLOGY

Dow AgroScience, LLC

It is a US company based in Indianapolis that developed the Concert™ Plant-Cell-Produced System as a leading edge platform for the production of vaccine antigen. In January 2006, the regulatory approval was received by them for the world's first plant-cell-produced vaccine against Newcastle disease virus in poultry from the USDA Center for Veterinary Biologics. The plant-derived poultry vaccine is the recombinant hemagglutinin-neuraminidase glycoprotein, one of the surface glycoproteins of the Newcastle disease virus and the major surface antigen that induces neutralizing antibodies. The vaccine was expressed in tobacco BY-2 cells.

Phyton Biotech, Inc.

Phyton Biotech, based in East Windsor, NJ, USA, with the proprietary plant cell culture fermentation (PCF™) platform, developed and commercialized products with applications in the pharmaceutical and biotech industries, such as paclitaxel and docetaxel. The significant commercial success for Phyton was developing a commercial production of paclitaxel with *Taxus* (*T. chinensis*) cell suspension culture, which is a secure, sustainable and environmentally-friendly source of paclitaxel for Taxol®, a mitotic inhibitor used in cancer chemotherapy. Later, Phyton expanded its PCF™ platform to include recombinant proteins.

Protalix BioTherapeutics, Inc.

Protalix is an Israel-based biopharmaceutical company that is leveraging its proprietary plant cell-based expression system, ProCellEx®, for the development and commercialization of recombinant biopharmaceuticals. In May 2012, Protalix partnered with Pfizer to commercialize taliglucerase alfa for injection, the world's first plant cell-produced human therapeutic protein approved by the FDA for marketing. Protalix's taliglucerase alfa is a recombinant active form of the lysosomal enzyme, β-glucocerebrosidase, used for treatment of Gaucher's disease. Protalix's development pipeline also includes the PRX-102, a modified version of α-galactosidase, for the treatment of Fabry disease, PRX-112, an orally delivered glucocerebrosidase enzyme that is produced and encapsulated within carrot cells for the treatment of Gaucher's disease, PRX-110, a DNase I enzyme for the treatment of cystic fibrosis. Protalix is currently the world's leader in development and commercialization of the plant cell-based production platform for biopharmaceuticals with great success.

Greenovation Biotech, GmbH

Greenovation Biotech is a German biopharmaceutical company that uses its proprietary moss (*Physcomitrella*)-based BryoTechnology™ for the commercialization of recombinant biopharmaceuticals. The moss, *P. patens,* has N-glycans that are generally free of the core α-1, 6-fucose, a sugar-structure typically present on N-glycans of mammalian-cell-

derived proteins. The absence of this sugar structure has been proven to drastically increase the efficacy of IgG-products by enhancing antibody-dependent cellular cytotoxicity. In addition, genome engineering in the moss, which is based on a homologous recombination, is straightforward and very effective compared with that in other organism.

PhytoCELLTECH

The company launched PhytoCELLTECH *Malus domestica* which is the first commercially available plant cell culture extract whose effect was studied on human skin cells and which claims to be derived from plant stem cells. It was established from the core of an endangered Swiss apple variety, which can be stored for a long time without becoming shriveled or losing flavor. The company has patented the manufacture and usage of apple cell culture extracts which originate from *Malus domestica* cultivar Uttwiler Spätlauber and which protect skin cells. PhytoCELLTECH *Malus domestica* entails numerous plant cell culture extracts which are used by leading cosmetics brands such as Dior, Lancôme, Guerlain, and La Prairie in their cosmetic formulations. The final products include facial serums, facial creams and facial masks, eye creams, make-up products, hair oils, hair serums, and hair conditioners.

CONCLUSION

Cell culture techniques continue to be a significant tool in the study of various areas of plant biology and biochemistry. These have acquired major importance and acceptance in molecular biology and biotechnology. Cell culture systems offer several advantages over the conventional cultivation of whole plants, few of which include production of various important compounds under controlled conditions that are independent of climatic changes or soil conditions, use of thoroughly sterilized cells in culture media, multiplication of cells of any plants, enhanced yield of specific metabolites, irrespective of their region of occurrence, reduction of labor costs as control of cell growth are automatic and rationally regulated leading to improved productivity. But as proposed by Smetanska (2008), developments in cell culture techniques are still done as several drawbacks are encountered that are needed to be solved before their application can be increased.

Thus we see, cell culture technique has evolved a lot through ages be it in terms of culture techniques, or bioreactor designs or introduction and manipulation of various factors, with a unified aim of enhancing the yield of products. Although a variety of culture techniques have been developed till date offering some marked advantage over one another, there is no single technique that can be used universally for culturing all types of cells. Thus a lot of trials and errors are needed to be done before designing a technique to culture a specific type of cell so as to obtain the maximum product yield. For instance, we see that for culturing both *C. roseus* and *N. tabacum* cells immobilized cell culture technique is preferred whereas in case of selecting bioreactors, for *C. roseus* cells stirred-tank or bubble column bioreactor is preferred and for *N. tabacum* cells air-lift or rotating drum bioreactor is preferred.

The companies designed based on the cell culture technique also keep on modifying the technique so as to meet the demand of the consumer. Hence a sound knowledge of plant science, economics and commerce is very important to increase the profit without compromising with the quality of the product and health of consumers and at the same time preventing environmental pollution or loss of habitat of the rare but important plants. Also as Hesami *et al.* (2020) proposed, different mathematical models combining factorial design, response surface methodology, artificial intelligence models and algorithms to enhance the productivity. Krasteva *et al*. (2020) mentioned that 3D printing has the potential to open new doors for enhanced production of high quality food and active ingredients of cosmetics using plant cell culture techniques. Today we see that this technique has been commercialized worldwide thus uplifting the economy as well as providing a source of livelihood to many people.

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UNVEILING THE MOLECULAR INTRICACIES OF MEDICINALLY IMPORTANT PLANTS ON SARS-CoV-2

ABSTRACT

The outbreak of the deadly disease caused by Severe Acute Respiratory Syndrome- related Coronavirus 2 or SARS-CoV-2 (COVID- 19) has caused severe impact health leading to lacs of human death toll. Natural products have been potentially exploited in India since ancient times and have manifested against several diseases. The review mainly focuses on the promising role of phytochemicals extracted from various plants on SARS-CoV 2 viral replication thereby inhibiting its multiplication and enhancing the immune system as a precautionary measure.

1. INTRODUCTION

Plants have been used in healthcare since a very long time **(Sofowora et al., 2013)**. The utility of plants to cure various human diseases has a very long history. Several plant parts like leaf, stem, bark, root etc. are used to prevent the symptoms or revert abnormalities back to normal **(Mintah et al., 2019)**. Recently, the new Corona Virus disease i.e. The Severe Acute Respiratory Syndrome-related Coronavirus 2 (SARS-CoV-2) or novel coronavirus (COVID-19) has caused global socioeconomic disturbances with a worrisome number of health issues and deaths. It belongs to the ß genus, order Nidovirales of the family Coronaviridae and is an enveloped, single (+) stranded RNA, with symmetric helical nucleocapsid **(Khan et al., 2020)**. The virus encodes four main structural proteins; S: spike; E: envelope; M: membrane; N: nucleocapsid, and various nonstructural proteins such as RNA dependent RNA polymerase (RdRp), coronavirus main protease (3CLpro), and papain-like protease (PLpro) etc. Though, the entry of several ß genus viruses like SARS-CoV and NL63 interaction with the ACE2 receptor resembles to that of SARS-CoV-2, however, some differences have been reported among these strains like, the length of the S protein and structure of the receptor binding.

Since the outbreak of Coronavirus disease, various traditional herbal medicines with promising results have been used to treat the infected patients either alone or in combination with other conventional drugs **(Benarba and Pandiella, 2020)**. Medicinal plants can be potential healers that can help people fight against this infection by either boosting their immune system or by applying viricidal effects **(Srivastava AK et al., 2020)**. Most virus-specific enzymes the main targets of antiviral medicines and hence, coronavirus-specific enzymes should be potential targets to treat the diseases **(Jang et al., 2020)**. The different herbal extracts and purified molecules may exert their anti-SARS-CoV-2 actions by directly inhibiting the virus replication or its entry. For allowing its attachment to human and bat cells and further replication, the angiotensin converting enzyme II (ACE2) was found to be an important functional receptor for the SARS-CoV-2 and therefore, some of the medicinal plant extracts may block the ACE-2 receptor or the serine protease TMPRRS2 that is needed by SARS-CoV-2 for infection **(Benarba and Pandiella, 2020)**. Several studies have proved that

polyphenols play an important role in controlling various human pathogens including severe acute respiratory syndrome-related CoV (SARS-CoV) of Southern China (2003), the Middle East respiratory syndrome-related CoV (MERS-CoV) of Saudi Arabia (2012), which are similar to COVID-19 **(Khalil & Tazeddinova, 2020; Mahmood et al., 2020)**.

It is observed that extracts isolated from medicinal plants and/or herbs including Traditional Chinese Medicines (TCM) such as *Andrographis paniculate, Artemisia annua, Agastache rugosa, Astragalus membranaceus, Amygdalus communis, Cassia alata, Curcuma longa, Ecklonia cava, Ephedra sinica, Gymnema sylvestre, Glycyrrhizae uralensis, Houttuynia cordata, Lindera aggregata, Lycoris radiata, Mollugo cerviana, Ocimum sanctum*, *Polygonum multiflorum, Punica granatum, Pyrrosia lingua, Rheum emodi, Saposhnikoviae divaricate, Thymus serpyllum*, *Tinospora cordifolia, Toona sinensis, Withania somnifera*, etc. have also shown promising inhibitory effect against the coronavirus **(Zahedipour et al., 2020 - Tin-Yun Ho et al., 2020)** . Various experiments are being done at molecular levels so as to get the proper medicine that can help in treating COVID-19 disease.

2. LITERATURE REVIEW

Till date there are various reports on the role of different active principles and their role on curing COVID-19 disease. This review explicates the biomolecular targets of the various secondary plant metabolites and their mode of antiviral action on this life-threatening disease (Table 1).

2.1 *Curcuma longa*:

Extracts from the roots of the plant *Curcuma longa* (family Zingiberaceae), contain a natural polyphenolic compound, curcumin that exhibits a variety of therapeutic properties including anti-microbial, anti-proliferative, anti-inflammatory, antioxidant, neuroprotective and cardioprotective. This yellow pigment of turmeric is also widely used in traditional Indian medicine to treat the great no. of infectious diseases since decades, and are now reported to have anti-viral activities against viruses such as HIV, HPV, HSV-2, Zikavirus, Influenza virus, Adenovirus and Hepatitis virus and therefore, can work as a potential therapeutic agent for COVID- 19 **(Manohar et al., 2020)**. In case of SARS-CoV, curcumin inhibited its replication and also 3Cl protease in Vero E6 cells. It also has an important protective function against the effect of SARS-CoV on Vero E6 cells **(Bababei et al., 2020)**.

Recent studies have shown that SARS-CoV and SARS-COV2 are similar in invading human host cells by targeting the Angiotensin Converting Enzyme 2 (ACE2) membrane receptor, which is present in the entry site i.e., mucous membrane of the coronavirus. Its binding to the S protein of virus, links the interaction of the virus with the membrane and the ongoing replication of the virus to the host **(Manohar et al., 2020)**. It was pointed out that human recombinant soluble ACE2 (hrsACE2) can inhibit the growth of SARS-CoV-2 in Vero-

E6 cells, kidneys and human capillaries that prevent them from entering host cells but, have not studied lung organoids which are major target organs of COVID-19 infection **(Bababei et al., 2020)**.

Studies have reported that catechin and curcumin can also prevent SARS-CoV2 infections. Curcumin binds directly to the RBD, i.e., receptor binding domain of the S Protein of virus, while catechin binds to amino acid residues adjacent to the RBD site of S Protein causing fluctuations in RBD amino acid residues and their close proximity. Both catechin and curcumin bind to the 'RBD/ ACE2-complex' complex link that causes the variability of alpha helices and beta-strands of protein complexes. Additional studies have demonstrated the ability of these two polyphenols to inhibit the formation of S Protein-ACE2 complex. Therefore, this computational study also predicts that these two polyphenols may have SARS-CoV2-resistant therapeutic properties **(Jena et al., 2021)**.

Therefore, curcumin can be regarded as a promising prophylactic, COVID-19 medicinal agent as it contains antiviral properties to many types of viruses, including SARS-CoV-2, in many ways i.e., by direct contact with membrane proteins -virus or prevent viral proteases or disruption of viral envelopes or create antiviral responses that are safe and well tolerated in healthy and sick people **(Thimmulappa et al., 2021)**.

2.2 *Andrographis paniculate*:

Andrographis paniculate has been reported to be used to treat a variety of ailments including liver disease, common cold, flu, high blood pressure, painful diarrhoea, hepatitis, leprosy, malaria, anti-inflammatory effects, diabetes, anti-cancer, etc. **(Rajagopal et al., 2020)**. By *in- silico* studies such as molecular docking, target analysis, toxicity prediction and ADME prediction, it has been shown that Andrographolide from *Andrographis paniculat*e can act as a potential inhibitor of the main protease of SARS-COV-2 (Mpro) **(Enmozhi et al., 2020)**. From *in silico* molecular docking experiments, it has been shown that some chemical compounds such as Andrographolide binds to the COVID-19 main protease active site by severely inhibiting SARS CoV-2 main protease and may be effective against COVID -19 in the ongoing process **(Rajagopal et al., 2020)**.

Between Andrographolide and 14-deoxy-11,12-didehydroandrographolide, 14-deoxy-11,12 didehydroandrographolide was expected to have a very high affinity with PLpro. Getting to this, test is done using Autodock 4.0. Molecular dynamics (MD) simulations may provide a deeper understanding of the interaction between ligand and protein amino acid residues at the atomic level. The MD simulation was performed with the software package AMBER18, and the results were analysed according to RMSD and RMSF of complex protein-ligand complexes. The above tests were performed and by these tests it was found that Andrographolide and 14-deoxy-11,12-didehydroandrographolide may have anti-viral properties over COVID-19, demonstrated by testing the binding affinity of these bioactive substances over PLpro, 3CLpro, and spike protein **(Khanal et al., 2021)**.

2.3 *Punica granatum*:

Punica granatum is a medicinal fruit plant that has been used around the world since many decades. The extraction of pomegranate has been in use in combating many viruses, such as influenza viruses, poxviruses, herpes viruses, and the human immunodeficiency virus. Phytochemicals like punicalagin, punicalin and pomegranate peel extracts (PoPEx) have also shown effects against viruses by preventing virus entry and its RNA transcription. further studies have shown that pomegranate peel extracts constituents such as punicalagin and punicalin targets and interacts with selected protein and thus may prevent the entry of the virus into the host cell **(Suručić et al., 2020)**.

Through docking study at the predicted active sites in S glycoprotein and ACE2, two molecules, namely, lopinavir and umifenovir were used as positive regulators. Chloroquine and hydrochloroquine shows inhibiting properties SARS-CoV-2 infection in vitro. While, on the other hand remedesivir-like molecules have recently been approved for the treatment of patients in hospitals with severe COVID-19 infections. Docking test results showed that no PoPEx complexes were more stable than those of lopinavir containing ACE2. Although it is seen as a novel treatment mechanism for SARS-CoV-2 infection by blocking ACE2 from any contact with S glycoprotein, making ACE2 one of the most important homeostasis regulators through the renin-angiotensin system. It encourages scientists to move forward in vitro studies and bioavailability in humans, which will enable us to test the potential of PoPEx components in the treatment or prevention of COVID-19 **(Suručić et al., 2021)**.

2.4 *Withania somnifera*:

Researchers from two different groups have found that the Withaferin A (WFA) which is a steroidal lactone, has anti-inflammatory properties, can bind to the SARS-CoV-2 spike protein i.e., (S-) protein. In addition, experiments have shown that Withaferin A does not alter the expression of ACE2 in the lungs of female rats and its reduction has recently been shown to increase the COVID-19 levels. Therefore, because of the tampering in S-protein of the virus to receptor binding in host and its absence of effect in ACE2 expression in the lungs, it can be argued that Withaferin A is potent as a therapeutic agent for COVID-19 **(Straughn, and Kakar, 2020)**.

Recently, various in- silico studies have reported several antiviral actions found in *W.somnifera* by inhibiting Viral protease (3CLpro and PLpro), host cell-producing protease (TMPRSS2), RNA polymerase (RdRp) and the S-protein interacting site with the ACE-2 receptor in host. In molecular docking, it has been shown (Kumar et al.) That withanone and the caffeic acid phenethyl ester also act as a potential inhibitor of SARS-CoV-2 main protease **(Kashyap et al. 2020)**. Also, according to YASARA scoring goals of Withanoside V and Somniferine, it is found that there is a strong binding relationship with SARS-CoV-2 Mpro **(Priya Shree et al. 2020)**.

The above reports indicate the potent phytochemicals present in the *W.somnifera* to aid in the treatment of COVID-19.

2.5 *Tinospora cordifolia*:

The various elements of *Tinospora cordifolia* show many therapeutic properties including anticancer, antitoxic, antimicrobial, antidiabetic, hypolipidermic, immunomodulation, wound healing, etc. Depending on the importance of the treatment, the chemical components of *Tinospora cordifolia* extract are categorised into various groups such as alkaloids, steroids, lignans, and terpenoids and are being investigated as a potential anti-COVID-19 agent. Computational molecular modelling (Auto dock), and their ADME / T properties have been used to demonstrate the components' ability to block the viral spike protein and human receptor ACE2 **(Jenal et al., 2021)**.

Tinocordiside, a newly discovered cadinane squiterpene glycoside reconstituted from *Tinospora cordifolia* has shown a very high affinity for SARS-CoV-2 Mpro according to a YASARA score **(Priya Shree et al. 2020)**. By pulling out the molecular docking for screening phytocompounds present in *T. cordifolia* against the ACE2-RBD complex, performing a MD i.e., molecular dynamics simulation, found that the tinocordiside was found to be well stabilized during MD simulation and is well positioned in the center of the ACE2-RBD complex interface. It shows that such tampering of electrostatic interactions between ACE2 and RBD, as well as increased complex global fluctuations, will further weaken or prevent the entry of SARS-CoV-2 and its infection.

It can therefore be argued that phytochemicals such as Tinocordiside may be undergo effective mechanisms for the prevention of entry of SARS-CoV-2 into host cells **(Balkrishna et al. 2020)**.

2.6 *Ocimum sanctum*:

Ocimum sanctum (Holy Tulsi) is known as an ancient and traditional medicine to treat common cold and respiratory illnesses in India and is considered one of the ingredients in the preparation of immune boosters. Since ethanolic extraction of aerial parts of Tulsi has been reported to contain polyphenolic acids and flavonoids, which have antiviral properties, and therefore, *in- silico* analysis of phytochemicals as inhibitors of SARS-CoV- 2 main protease was performed **(Mohapatra et al. 2020)**.

According to YASARA scoring goals, Vicenin, Isoorientin 40-O-glucoside 200-O-p-hydroxybenzoagte and Ursolic acid showed significant affinity of binding to SARS-CoV-2 Mpro **(Priya Shree et al. 2020)**. *In- silico* analysis of the many flavonoids and polyphenolic acids of *Ocimum* shows potentiality to be inhibitors of Mpro and is reported to be potent to have antiviral effects to some viruses **(Weng et al, 2019; Jo et al., 2019).** It has also been reported that flavonoids are known to inhibit SARS-Cov 3CL (homologous to Mpro SARS-CoV-2).

Although all the studies conducted are entirely theoritical, these studies may show the potential for phytochemicals to treat or prevent SARS-CoV-2 **(Mohapatra et al. 2020)**.

2.7 *Toona sinensis*:

The Traditional Chinese medicines (TCM) have a history of more than 2000 years and is used to treat various diseases. During the SARS outbreak, it was reported that the TSL1 aqueous extract component from the tender leaf of *Toona sinensis* Roem was identified as an effective agent that may combat SARS-CoV in vitro **(Kwong et al. 2020)**.

A Taiwanese group of scientists has reported that leaf extracts of *Toona sinensis* Roem (TSL) have a strong anti-SARS-CoV effect by taking a fraction of the nanoparticulated extract (TSL-nm) and crude extract (TSL-1) of leaves prepared by boiling and conventional techniques and tested separately. Through experiments they concluded that the components of TSL have a promising function against SARS-CoV. Although their antiviral activity has been proven but active TSL components responsible for those functions have not been identified **(Orhan et al. 2020)**.

As, research shows that the tender leaf of *Toona sinensis* Roem, can prevent SARS-CoV, so it is expected to prevent SARS-CoV-2 as well **(Mahmood et al., 2021)**.

2.8 *Amygdalus communis* and *Ephedra sinica*:

Numerous studies have shown that Traditional Chinese Medicines (TCM) are functionally effective in treating many diseases. The effective combination of plants among those TCM herbs or herbal pairs are considered to be, which are widely used in the treatment of diseases. Since the outbreak of the COVID-19 problem in China, Chinese health authorities have brought in top TCM specialists to evaluate the formulations and development of anti-SARS-CoV-2 coronavirus drugs **(Xia et al., 2021)**.

After categorising the TCM plants for its use in treating COVID-19, recommended by leading experts, the association rule approach was employed to evaluate its compatibility and its distribution, in order to determine the most important remedies. Finally, the major compounds that work in pairs are selected after analysing the potential mechanisms of active ingredients in the selected pairs of anti-COVID-19 for molecular docking with SARS-COV-2 3CLpro and ACE2 i.e., angiotensin converting enzyme II. The results showed that the pair of *Amygdalus Communis* Vas (ACV) which is part of the bitter apricot kernels and *Ephedra sinica* Stapf (ESS), a variety of plant species native to Russia, Mongolia, northeast China and parts of northern Thailand can be used in almost all phases of COVID-19 disease, and hence, ACV and ESS are selected as the most important herbal remedies **(Xia et al., 2021)**. The components that are functional against COVID-19 were kaempferol, quercetin, luteolin. The targets of these components were Interleukin 6 (IL-6), MAPK8, Interleukin-1β (IL-1β), MAPK1, and Nuclear factor kappa -light-chain- B-activated B-enhancer (NF-kB) p65 subunit (RELA) The molecular docking derived reports have shown that it has a very good relationship with SARS-COV-2 ACE2 and 3CLpro, therefore, the combination of ACV and EAS could be an important component of the COVID-19 therapeutic drug.

2.9 *Glycyrrhiza uralensis*:

Glycyrrhiza uralensis or licorice root has biological properties, including anti-inflammatory, antitumoral, and antiviral effects. Glycyrrhizic acid (glycyrrhizin) and its aglycone glycyrrhetinic acid (GLA) are effective against viruses, including SARS coronavirus (SARS-CoV) in vitro **(Mahmood et al., 2021)**.

Studies have been performed on aqueous *Glycyrrhiza uralensis* extract for its antagonistic activity against SARS-CoV-2 in vitro, and reported that glycyrrhizin is responsible for the process of neutralization, which strongly inhibits the viral main protease, neutralising SARS- CoV-2 19 **(Sand et al. 2020)**. An *in- silico* simulation study was performed to investigate the activity of neutralization. It has been shown that the human transmembrane serine protease (TMPRSS2) cleaves the SARS-CoV-2 spike protein S, which facilitates the entry of the virus into the cell. From the experiments, they concluded that glycyrrhizin neutralizes the virus by an activity that is different from TMPRSS2 inhibition and targets SARS-CoV-2 main protease (Mpro), which is very important for viral replication, as it is responsible for processing viral polyproteins translated from viral RNA. Therefore, Glycyrrhizin can be considered a promising antiviral compound to be studied for its use in the treatment of COVID-19 **(Sand et al. 2020)**.

2.10 *Rheum emodi*:

Since many years, traditional healing plants have been used to treat various ailments **(Rolta et al., 2020)**. *Rheum emodi* is a medicinal plant with a variety of medicinal properties and has been used in many herbal remedies, in the treatment of diseases, such as in regulation of blood fat, cancer etc. **(Zargar et al., 2011)**.

Studies have reported that emodin from *Rheum emodi* acts as an inhibitor of the corona SARS-CoV 3a ion channel and is a potent inhibitor of 3a channel. Emodin shows inhibition of viral release by reducing the viral RNA of cells outside the cell **(Rolta et al., 2020)**.

The *in- silico* method was adopted to determine whether unique phytocompounds such as emodin from *Rheum emodi* could physically bind COVID-19 binding proteins such as SARS-CoV-2 spike glycoprotein, SARS coronavirus spike receptor-binding domain, with spike ectodomain structure of the SARS-CoV-2 and then by inhibiting COVID-19 binding to the receptor ACE2 **(Rolta et al., 2020)**. It has been reported that anthraquinone compound extracted from this plant, emodin significantly inhibited the interaction of S protein with ACE2. Those reports suggest that emodin could be considered a leading treatment solution in the curing SARS **(Tin-Yun Ho et al., 2007)**. Therefore, further studies on emodin should be continued for its potential in the treatment of SARS-CoV2.

2.11 *Thymus serpyllum*:

In the study of in vitro and in vivo of traditional medicinal plants, the phytochemical found in *Thymus serpyllum*, thymol showed many medicinal properties such as anti-fungal, anticancer, anti-oxidant, anti-bacterial, anti-inflammatory, vasorelaxant, spasmolytic and hepatoprotective **(Javed et al., 2021)**.

The *in-silico* approach was adopted to determine whether thymol and carvacrol could physically bind COVID-19 proteins such as SARS-CoV-2 spike glycoprotein, SARS coronavirus spike receptor-binding domain, and SARS -CoV-2 spike ectodomain structure and then blocking COVID-19 binding to host receptor ACE2 **(Rolta et al., 2020)**. Kulkarni et al. (2020) reported that carvacrol may inhibit viral spike (S) glycoprotein binding to host cells. Tests were performed by docking carvacrol against the spike protein S1 receptor binding domain, which is the main target of antiviral drugs, to identify their inhibitory effects **(Javed et al., 2021)**.

Thymol, derived from thyme oil, is commonly used in pesticide products such as fungicidal products, tuberculocidal, antimicrobials, and virucides. It can be used in Sanitizers and cleaning products that effectively kill SARS-CoV-2 **(Pedreira, Ta¸skın and García, 2021)**.

2.12 *Artemisia annua*:

The active compounds present in *Artemisia annua* have a significant effect on many viral infections such as the hepatitis B virus, Epstein - Barr virus, bovine viral diarrhoea virus and SARS coronavirus **(Haq et al., 2020)**. The *in- silico* approach reported that Artemisnin derived from *Artemisia annua* could physically bind COVID-19 target proteins such as SARS-CoV-2 spike glycoprotein, SARS coronavirus spike receptor-binding domain, and SARS-CoV-2 spike ectodomain and inhibits COVID-19 binding to the ACE2 receptor **(Rolta et al., 2020)**.

A series of studies concluded that the extracts of *Artemisia annua* showed anti-SARS-CoV-2 activity in Vero-E6 cell-based cytopathic effect screening and may prevent SARS-CoV induced cytopathy **(Kapepula et al., 2020)**. Since, during SARS-CoV-2 infection, active pulmonary fibrosis, which is linked to Interleukin-1 (Conti et al., 2020), that appears to be associated with oxidative stress and natural antioxidant use are effective in this condition (Day, 2008), Studies have shown that the extract of *A. annua* shows significant antioxidant activity, hence the promising drug for the prevention of pro-fibrotic molecules associated with pulmonary fibrosis and for the treatment of pulmonary fibrosis to fight against SARS-CoV-2 **(Haq et al., 2020)**.

Among the many phytocompounds such as emodin from *Rheum emodi*, thymol and carvacrol extracted from *Thymus serpyllum* and artemisnin found in *Artemisia annua*, molecular docking studies using AutoDock / Vina software have shown that best binding affinity of SARS-CoV-2 spike glycoprotein, SARS coronavirus spike receptor-binding

domain, and ectodomain structure of SARS-CoV-2 exhibited by artemisnin **(Rolta et al., 2020)**.

2.13 *Piper nigrum:*

Piper nigrum is used as spice on daily basis and is known for piperine, a pungent compound present in it **(Meghwal and Goswami, 2013)**. A team of Indian scientists have identified black pepper, as part of an anti-coronavirus medicine.

Computational studies conducted by researchers at the Indian Institute of Technology (Indian School of Mines), Dhanbad found that piperine, found in black pepper, is able to bind and prevent the SARS-CoV-2 virus that causes the disease. It was revealed that an oleoresin compound piperin in black pepper (*Piper nigrum*) can be a potential inhibitor of SARS-CoV-2. In this experiment, inhibitor of SARS-CoV-2. In this experiment, 26 compounds examined found in various anti-SARS-CoV-2 compounds through molecular docking and showed that piperine has a higher affinity than adenosine monophosphate in relation to the RNA-binding pocket of nucleocapsid Molecular dynamics simulation of the docked compounds has confirmed the stability of piperine that is trapped in the nucleocapsid protein as an inhibitor of the RNA binding site.

Thus, piperine appears to have the potential to inhibit the RNA packaging into the nucleocapsid and therefore, inhibit the spread of the virus **(Choudhary et al., 2020)**.

2.14 *Syzygium aromaticum:*

Syzygium aromaticum or clove is among the most important herbs in traditional medicine and is well-known for its use in traditional medicine since centuries for many ailments **(Vicidomini et al., 2021)**. The major phytochemicals found in clove oil are mainly eugenol (76.8%) followed by eugenyl acetate (1.2%) and β-caryophyllene (17.4%) which leads to biological benefits such as antibacterial, antifungal, insecticidal, antioxidant, anticarcinogenic capacities **(Jirovetz et al., 2006)**. Docking scores have shown that these compounds have affinity of binding to the spike protein of SARS-CoV-2, main protease (Mpro), RNA-dependent RNA polymerase and human ACE-2 proteins **(Asif et al., 2020)**.

The combination of clove oil, wild orange oil, and cinnamon oil has shown significant antiviral activity with a 90 percent viral particles reduction. The combination of oils also reduces the risk of infection. All essential oils can be used as a treatment for the disease such as by using essential oil or Nebulizer, inhaling Essential Oil, so that the active ingredients reach the bloodstream through Lungs **(Wu S et al., 2010; Patne et al., 2020)**.

Essential oils have anti-viral activity compared to SARS-CoV-1. Based on the genetic similarity between SARS-CoV-1 and SARS-CoV-2, these essential oils can be functional against Covid-19 disease **(Patne et al., 2020)**.

2.15 *Cinnamomum verum*:

In patients with severe COVID-19, a cytokine defect has been identified, characterized by exacerbated inflammation and acute respiratory stress syndrome (ARDS). This type of vascular-induced inflammation is not limited to the lungs damaged by COVID-19, but also involves the ongoing changes in inflammation of the liver, brain, stomach, and heart, among other organs **(Lucas et al., 2021)**.

Cinnamon is a spice of Chinese and Ceylonian varieties. Its constituents have anti-inflammatory activity, which counteracts the activity of TLR2 and TLR4 and activates NRF2. It also prevents angiotenesis **(Lucas et al., 2021)**.

A previous experiment examined a panel of 99 ethanolic herbal extracts having anti-inflammatory properties. Ceylon cinnamon (*Cinnamomum verum*, bark) and Hop (*Humulus lupulus*, cones) have been found to have a significant reduction in the activity of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), the main regulator of pro-inflammatory cytokines. In conclusion, it can be recommended for future trials on Ceylon cinnamon to test their potential in maintaining the immune response to COVID-19 **(Lucas et al., 2021)**.

SCIENTIFIC NAME WITH FAMILY	ACTIVE COMPONENT	ANTIVIRAL ACTIVITY	REFERENCE
Curcuma longa Family: Zingiberaceae	Curcumin	Curcumin is found to prevent SARS-CoV replication and to block 3Cl protease in Vero E6 cells.	(Bababei et al., 2020).
Andrographis paniculate Family: Acanthaceae	Andrographolide	Andrographolide is found to be as effective as possible inhibitor of SARS-CoV-2 main protease (3CLpro) via in- silico approach.	(Khanal et al., 2021).
Punica granatum Family: Lythraceae	Punicalagin, punicalin	PoPEx polyphenols are a promising candidate that demonstrates potential preventive activities against SARS-CoV-2 and its in vitro studies which is demonstrated by molecular docking study.	(Suručić et al., 2021).
Withania somnifera Family: Solanaceae	Withanoside V, Withaferin A, Withanone and Somniferine	According to YASARA scoring Withanoside V and Somniferine is found significantly in the affinity of binding of SARS-CoV-2.	(Priya Shree et al. 2020).
Tinospora cordifolia Family: Menispermaceae	Tinocordiside	Tinocordiside has been shown to be stable during MD simulation and is docked in the center of the interface of the ACE2-RBD complex indicating electrostatic interactions between RBD and ACE2 and increased fluctuations, which would	(Priya Shree et al. 2020).

		further weaken or prevent SARS- CoV-2 and its infection.	
Ocimum sanctum Family: Lamiaceae	Vicenin, Isoorientin 4'-O-glucoside, Ursolic acid	These compounds have been shown to have prominent SARS-CoV-2 interactions with Vicenin that have the highest binding effect among them and can therefore be used in the treatment of the disease.	(Priya Shree et al. 2020).
Toona sinensis Family: Meliaceae	TSL	TSL-1, the extract of the *Toona sinensis* Roem tender leaf can prevent SARS-CoV and thus is expected to block SARS-CoV-2 as well.	(Mahmood et al., 2021)
Amygdalus communis Vas (ACV) Family: Rosaceae and *Ephedra sinica* Stapf (ESS) Family: Ephedraceae	Quercetin, kaempferol, luteolin	The combination of ACV and EAS (AE) herbs can have therapeutic effects against COVID-19 by affecting immune responses, hypoxia damage and other pathological processes through multiple factors, cell apoptosis, targets and methods.	(Xia et al., 2021).
Glycyrrhiza uralensis Fisch. Family: Fabaceae	Glycyrrhizin	Glycyrrhizic acid (glycyrrhizin) and its aglycone glycyrrhetinic acid (GLA) are effective against SARS coronavirus (SARS-CoV) in vitro.Glycyrrhizin activates SARS-CoV-2 by blocking the main protease Mpro of the virus. This suggests that glycyrrhizin can be a promising compound that can be further studied for use in the treatment of COVID-19.	(Mahmood et al., 2021)
Rheum emodi Family: Polygonaceae	Emodin	By using the in-silico approach emodin, an anthraquinone compound can block the interaction of S protein and ACE2.	(Rolta et al., 2020).
Thymus serpyllum Family: Lamiaceae	Thymol and carvacrol	These compounds show potential effects by physically binding COVID-19 proteins such as SARS-CoV-2 spike glycoprotein, the SARS-CoV-2 ectodomain structure, and the spike receptor-binding domain of SARS and blocking the binding of COVID-19 to host ACE2 host.	(Rolta et al., 2020).
Artemisia annua Family: Asteraceae	Artemisnin	Researchers in the United States have shown that extracts of *Artemisia annua* prevent the replication of SARS-CoV-2 viruses.	(Rolta et al., 2020).
Piper nigrum Family: Piperaceae	Piperine	A team of Indian scientists have identified black pepper, a daily kitchen spice, as part of an anti-coronavirus medicine through computational studies.	(Meghwal, and Goswami , 2013).
Syzygium aromaticum Family: Myrtaceae	Eugenol	Essential oils have been known to have antioxidant, anti-inflammatory, immunomodulatory, and antiviral properties and are also	(Asif et al., 2020).

		proposed to have anti-SARS-CoV-2 activity. Molecular docking has indicated that eugenol have affinities to bind SARS-CoV-2 protein spike, main protease (Mpro), RNA-dependent RNA polymerase and human ACE-2 proteins, (Silva et al. 2020).	
Cinnamomum verum Family: Lauraceae	trans-cinnamaldehyde, cinnamic acid, cinnamyl alcohol	Ceylon cinnamon is used as anti-inflammatory agent which is an important criterion for anti-SARS-CoV-2 medicine.	(Lucas et al., 2021).

Table1: List of some plants having antiviral properties exploited against SARS-CoV-2

CONCLUSION

Plants have been used in traditional as well as commercial remedies since ancient times. In India, plants are used in ayurvedic medicines traditionally to treat diseases naturally. The extracts of medicinal plants contain phytochemical compounds that are significantly used to treat bacterial, fungal, viral or other microbial diseases either alone or in combination.

The above review is based on medicinal values of plants and their constituents that have anti- viral properties and may have ability to cure or prevent the new COVID- 19 disease in a molecular level. A very common way to ensure the plant that can be used to treat the disease along with the components is Molecular Docking and in- silico screening. This review shows several plants that have undergone such experiments to identify the component present in the plants that can be further studied in order to provide medicine for COVID- 19.

Several plants were recognised to have potential to be used in preparing the medicine and are still being studied so as to get proper remedy against SARS-CoV-2.

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"SILICON : A SUSTAINABLE TOOL IN STRESS TOLERANCE IN PLANTS"

ABSTRACT

After oxygen, silicon (Si), is the second most abundant element on earth surface at 28% and is rapidly gaining attention in agriculture because of its many beneficial effects for plants. Near about hundreds of studies that have been performed over the time with several plant species and under diverse growth conditions have demonstrated the favorable benefits of Si fertilization, particularly in alleviating biotic and abiotic stresses. Biotic as well as abiotic stress adversely affects the agricultural productivity leading to physiological and biochemical damage to crops. Therefore, the most effective way to maintain a healthy agricultural productivity is to increase the resistance to stresses. Si plays an important role in reducing the effects of abiotic and biotic stresses (drought, heavy metal, salt stress, diseases and parasite infection) on plants. Si is accumulated in the cell walls and intercellular spaces and thus it has beneficial effects on disease infestations in especially small grains. The application of Si may reduce the effects of environmental stresses on plants while making effective use of plant nutrients such as nitrogen and phosphorus. Again, silicon may reduce the toxic effects of heavy metals in soil. It may protect the foliage, increase light uptake and reduce respiration. Therefore, in this review, we discussed the effects of abiotic and biotic stresses in plant and the crucial role of Si in enhancing a sustainable plant stress resistance.

REVIEW OF LITERATURE

INTRODUCTION

Stress in plants refers to some external conditions that adversely affect the growth, development or productivity. Stress can trigger a wide range of plant responses like altered gene expression, cellular metabolism, changes in growth rates, crop yields, etc. Plant stress usually reflects some sudden changes in environmental condition and this can be divided into two primary categories namely abiotic stress and biotic stress. Abiotic stress that are imposed on plants by environment may be either physical or chemical, while as biotic stress that are exposed to the crop plants is a biological unit like diseases, insects, etc. Biotic stress is caused by living organisms, specially viruses, bacteria, fungi, nematodes, insects, arachnids and weeds in plants. The agents causing biotic stress directly deprive their host of its nutrients and can therefore lead to death of plants. Whereas the abiotic stress is totally different from the biotic stress and is imposed on plants by non-living factors such as salinity, sunlight, temperature, cold, floods and drought having negative impact on crop plants. It is the type of climate in which the crop survives, decides what type of biotic or abiotic stress may be imposed on the plants and it also depends on the ability of the crop species to resist that particular type of stress (Gull et al., 2019).

A major concern across the world is stress that alters the growth and development of plants. Both biotic stress and abiotic stress cause a huge loss in crop yield and productivity. On the other hand, plants also have evolved several mechanisms to survive under the stress conditions; and the plants that are healthy can sustain themselves or survive better under the stress. In other words, plant nutrition plays an important role in maintaining healthy growth as well as in enhancing the stress tolerance. The role of micronutrients in providing tolerance to plant against various stresses has been demonstrated in several studies (Zargar et al., 2019; Bradacova et al., 2016; Vanderschuren et al., 2013). Silicon (Si) is one such nutritive element which is gaining increasing attention of plant researchers due to its observed properties in enhancing plant tolerance against biotic and abiotic stresses (Ma et al., 2004). Ample amount of Si is present in the earth's crust and is considered as the second most abundant element after oxygen (O_2). The source of Si are water-soluble chemical compounds, including silicic acids and potassium, sodium, calcium and ammonium silicates (Soundararajan et al., 2013; Kamenidou et al., 2010;

Gorecki and Danielski-Bush, 2009; Reezi et al., 2009) as well as organic compounds, including rice husk ash (Kamenidou et al., 2008). Si is also present in certain fertilizers such as Actisil, where orthosilicic acid H_4SiO_4 is established by choline. However, most of the Si present in the soil is in the form of silicon dioxide (SiO_2) that plants cannot uptake directly. Besides having abundant availability, the plant-available form (PAF) of Si (silicic acid) in the soil is mostly a limiting factor.

Si has been considered as a multi-talented micronutrient because of its versatile role in providing several benefits for plant growth particularly under stress conditions (Zargar et al., 2012). Si plays many important role in plants, some of which are enhancing growth, yield and crop quality, photosynthesis, nitrogen fixation and providing tolerance against abiotic and biotic stresses such as extreme temperature, UV radiation, metal toxicity, nutrient deficiency, drought, salinity, pathogen and fungus attack (Van Bockhaven et al., 2013; Guntzer et al., 2012; Cooke and Leishman, 2011; Zargar et al., 2012; 2010; Epstein, 2009; Liang et al., 2015, 2007; Ma et al., 2004; Richmond and Sussman, 2003). Earlier, Si was assumed to be a non-essential element for plant growth (Arnon and Stout, 1939; Sachs, 1860) but numerous (over 100) studies that have been performed during the last couple of decades has confirmed the impact of several Si-derived benefits in crop plants (Liang et al., 2015). Since the Si-derived benefits are more applicable under stress condition, it is widely considered as a quasi-essential element (Liang et al., 2015). Si makes the plant tissues stronger and rigid and hence provides strength to the plant (Marxen et al., 2015). Soluble Silicon enhances resistance to diseases by interacting with several key compounds of the stress signaling system of the plant (Rodrigues et al., 2004; Fawe et al., 1998) and its accumulation further leads to the production of phenolics and phytoalexins that provides tolerance against the various plant pathogens (Datnoff et al., 1997; Miyake and Takahashi, 1982a, b). Si enhances the resistance of plant to diseases and by the formation of physical barriers on the tissue surface (Frew et al., 2018; Silva et al., 2010; Kim et al., 2002). It has also shown to improve abiotic stress tolerance such as extreme temperature, drought, salinity, and metal toxicity (Zargar et al., 2019). Moreover, some of the physiological processes such as photosynthesis, respiration, translocation, ion uptake, transpiration rate, root hydraulic conductance, stomatal behavior and conductance, seed germination, mineral nutrition, and plant water relation are enhanced by Si (Zargar et al., 2019; Luyckx et al., 2017). In this manner, a double cuticle Si complex provides mechanical resistance and strength to plants (Ligaba-Osena

et al., 2020). Further, it also forms complexes with organic compounds within cell walls of epidermal tissues, thereby enhancing their tolerance towards degradation with the aid of enzymes (Zarger et al., 2019). Some investigations carried out, also focus on the primitive role of Si in plants as crucial members during association of phenolics, phytoalexins, peroxidases, β-glucanases, PR1 proteins with colonizing fungal pathogens (Etesami and Jeong, 2017; Rodrigues et al., 2003). This review is a genuine attempt to assemble published information on this topic, with a particular focus on the impact of combined drought and pathogen stresses on crop productivity. In addition, this review outlines potential role of Si in crop production and stress resistance.

EFFECT OF STRESSES ON PLANT GROWTH

Global warming leads to the concurrence of a number of abiotic and biotic stresses, thus affecting agricultural productivity. Occurrence of abiotic stress enhances host plant susceptibility to pathogenic organisms, insects, reduces competitive ability with weeds and hence alters plant-pest interactions. On the contrary, some pests may alter plant response to abioticstress factors (Pandey et al., 2017). Therefore, to understand the effect of concurrent abiotic and biotic stress conditions on crop productivity, systematic studies are pivotal. Due to global warming, and the potential climatic abnormalities associated with it, crops typically encounter a large number of abiotic and biotic stress combinations, which severely affect their growth and yield (Mahalingam, 2015; Pandey et al., 2015a; Ramegowda and Senthil-Kumar, 2015; Suzuki et al., 2014; Atkinson et al., 2013; Narsai et al., 2013; Prasch and Sonnewald, 2013; Prasad et al., 2011; Mittler, 2006). Concurrent occurrence of abiotic stresses such as drought and heat has been shown to be more destructive to crop production than these stresses occurring separately at different crop growth stages (Prasad et al., 2011; Mittler, 2006). Drought, high and low temperature and salinity are such abiotic stress conditions that are known to influence the occurrence and spread of pathogens, insects and weeds (Peters et al., 2014; Ziska et al., 2010; McDonald et al., 2009; Scherm and Coakley, 2003; Coakley et al., 1999). The affect of combined stress factors on crops is not always additive, because the outcome is dictated by the nature of interactions between the stress factors (Choudhary et al., 2016; Ramu et al., 2016; Pandey et al., 2015a,b; Atkinson et al., 2013). Plants put their responses to combined stress

factors and exhibit several unique responses from it, along with some other common responses. Mittler and colleagues developed a "stress matrix" to compile the interactions among various abiotic and biotic stresses on plant growth and productivity (Pandey et al., 2017; Suzuki et al., 2014; Mittler, 2006). This matrix clearly illustrates that the stress combinations can have negative as well as positive effects on plants. Therefore, development of plants with enhanced tolerance to combined abiotic and biotic stresses involves identification of physio-morphological traits that are affected by combined stress.

ABIOTIC STRESS :-

Plants are encountered by number of abiotic stresses worldwide which impacts the crop productivity. These abiotic stresses are interconnected with each other and may occur in form of osmotic stress, malfunction of ion distribution and plant cell homeostasis (Gull et al., 2019).

1. **Cold :-**Cold stress has proved to be the main abiotic stresses that decrease productivity of agricultural crops by affecting the quality of crops and their post-harvest life. Plants being immobile in nature must always modify their mechanisms in order to prevent themselves from such stresses. In temperate conditions plants are encountered by chilling and freezing conditions that are very harmful to plants as stress. Cold affects the cellular functions of plants in every aspect. Several signal transduction pathways are there by which these cold stresses are transduced like components of ROS, protein kinase, protein phosphate, ABA and Ca^{2+}, etc. and among these ABA is the best.
2. **Salt :-**Salinity of soil poses a global threat to world agriculture by reducing the yield of crops. Salt stress reduces the crops growth and yield in many ways of which the two primary effects imposed on crop plants by salt stress includes osmotic stress and ion toxicity. The presence of more salt exceeds the osmotic pressure under salinity stress in the soil solution than the osmotic pressure in plant cells, and thus, the ability of plants to take up water and minerals like K^+ and Ca^{2+} from the soil becomes limited. These primary effects of salinity stress causes some secondary effects like assimilate production, reduced cell expansion and membrane function as well as decreased cytosolic metabolism.
3. **Drought :-**Nowadays we experience a lot of climatic change all around the globe by the continuous increase in temperature and atmospheric CO_2 levels. The distribution of

rainfall has become uneven due to the change in climate which acts as an important stress as drought. The soil water available to plants steadily increases due to severe drought conditions and cause death of plants prematurely. As the first response after drought is imposed on crop, growth arrest is subjected on the plants. The growth of shoots and their metabolic demands are reduced under drought conditions.

4. **Heat :-** Increase in temperature throughout the globe has become a great concern, which not only affect the growth of plants but their productivity as well especially in agricultural crop plants. When heat stress is encountered on plants, it results in the declination of the percentage of seed germination, photosynthetic efficiency and yield. During the reproductive growth period, the function of tapetal cells is lost, and the anther becomes dysplastic.
5. **Toxin :-** The increased use of chemical fertilizers on agriculture, sewage waste water irrigation and rapid industrialization has added toxic metals to agricultural soils causing harmful effects on soil-plant environment system.

BIOTIC STRESS :-

Plants are immobile living organisms which struggle with many kinds of biotic stresses caused by other living organisms like fungi, virus, bacteria, nematodes, insects etc. These agents imposing biotic stress on plants cause various types of diseases, infections and damage to crop plants and ultimately affect the crop productivity. The biotic stresses imposed, depends on the climate where the organism lives and also on its ability to resist particular stresses. The damage caused by various living and nonliving agents can appear similar, even when observed closely, accurate diagnosis can be difficult. For example, browning of leaves on an oak tree caused by drought stress appears similar to leaf browning caused by oak wilt. Biotic stresses caused to cash crops results to vast economic losses. Economic decisions as well as practical development is affected by the relationship between biotic stress and plant yield. Biotic injury on crop yield impacts population dynamics, plant-stressor coevolution, and ecosystem nutrient cycling. It also impacts horticultural plant health and natural habitats ecology and also has dramatic changes in the host recipient. Although there are many kinds of biotic stress, the most common and majority plant diseases are caused by fungi. *Arabidopsis thaliana* is often considered as a model plant while studying the responses of plants to different sources of stress (Singla and Krattinger,

2006). Biotic stresses may also lead to huge repercussions for humanity; an example of this is the potato blight, an oomycete which caused widespread famine in England, Ireland and Belgium in the 1840s. Another example is Great French Wine Blight caused by grape phylloxera coming from North America in the 19th century. Many biotic stresses also affects photosynthesis, as insects chew leaf which reduces leaf area and infections by virus reduce the rate of photosynthesis per leaf area.

BENEFICIAL EFFECTS OF SILICON IN STRESS TOLERANCE

Si can play an important role in overcoming the effects of various environmental stresses. It reduces manganese (Mn), cadmium (Cd), arsenic (As), aluminium (Al), zinc (Zn) and phosphorus (P) deficiency and, increases the resistance to lodging, diseases and insects. Besides, Si increases plant resistance to abiotic stresses, such as drought and salt stress. The positive effects of silicon on plant growth, development, yield and disease resistance are observed in both monocots and dicots. Si application influences the nutrient content of sunflower by increasing the accumulation of both macro and micro nutrients (Savic and Marjanovic-Jeromela, 2013).

In relation to plant structure, it was explained by (Taiz and Zeiger, 2002) that silicon is found at different concentrations within plant tissue and it improves growth and fertility of plants. Primarily, Si is founded in the endoplasmic reticulum, cell walls (Raven, 2003) and intercellular spaces. As an easily understandable mechanism, it plays an important role in the support of cell walls by forming many complexes with polyphenols. Moreover, Si reduces the adverse toxic effects of heavy metals. After external Si application, leaves would stand up and benefit from sunlight more efficiently. Thus, plant performs better photosynthesis and better yield (Ding et al., 2007). Silicon stored in the bulliform cells and dumbbell cells improves the strength and rigidity of cell wall, and accordingly increases the resistance of rice to diseases, pests, and lodging (Jones, 2012; Epstein, 1999). Especially bulliform cell are located near the midrib of grass leaf such as rice. The cell group that affects leaf folding and, leaves are less exposed to sunlight during drought (Mauseth, 2017).

Currie and Perry, 2007 stated that Si as an organically dynamic component activating natural defense mechanism and, reviewed the transport of the Si in plants such as rice, known as Si-

accumulator. Two different Si mechanisms were emphasized. These are low affinity transporter responsible for the uptake of silicic acid from the soil to the root cortical cells and, second transporter is responsible for xylem loading via passive diffusion (Ma, 2004).

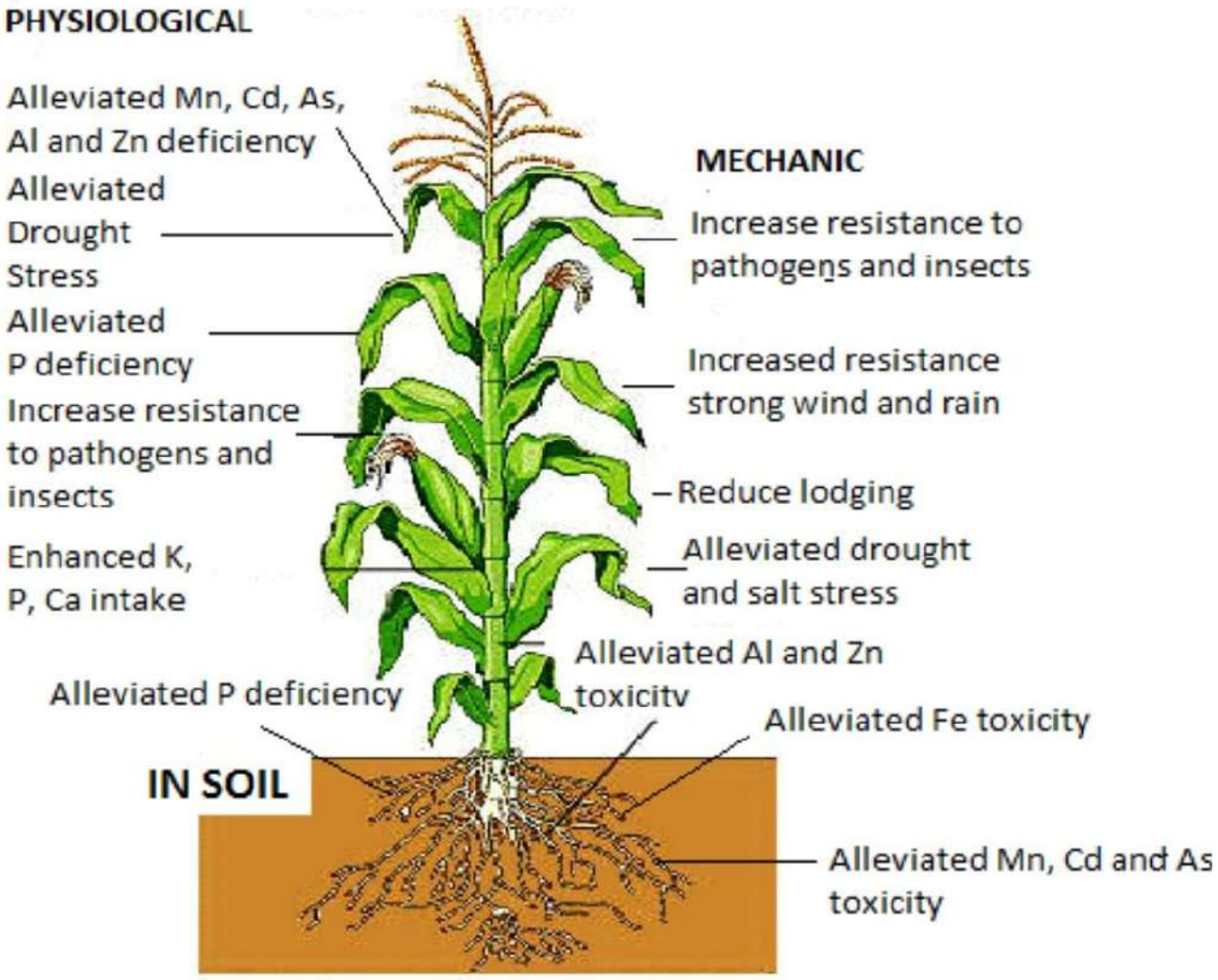

Fig1 : Beneficial effects of Silicon (Yavas and Unay, 2017; Modified from Mcginnity, 2015)

UPTAKE, TRANSPORTATION, AND ACCUMULATION OF SILICON IN PLANTS

Abundant, Si is never found in a plant in available form and is always combined with other elements, usually forming oxides or silicates (Gunes et al., 2007b). Siis absorbed by plants in the form of uncharged silicic acid, $Si(OH)_4$, and is ultimately irreversibly precipitated throughout the plant as amorphous silica (Ranganathan et al., 2006). Therefore, although Si is plentiful, most sources of Si are insoluble and in a plant-unavailable form. Si in the form of silicic acid [$Si(OH)_4$] or mono silicic acid [H_2SiO_4] can cross the plasma membrane of root at physiological pH (Raven, 2001). The concentration of silicic acid in soil solution below pH 9 ranges from 0.1 to 0.6mM (Knight and Kinrade, 2001). In the plant leaves, Si concentration varies from 0.1 to 10% on dry weight basis (Richmond and Sussman, 2003; Ma et al., 2001; Epstein, 1999).

The wide variation in Si concentration in plant species is attributed mainly differences in the characteristics of Si-uptake and transport. Active Si-uptake has been well demonstrated in Graminaceous species such as rice (Ma et al., 2001b), wheat (Rains et al., 2006), ryegrass (Jarvis, 1987), and barley (Barber and Shone, 1966). However, some of the Gramineae plants such as oats take up Si passively (Jones and Handreck, 1967). Passive Si-uptake has been demonstrated in some dicots such as cucumber, melon, strawberry and soyabean (Liang et al., 2005). Unfortunately, molecular mechanisms underlying Si uptake in some of these plants are unknown (Ma and Yamaji, 2006).

Different plants have different uptake mechanisms and the particular ability of the roots to uptake Si is considered to be the reason for the differences in Si accumulation in different plants (Ma and Yamaji, 2006). Higher accumulation of Si was observed in Bryophyta, Lycopsida, and Equisetopsids (Pteridophyta), whereas in Filicopsida (Pteridophyta), Gymnospermae and most Angiospermae there is a low Si concentration (Hodson et al., 2005; Ma and Takahashi, 2002; Ma et al., 2001). Investigations of the different mechanisms by which Si is absorbed into the plants conducted by (Parry and Kelso, 1975) showed that Si interacted with polyphenols in xylem cell walls and has affected lignin deposition and biosynthesis. In rice, under water deficit induced by polyethylene glycerol, addition of Si decreased the transpiration rate and membrane permeability (Agarie et al., 1998). Rice roots have the capacity of uptaking 90% of the Si present in the soil and translocates it to the shoots (Ma and Takahashi, 2002). Research performed using rice

mutants for Si-uptake have identified two different types of Si-transporters namely *OsLsil*(Si-transporter AQPs, influx) and *OsLsi2* (efflux, Si-transporters) (Ma et al., 2006, 2007). Furthermore, an increase in Si deposition in the silicified epidermal cells of leaf blades and sheaths and increase excretion of Si in guttation fluid was reported in rice (Ma et al., 2011). Other monocots such as rice can accumulate Si up to 10% of the plant mass while most of the dicots accumulate very less. The high accumulation of Si in rice can be attributed to efficient Si-transporters as well as specialized silica cells. Dicots do not have silica-cells, and also several dicot families have lost the Si-transporter AQPs (Deshmukh et al., 2015). The difference in the mechanism of Si uptake in monocot and dicot plant species makes the monocot species more tolerant against frost stress and certain metabolic inhibitors as because the later involves concentration independent process for Si uptake. Thus, having the complete knowledge of the difference between two uptake mechanisms might be useful in generating genetically modified species with improved Si uptake and assimilation properties.

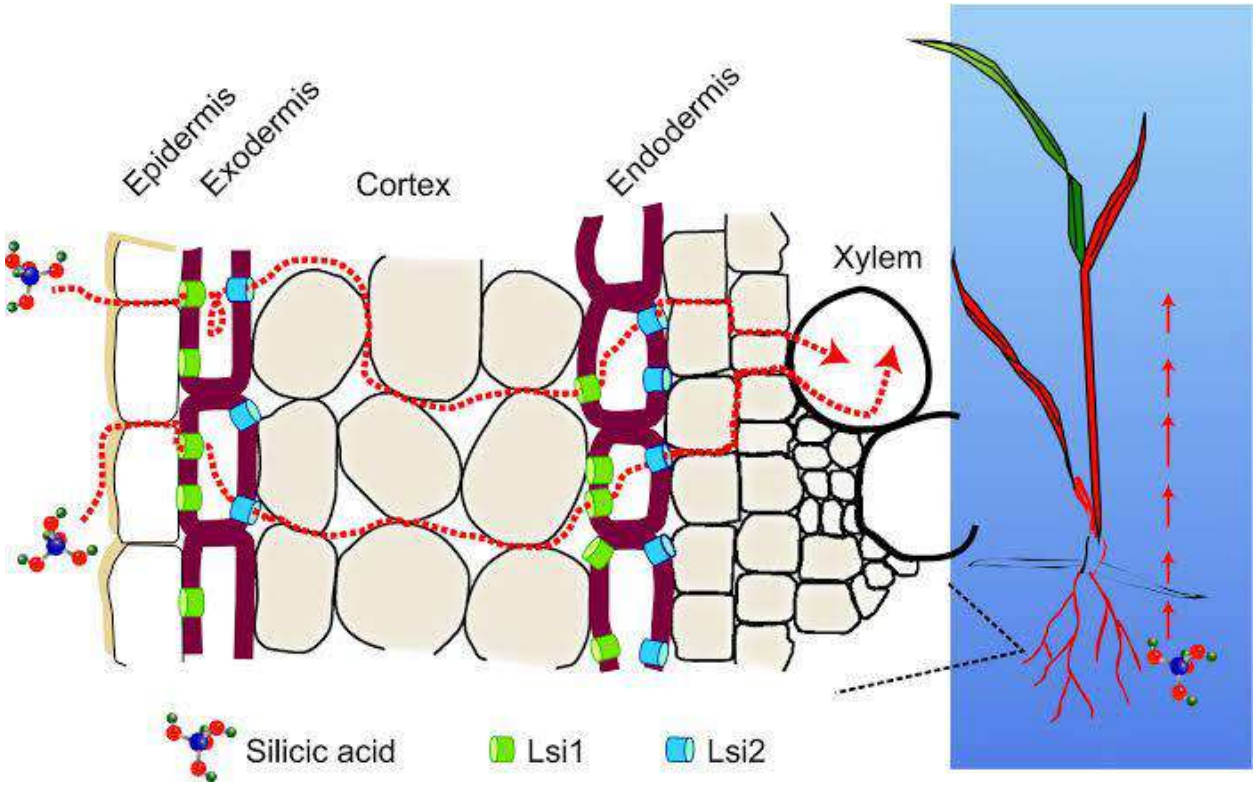

Fig2 : Silicon uptake mechanism of plants (springerlink.com)

POTENTIAL ROLE OF SILICON IN ABIOTIC STRESS TOLERANCE

Abiotic stress is one the most severe constraints for crop cultivation all over the world. Because of climate change and unpredictable weather, abiotic stresses have become more common and challenging. Plants generate reactive oxygen species (ROS) as a first response to most abiotic stresses like salinity, drought, thermal, and heavy metal stress. This response is known to cause severe damages to cell structure and organelles, and to alter normal cell function (Deshmukh et al., 2017).

- A study conducted by (Hasanuzzaman et al., 2018) has shown that plants growing under heavy metal stress (excess of cadmium) has reduced ROS contents when supplemented with Si compared to control plants. The improved antioxidant defense mechanisms against Cd stress with Si supplementation was found to be associated with an efficient augmentation of antioxidant components, associated with an increased activity of AsA-GSH and glyoxalase pathways.
- Pontigo et al., 2015 observed that Si-derived aluminium (Al) stress tolerance in ryegrass was associated with a change in ROS profile and reduced uptake of Al by plants from the soil. Incidentally, a review article by (Kim et al., 2002) discusses the role of Si in abiotic stress and its possible implication in regulating the generation of ROS.
- Abiotic stress significantly affects physiological processes leading to altered metabolic activities and overall health of plants. Grasses are well-known high accumulators of Si, and, therefore, serve as an excellent model to investigate the passive and active regulation of Si transport.
- (McLarnon et al., 2017) evaluated some physiological parameters in three genotypes of forage grass differing in their ability to accumulate Si. Their results suggest that the varietal differences are attributed to stomatal conductance and transpiration, particularly when plants are grown under control conditions. However, under stress (wounding), an increased level of Si was noticed in all three genotypes, a reaction attributed to a higher expression of Si transporter genes.

- (Soundararajan et al., 2014) observed an improved stomatal development in tissue-cultured carnation plants supplemented with Si. This was correlated with a differential expression of proteins linked to photosynthesis, ribosomes, oxido-reduction, hormone signaling, metal ion binding, and defense responses.
- (Manivannan and Ahn, 2017) have critically reviewed several such studies suggesting a role of Si in regulating physiological processes in plants. Similarly, based on several studies conducted over the last decade, Rios et al., 2017 proposed a model explaining how Si could improve stomatal functioning and enhance root hydraulic conductance through the regulation of aquaporins.

Nevertheless, there is still no conclusive evidence for the direct active involvement of Si in any metabolic processes that can explain systematically how Si regulates cellular processes (Manivannan and Ahn, 2017; Rios et al., 2017).

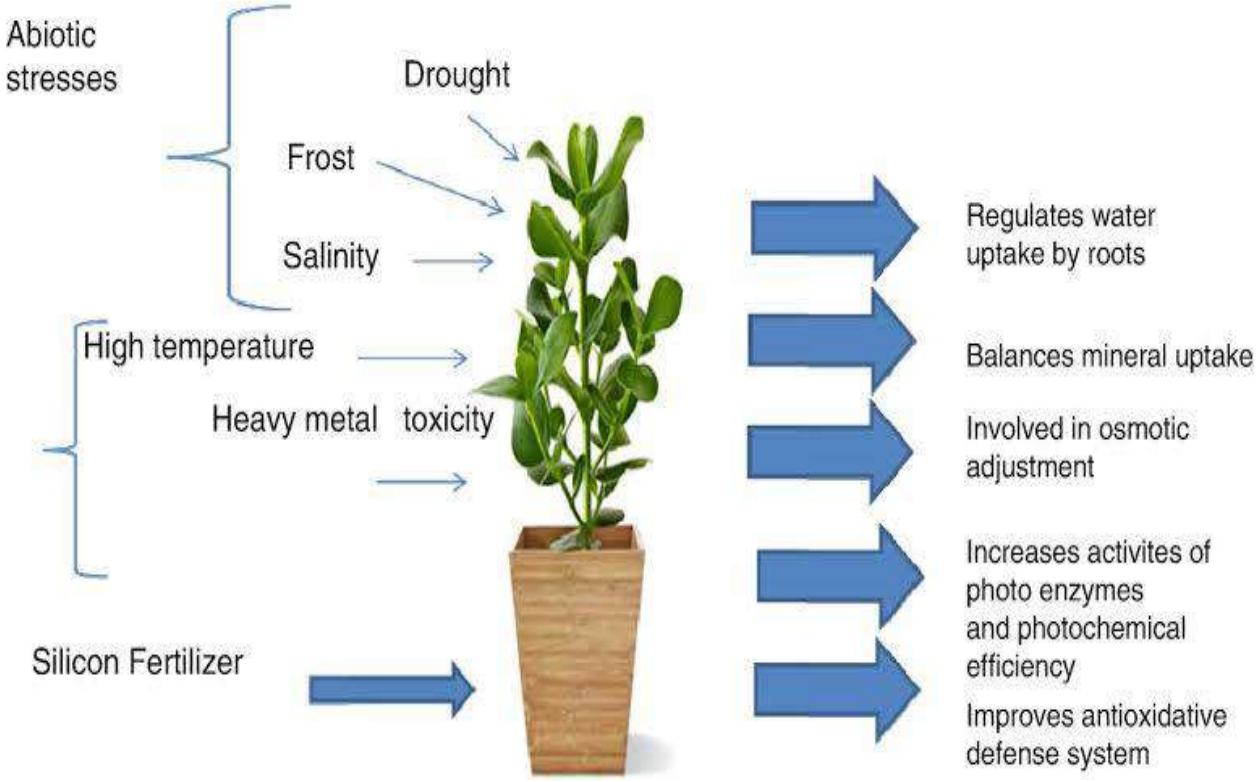

Fig 3 : Role of Silicon in Abiotic Stress (springerlink.com)

POTENTIAL ROLE OF SILICON IN BIOTIC STRESS TOLERANCE

The beneficial effects of Si in improving tolerance against diseases and pests are arguably the most commonly described. A review article by Wang et al., 2001 provides a catalogue of many significant studies and discusses models explaining the role of Si. For a long time, Si-derived resistance to pathogens and insects was thought to be the result of a mechanical barrier formed by the deposition of Si along the cell wall thus hindering their progression. However, studies performed in the 90's associated the presence of Si with specific defense responses in plants. (Fawe et al., 1998; Cherif et al., 1994, 1992), a phenomenon that has since been shown in many host-pathogen interactions (Fauteux et al., 2005).

- In a recent study, Si was further shown to interfere with host-pathogen recognition, probably by preventing effectors and signaling molecules from finding their specific targets (Vivancos et al., 2015).
- Si was also suggested to induce indirect defense mechanisms by altering the composition of herbivore-induced plant volatiles (HIPV) (Liu et al., 2014). The HIPV compounds play an important role in attracting parasitoids to infested rice plants.
- The evaluation of different sources of Si is a critical aspect to optimize the practical use of Si fertilization. In this context, (Ouellette et al., 2017) tested different Si fertilization regimes under high tunnel and field conditions for strawberry production. Under high tunnel, strawberry plants accumulated as much as 3% dry weight of Si, resulting in significant reduction of powdery mildew severity and higher yields. On the other hand, strawberry plants grown in soil, were unable to absorb Si, whether amended in liquid or solid form.
- Similarly, keeping tested several sources of Si including fused magnesium (thermo) phosphate, volcanic rock dust, magnesium silicate, calcium silicate slag, and granular potassium silicate for sugarcane plant growth. Only the later source led to a significant increase in Si accumulation.

These studies suggest that Si sources and modes of application will greatly influence Si accumulation in different plant species. Therefore, more extensive efforts are required to better understand the relationship between Si sources and

soil properties to obtain higher level of plant available Si. Apart from these technological sources, nano-technological advances are also being used to explore possibilities for the application of Si nanoparticles as a source to elevate stress tolerance in plants (Luyckx et al., 2017).

ROLE OF SILICA NANOPARTICLES IN PLANTS

Sustainable agricultural models and food scarcity have long-standing importance, and silica nanoparticles may play an important role to that end. Through a controlled release into the soil, silica nanoparticles and their counterparts combine to yield healthier, stronger, bigger plant

In certain plants, this can impact germination, root growth, chlorophyll levels, and more. More research needs to be done in this area, but the current findings show promise. Some papers in the literature have studied the effects of silica nanoparticles (SNPs) on plant physiology.

- Mesoporous SNPs (MSNPs, 20 nm in size) coupled to FITC were shown to be taken up by three important crops (lupin, wheat, maize), as well as *Arabidopsis* protoplasts and to be translocated to the aerial parts following the xylematic flow after entwring the roots via symplastic/apoplastic routes (Sun et al., 2014).
- Mesoporous SNPs were shown to boost the growth, total protein content and photosynthesis of lupin and wheat seedlings and to induce no changes in the activity of antioxidant enzymes (Sun et al., 2016).
- Silica nanoparticles were shown to protect wheat seedlings against UV-B stress by stimulating the antioxidant defense system (Tripathi et al., 2016).
- Silicon nanoparticles also conferred protection via mitigation of oxidative stress in pea seedlings treated with Cr (VI): the activities of enzymes such as superoxide dismutase, ascorbateperoxidase increased significantly in the presence of SNPs, while catalase, glutathione reductase and dehydroascorbate were less inhibited by Cr(VI) in the presence of SNPs (Tripathi et al., 2015b).
- Silicon nanoparticles (12nm) were also found to improve germination in a known Si-excluder, tomato; at a concentration of 8 g/L, SNPs improved seedling germination, as

well as fresh and dry weight by 116.6 and 117.5% respectively (Siddiqui and Al-Whaibi, 2014).

- Nanostructured SiO_2 was shown to be valuable in larch seedling production, because, when applied to the roots of 1-year-old seedlings via soaking for 6 h, it promoted lateral root growth, main root length and chlorophyll content (Bao-shan et al., 2004)

Si is an abundant element on Earth and its positive effects on plants make it important in agriculture. Si has numerous functions on plant physiology, and its most significant effects are focused on cell wall. The presence of Si in the cell wall increases their strength, resistance to salinity, drought tolerance and photosynthetic activity. It supports root and foliage growth and leads to prevention of oxidative stress by antioxidant enzymes. The other important role of Si in reducing the adverse effects of stress may be by improving soil conditions. Therefore, Si could be used as a growth regulator to improve plant growth and resistance under stress conditions. The result of these studies illustrate that Si could be used as a potential growth regulator to improve plant growth and resistance under stress conditions, they also suggest that the entry of silicon to plant tissues leads to inhibition of the oxidative destruction processes that is accompanied with increasing activity of some antioxidant enzymes that neutralize ROS. This may be a promising new strategy for improvement of soil properties in agriculture. It is obvious that most of the effects of Si were exposed through Si deposition on the leaves, stems and hulls. The more Si accumulated in the shoots, the larger the effect (Ma, 2003). However, Si accumulation in the shoot varies considerably with the plant species and most plants are unable to accumulate high levels Si in the shoots. The difference in Si accumulation was attributed to the ability of the roots to take up Si. Therefore, although Si is abundant in soil, since most plants especially dicots are unable to take up a large amount of Si from soil, they do not benefit from Si.

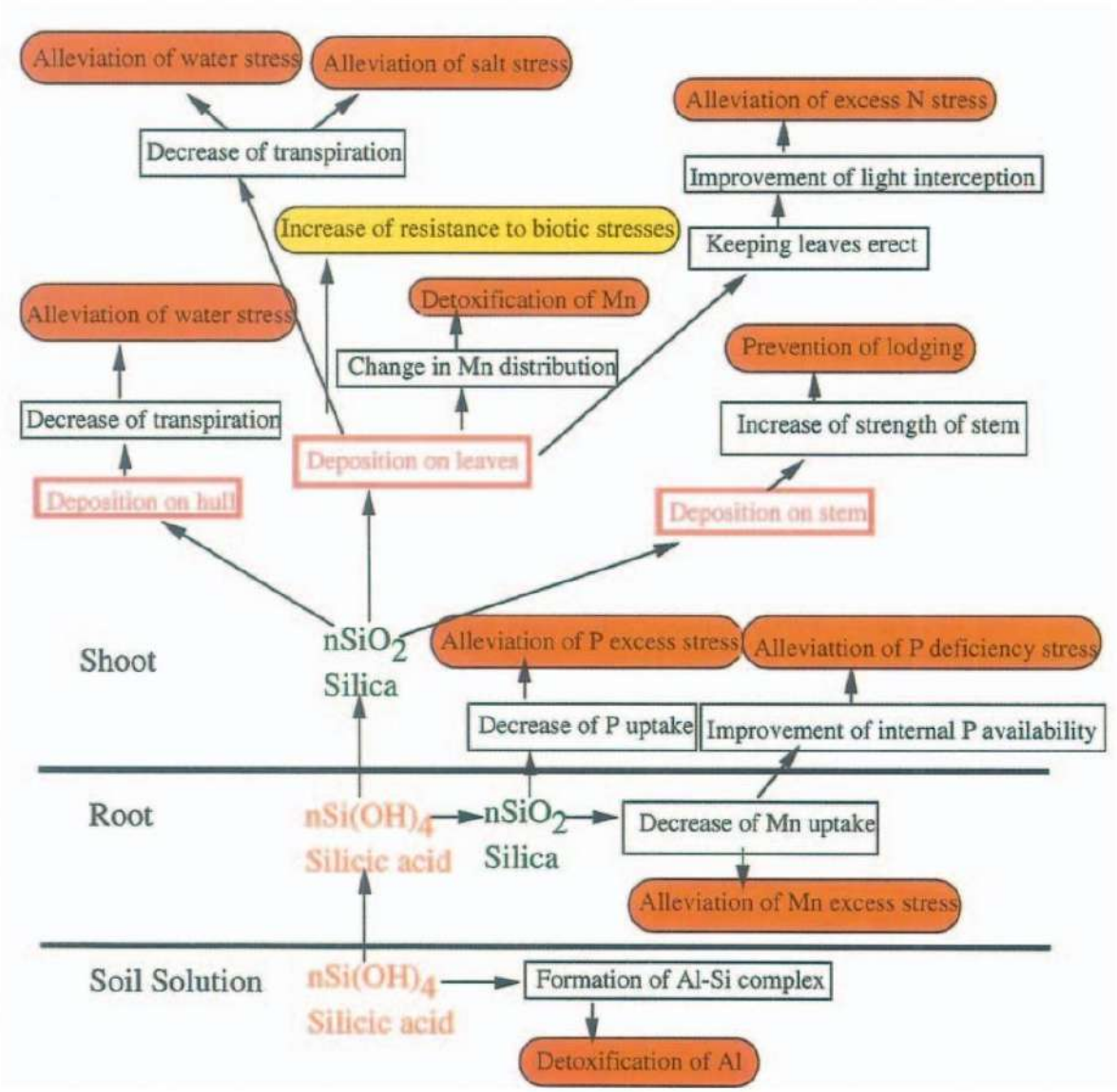

Fig 4 : Beneficial effects of Si under various stresses [Ma, 2003; Ma and Takahashi, 2002; Ma et al., 2001]

One approach to enhance the resistance of plants to multiple stresses is to genetically modify the Si uptake ability.

ABBREVIATIONS

Abbreviations	Full Forms
Si	Silicon
ROS	Reactive Oxygen Species
Cd	Cadmium
AsA-GSH	Ascorbate-Glutathione
Al	Aluminium
H_2SiO_4	Mono Silicic Acid
SiO_2	Silicon Dioxide
PAF	Plant Available Form
$Si(OH)_4$	Silicic Acid
Mn	Manganese
As	Arsenic
Zn	Zinc
P	Phosphorus
HIPV	Herbivore-Induced Plant Volatiles
SNPs	Silica Nano-Particles
FITC	Fluorescein Isothiocyanate
ABA	Abscisic Acid
Ca^{2+}	Calcium ion
CO_2	Carbon-dioxide
AQP	Aquaporin

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CONTENT

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Abstract:

In recent years, genome editing is growing exponentially and has become the dominant technology in order to manage the abiotic and biotic stress in plants. The Clustered regularly interspaced short palindromic repeat (CRISPR) – Cas (CRISPR-associated protein) system is one of the most efficient, convenient and less time consuming methods to achieve successful manipulation of the targeted gene. The RNA guided nuclease i.e., the Cas9 protein, in the DNA is induced to break the double strand and are also repaired by the DNA repairing mechanism initiating genome mediated modulation. The drastic change in climate demands the use of traditional CRISPR/Cas9 RNA guided DNA endonuclease incorporating the identification and alteration in Cas9 variant, multiplex editing and base editing for specific genome editing and new and improved stress tolerant varieties can be developed. Plant are vulnerable to phytopathogens like insects, bacteria, virus, fungi etc. and against these phytopathogen, the plant undergoes two levels defense mechanisms. PAMP-triggered immunity (PTI), and Effectors-triggered immunity (ETI). CRISPR/Cas9 technology have concluded the complex plant defense mechanism and edited the genome in order to improve the plant immunity system and obtaining a long duration resistivity against the pathogen. This review explicates the role of CRISPR–Cas mediated genome editing in plants against different biotic and abiotic factors thereby leading to improved plant vitality and resistivity.

Keywords: CRISPR/Cas technology, Abiotic Stress, Biotic Stress, Genome editing, Mutagenesis, Plant resistance, Plant immunity, Susceptible gene.

Abbreviations:

AITRs- ABA-induced transcription repressors

Cas- CRISPR-associated protein

CRISPR- Clustered regularly interspaced short palindromic repeat

ETI- Effectors-triggered immunity

NPR- Non-expresser of pathogenesis related gene

PTI- PAMP-triggered immunity

QTL- Quantative trait loci

UGT- UDP-glucosyltransferase

1.1 Introduction:

Since the origin of life form, prokaryotic organism has co-evolved defense strategies against their predators. And in the past decade their complicated defense mechanism against phage predation was discovered by an author Y. Ishino et al. (1987) in *Escherichia coli* in his review. CRISPR is an interspaced clustered short palindromic repeats of DNA sequence that endows the adaptive, heritable immunity against virus and plasmids in vitro. The CRISPR is composed of 25-50bp separated by similar length of unique sequence spacer (Alexander Bolotin et al. 2005). The pivotal protein that is associated with the system is called CRISPR associated system (cas) protein. This system is highly specific and has high target efficiency records all the information involved in combating the biotic and abiotic agents thereby and deploying the RNA for sequence silencing phage and other foreign genetic materials. Initially four cas genes were discovered and later on 13182 cas protein came into notice. The cas1 and cas2 are the most important protein present in all CRISPR/cas system (Makarova et al. (2011). Cas1 and Cas 2 are both metal dependent DNase where the role of Cas 1 has been found out to be helping the CRISPR locus to integrate the DNA (Wiedenheftet al. 2009). However the role of Cas 2 is still to be elucidated (Wiedenheftet al. 2009). Among other proteins Cas 3 includes HD domain (Makarova et al. 2006), Cas4 is a RecB-like nuclease who helps in spacer acquisition (Makarova et al. 2006), the cas5 and cas6 is termed as repeat association mysterious proteins (RAMPs) they includes a glycine rich loop and at least a RNA recognition motif (Makarova et al. 2006). Beyond all expectation, this CRISPR-Cas mediated genome editing has distorted the complete scenario of plant molecular biology for achieving knockout gene in the cell. The RNA guided enzymes have the potential to manipulate precise editing of genetic loci in cultured cell of crops has provided opportunities of economically as well as social benefits in sustainable environment.

The cas9 nuclease which further binds with the DNA, and cleave the targeted site with the help of typical short RNA molecule called the CRISPR RNA or (crRNA) and trans-encoded RNA (tracrRNA), the typical short molecular RNA sometimes fuse together to form a

chimaeric RNA molecule called single guide RNA (sgRNA) (Makarova et al. 2011), the typical damaged DNA undergoes either of the pathway:

1. The error prone nonhomologous end joining (NHEJ)
2. The high fidelity homology directed repair (HDR) pathway

The CRISPR-cas system can be further classified into three categories**:** Type I, II, III (Makarova et al.(2011)**.** Based on the specific signature of cas protein bacteria and archaea both have type I CRISPR-cas system in common. Here with the help of endonuclease activity of cas3 protein, it binds with the DNA sequence (Makarova et al. 2011). Type II CRISPR-cas system is only been discovered in bacteria, it is the simplest system with only four pair of protein namely Cas1, Cas2, Cas4/Csn2 proteins along with a multifunctional protein i.e., cas9. The type III CRISPR-cas system targets both RNA as well as DNA and basically found in archaea and sometimes in bacteria as well. It is recognized by the presence of cas10 as well as cas6 protein along with RAMPs. The processing of crRNA is conducted by cas10 protein which ultimately targets the DNA cleavage. The type III is sometimes present in bacteria too (Makarova et al. 2011). The cas9 i.e., type II CRISPR system is adopted much more widely from *Streptococcus pyogenes* (SpCas9) (Zhang et al. 2019b). This technology basically targets the negatively regulating genes.

The first gene targeting experiment in plant was done in the year 1988 in tobacco (*Nicotiana tabacum*) protoplasts by Paszkowski J et al. Early research of genome editing was mainly based on Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) hybrid protein (Weinthalet al.2010; Miller et al. 2011; Li et al. 2013; Shan et al. 2013a; Voytas, 2013). The complex interaction of amino acid residues with the base pair is a complex mechanism in ZFNs. Moreover, licensing fees restricts the use of ZFNs in plant in broader sense by prohibiting the access of designing tool developed by the companies. The TELENs technology also requires very sophisticated design. Here for each targeted DNA sequence of DNA binding protein assemblage is very difficult.

The CRISPR/Cas system can organize into three divisions. The first stage is the insertion of new spacers in the locus; the second stage is the expression of cas gene and transcribing the precursor CRISPR RNA (pre-crRNA). This leads to maturation of pre-crRNA by cas protein. The final stage comprises the activity of combined effect of crRNA and cas protein on the targeted nucleic acid degradation (Devashish Rath et al. 2015).

1.2 Literature Review:

Plants are sessile in nature and hence adapts to signaling pathways in order to achieve cellular stability and water homeostasis against the stress conditions. Due to repetitive stress orders, plants develop active immunity, resulting in a huge loss of yield in due course of time. This sort of stress negatively impacts the global world (Pandey et al. 2017). The modern studies of genetical engineering have approached plant breeding innovations, used during past few decades to generate stress tolerant plant (Mushtaq Muntazir et al. 2018). The application of CRISPR-cas technology was implicated on plants in the year 2013 bringing significant trait enhancement (Shan et al. 2013; Nekrasov et al. 2013; Liet al. 2013). Plant undergoes a wide range of environmental stresses which limits the proper growth for plant. The two main types of stress which are encountered by the plant are: Abiotic stress and biotic stress.

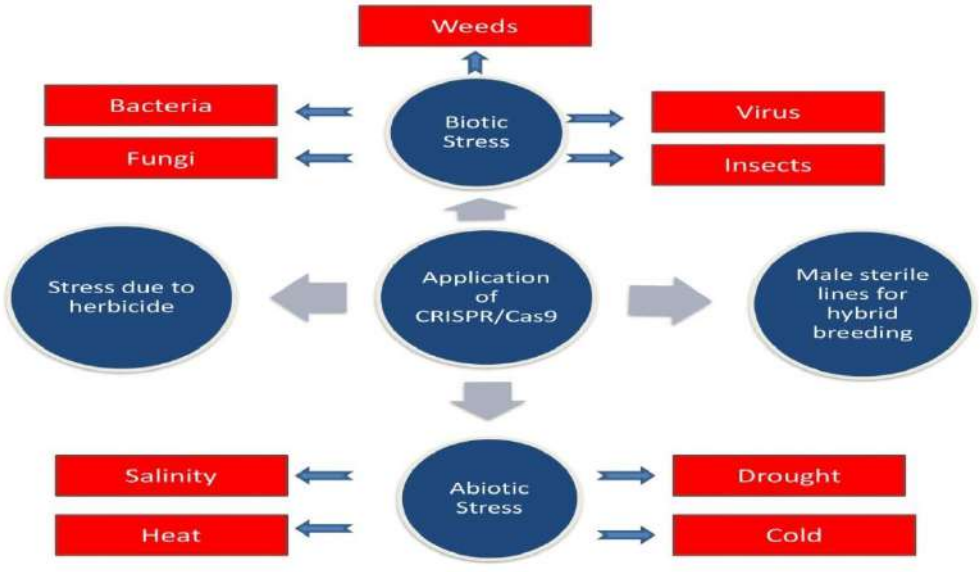

Fig 1: Types of biotic and abiotic stresses in plants.

1.2 Abiotic Stress:

Climate change is the most significant factor for abiotic stress, which is caused due ion toxicity, salinity, drought, heat, flooding and radiation leading to more than 50% crop loss annually (Pandey et al. 2017). Excessive induction of green house gas results in the increment of temperature will further increase crop loss by 20% annually. The improvement of transgenic plant to work against the abiotic stress may also increase the susceptibility of the resistant transgenes in the mutant variation (Parmar et al. 2017). Therefore the advert effect of CRISPR technology has widened up further possibilities in resolving the issues in abiotic stress in a plant.

1.3.1 Drought Stress Tolerance:

The drought is the most frequent abiotic stress that is effective for global warming having a serious effect on the plant community. Since last few years, extensive studies lead to adaption and tackling of drought stress in plant community with the help of CRISPR-cas9 technology. Drought tolerance QTLs (Quantative trait loci) have been mapped in may crops like *Zea maize* (Hao et al. 2010; Almeida et al. 2014; Trachsel et al. 2016), *Triticum* (Kirigwi et al. 2007; Mathews et al. 2008; Pinto et al. 2010; Gahlaut et al. 2017), *Oryza sativa* (Kamoshita et al. 2008; Khowaja et al. 2009; Swamy et al. 2011; Suji et al. 2012), etc. The stress hormone that plays a crucial role in regulating drought and salinity stress is the abscisic acid (ABA). The abscisic acid leads to stomatal closure thereby reducing the loss of water in a plant. SNF 1-related protein kinase 2 (SnRK2) plays an important role in ABA-dependent development; it is basically a hyper osmotic stress signaling growth in plants. It has been found that rice plants become more susceptible to drought if there is CRISPR/Cas9 dependent loss-of-function mutation in osmotic stress/ABA-activated protein kinase 2 (SAPK 2). Drought tolerance in OsSAPK 2 gene induces by the expression of gene OsRab21, OsbZIP23, OsLEA3, and OsDREB1 (Lou et al. 2017).

A negative regulator ARGOS8 of the ethylene response pathway having low sensitivity against ethylene causes amplification of drought tolerance in maize (A. Jain et al. 2017).

Many natural genotypes for drought resistance have been diagnosed against ARGOS8 but its expression was of very low level. Hence, under less expressing constitutive promoter GOS2, CRISPR-Cas9-dependent breeding technique has been established in order to express ARGOS8 (A. Jain et al. 2017).

After many experiments it has been concluded that in tomato plants, the mutation by CRISPR/Cas9 dependent technology in SINPR1 gene results in reduction of the drought stress. It has also been concluded that the drought stress reducer SINPR1 gene controls SIGST, SIDHN and SIDREB gene activity at the same time (Li et al. 2019a). In tomatoes, mitogen-activated protein kinases (MAPKs) editing with the help of CRISPR/Cas gene demonstrated that SlMAPK3, is a drought stress modulator (Wang et al. 2017). The third exon of SlMAPK3 helps in generating its mutant line which exhibits lower antioxidant enzyme activities, higher hydrogen peroxide content, more wilting, and suffered more membrane damage under drought stress. The SlMAPK3 in general modulates the genes those are related to stress and also protects the cell membranes from oxidative injuries.

In *Arabidopsis* plant, the gene OPEN STOMATA 2 (OST 2) plays a key role in drought tolerance stress with the help of CRISPR/Cas9 technology (Osakabe et al. 2016). Some studies have also revealed that ABA-induced transcription repressors (AITRs) are involved in the ABA-regulating signals and also in stress tolerance, *Arabidopsis* aitr2aitr5aitr6 (aitr256) triple mutant express their roles in drought and salt tolerance and hence by using CRISPR/Cas9 technology quintuple mutants have been created in order to enhance the tolerant capacity for stress in the plants.

1.3.2 Salinity Stress Tolerance:

According to Shahid et al. (2018), due to salinity stress there is a loss of yield of 5000 ha in arable land every single day. Due to sea water drift, transpiration or evaporation of water there is accumulation of salts in soil, which suspend the nutrition uptake system via roots thereby affecting the growth and development of the plant. The salinity tolerance according to Schmöckel (2014) is the capability of a plant to maintain the equilibrium of biomass and/or yield under salt stress conditions. Anthropogenic activities in the field such as use of

fertilizers, irrigation of saline water also causes salinity in the soil. And in its defense, the plant exhibits three types of tolerance mechanism viz., ion tolerance, osmotic tolerance and tissue tolerance (Roy et al. 2014).

In *Oryza sativa*, the OsRR22 gene plays the key role in regulating cytokinin signal transduction. The decrement of OsRR22 transcription leads to the increase of salinity tolerance (Takagi et al. 2015). So with this information, Zhang et al. (2019a) edited the OsRR22 gene using CRISPR/Cas9 technology and concluded that the two homologous T_2 generations enhanced the salinity tolerance with no significant difference the edited and the wild type lines.

Pumpkin exhibits high expression of NADPH oxidase (RBOHD), plasma membrane H+-ATPase (AHA1), 14-3-3 protein (GRF12), and potassium transporter (HAK5) that induces salt tolerance in this plant. The knocking out of the NADPH oxidase (RBOHD) sequence by CRISPR/Cas9 technology initiated salt sensitive tolerance in the plant. It was then considered that, for pumpkin the RBOHD dependent H_2O_2 signaling is important in the root apex for the salt tolerance (Huang et al. 2019).In some plants the microRNA (miRNA) plays a vital role in salt stress tolerant mechanism (Gao et al. 2011). Hence the miRNA, a negatively regulating gene can be further edited and can be applied in CRISPR/Cas mechanism for salinity stress tolerance.

1.3.3 Cold Stress Tolerance:

Cold stress in plants can be further classified into two types those are the chilling stress (0-20°C) and freezing stress (0°C or below) effecting the growth and development of the plant (Kazemi-Shahandashti et al. 2018). When plants are exposed to cold stress they show various symptoms like rapid wilting, sunken pith forming necrotic patches of tissues (Rasool et al. 2015).

Using knock-out approach of CRISPR/Cas9 in tomato, Li et al. (2018) explained that *Solanum lycopersicum* C-repeat binding factors (*sl*CBFs) enhance chilling injuries in wild type. The chilling stress in rice can be enhanced by silencing the thermosensitive chlorophyll deficient

mutant 10 (tcd 10) using CRISPR/Cas9- approach (Wu et al., 2016). The knock-out gene *Os*Ann3 generates chilling tolerance in rice by and this is achieved by CRISPR/Cas9 approach (Shen et al. 2017). Concluded that proline-rich proteins (PRPs) help to tolerate the low temperature. The *Os*Prp1 lowers nutrient leakage, increases antioxidant activity, helps in chlorophyll synthesis. Knocking out *Os*Prp1 gene by CRISPR/Cas9 technology maintains chilling sensitivity by external treatment with salicylic acid (SA) (Nawaz et al., 2019). Simultaneously, Shen et al. (2017) concluded that knocking of *OsAnn3* gene from rice plant via CRISPR/Cas9 technology also reduces chilling tolerance. The ABA signaling pathway for enhancing the cold stress tolerance is activated by over expression of the gene *PtPYRL1* and *PtPYRL5* (Yu et al. 2017).

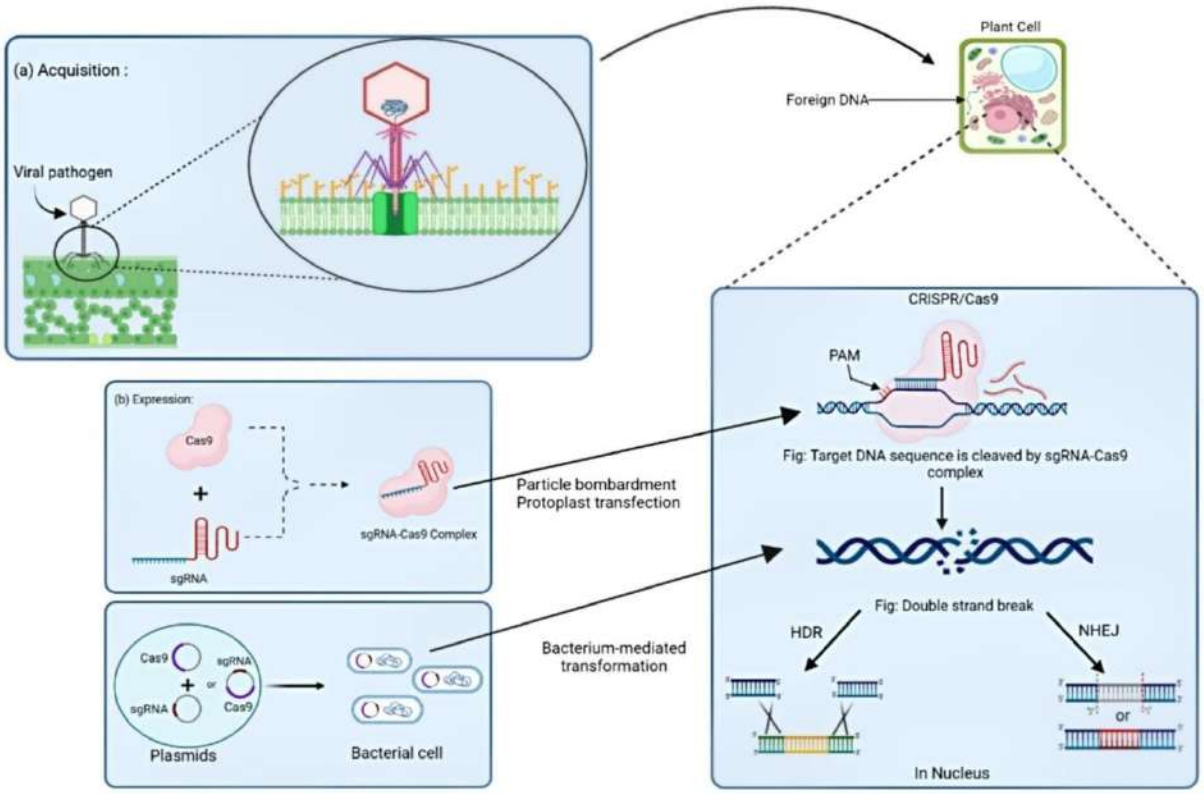

Fig 2: Plasmids of the bacteria contains the gene for cas protein that are inoculated into the cell through transformation process. The cas endonuclease protein binds with sgRNA forming a RNP complex that cleaves both the strand of a DNA. The PAM sequence consisting of 2-6 nucleotides, is present on the non-complementary strand of the DNA i.e., downstream the targeted sequence is present. The PAM will be recognized by the cas9 nuclease. Once the double stranded break is generated, DNA repair mechanism is triggered.

1.3.4 Heat Stress Tolerance:

Heat stress is one of the major issues that negatively impact the growth and development of the plant (Hemmati et al. 2015). According to Hall (2012), temperature more than 10°C - 15°C is considered as heat shock. The heat stress not only effects the growth of the plant but also affect phonological stages like grain filling and anthesis (Hasanuzzaman M et al., 2013).

Heat tolerant plant can be achieved by over expressing heat shock transcription factor like *hsps* gene thereby increasing the levels of osmolytes and preventing cell protein damage (D. Jaganathan et al. 2020). Synthesis of glycine betaine by genetic engineering approach have resulted in heat stress tolerant *Arabidopsis* (Hayashi et al. 1998) According Liu et al., (1998) suppression of gene *OsMDHAR4* in *Oryza sativa* increases its heat tolerant capacity. The transcription factor *OsbZIP46CA1* and SAPK6 (protein kinase) in rice also enhances the heat stress tolerance activity (Chang et al. 2017). Protein disulfide isomerase gene isolated from *Methanothermobacter thermautitrophicus,* increases the heat stress tolerance of the rice plant (D. Jaganathan et al. 2020). Esmaeili et al. (2019) has justified that co-over expression of *AVP1/OsSIZ1* provides better stress tolerance against drought, heat and salinity.

1.3.5 Multiple Abiotic Stress:

A variety of abiotic stresses undergoes same physiological changes due to common pathways of gene interaction in plants. In an experiment, Njuguna et al. (2017) stated that the knock down of poly-ADP-ribosylation protein pathway, poly (ABP-ribose) polymerase (PARP) mediated by CRISPR/Cas9 and RNAi-mediated down regulation and ADP-ribose specific Nudix hydrolase (NUDX) play a novel role in oxidative, drought and genotoxic related stress in some plants like *Zea maize* and *Arabidopsis*. In negatively regulated gene by CRISPR/Cas9 mediation, a superfamily protein AP2/ERF, ethylene response factor, induces multiple stress tolerance like drought, salinity, cold and heat as revealed by Debbarman et al. (2019). Huang et al. (2018) concluded in his studies that rice 9-cis-epoxycarotenoid dioxygenase 3 (OsNCED3) plays a crucial role in salt stress, drought stress and oxidative

stress tolerance. In *Arabidopsis*, the UGTs mainly the UGT79B2 and UGT79B3 transfer the sugar moiety to numerous small molecules controlling various abiotic stresses like salt, cold, heat and drought (Li et al. 2017). A R2R3-MYB transcription factor, GmMYB12B2, affected the expression level and involved in the multistress tolerance i.e., salt stress, chilling stress, drought stress etc in mutated plant species of *Arabidopsis* (Li et al. 2016). The MYB transcription factor is a novel point of interest, as it is affectively involved in inhibiting multiple abiotic stresses in the plants.

1.3 Biotic Stress:

The plants often get infected by many pathogens causing diseases. Although different types of herbicides, pesticides, fungicide can be used as an alternative, but they remain active for a longer period of time in the environment polluting nature both by direct and indirect means (Isman and Grieneisen, 2014; Law *et al*. 2017; Yoon *et al*. 2013). These chemicals also affect the metabolic pathway of the plant (Aktar*et al*. 2009; Kim *et al*. 2017). Hence CRISPR/Cas9 technologies are exploited to induce plant resistance against pathogens like bacteria, virus, fungi, nematodes, insects and also weeds.

1.4.1 CRISPR/Cas on weeds:

Weed causes maximum damage to the plants. They constantly increase the competition for food, space and shelter, sunlight, water and fertilizers that inhibits proper growth and development of the plant. There are some pathogenic plants that invade the host plant and infect the roots as well as the aerial part of the host. *Cuscuta*sp, *Arceuthobium* sp, *Orobanche* and *Phelipanche aegyptiaca,* are the example of some parasitic plant that penetrates the plant and causes huge damage in agro economic society. Many weed pollens and the seed contain toxic chemicals that directly or indirectly causes effects the host plant. Many cost effective herbicides are used against the weeds, although the reuse of same type of herbicide the weeds developed their resistivity against them (Shaner, 2020). And hence it is very important to develop herbicide resistant as well as weed resistance crop, and in turn phytotoxicity of crop will be reduced due to the application of herbicides.

Zheng et al. (2018) edited the uORF of LsGGP2, and obtained the paraquat-resistant lettuce to modify a gene creating maximum ascorbic acid that leads to the resistance against oxidative stress and it also showed measurable effect as an herbicide resistant crop. In *Arabidopsis* sp. deleting of a base pair and addition of a new bp in that return modified the plant into a ALS-inhibiting herbicide and ACCase-inhibiting herbicide (Yang et al. 2020). By inducing point mutation in the 548th and 627th amino acid positions of the rice ALS gene, the researcher were able to initiate resistance with bispyribac-sodium (Li et al. 2015 and Svitashev et al. 2015).*Orobanche* and *Phelipanche*are the obligate parasitic weeds that infect the root of the host plant of almost all the economically important plant like Fabaceae, Solanaceae, Apiaceae, Brassicaceae etc (Westwood et al. 2010 and Joel et al. 2006). Strigolactones (SLs) is the plant hormone which when altered with the help CRISPR/Cas9 technology in the tomato crop, observation was made that it provides resistance against the seed germination of parasitic plant by editing the genome sequence; a non-transgenic *MAX1* mutant was developed in tomato that provided resistance against *Phelipanche aegyptiaca.* (Bari et al. 2019). In rice, intron targeting mutation was developed by NHEJ using CRISPR/Cas9 method, and an herbicide resistant plant was developed (Li et al. 2016). Further analysis and experiments are required in this field of grafting for better resistance against weeds and herbicides.

1.4.2 Pathogenesis by insects:

The lack of guidance in genomic sequence of the insect along with the unavailability of embryonic microinjection, the development of insect resistivity against plant through CRISPR/Cas9 mechanism is difficult. Bu *et al*. (2006) and Liu *et al*. (1996 concluded that, *Arabidopsis* plant, when knocked-in with the potato protease inhibitor II (pinII) gene with the help of CRISPR/Cas9 method provided resistivity against insect pathogen. Bu *et al*. (2006) also stated that in maximum plants potato protease inhibitor II (pinII) induces resistance and so it is ideal promoter. By using CRISPR/Cas9 approach, the abdominal-A (*sl*abd-A) in *Spodoptera litura* was targeted in an experiment by Bi et al., (2016) that resulted in determining the abdominal section of insects. Zhu-Salzman *et al*. (2004) targeted the transgenic peanut and induced a promoter PR1-a expressing transgene Cry1Ac, adapted

from *Bacillus thuringenesis* providing resistance against *Spodoptera litura* pathogen. The PR1 promoter is considered as the prominent promoter for inducing the aphid resistance in transgenic plant (War *et al*. 2012). The Cry1Ab provides resistance in broccoli under the promoter PR-1a against the pathogenic insect *Plutella xylostella Linn.* (Cao *et al*. 2001).

1.4.3 Bacterial infection:

Primarily it was quite difficult to deal with the pathogenicity of bacteria in plants due to absence of appropriate chemical control, constant genetic evolution and late detection of the disease; the phytopathogenic bacteria cause's diseases like mosaic, spots, blight disease, etc (Vale FXRD et al. 2001; Zeng et al. 2010).

In rice, the sucrose transporter gene OsSWEET13, by CRISPR/Cas9 editing method has been transformed in rice providing resistance against *Xanthomonas oryzae pv. oryzae***.** According to Zhou et al. (2015) OsSWEET13 allele of *Oryza sativa subsp. indica* IR24 when transferred to *Oryza sativa subsp. japonica* by CRISPR technology induced resistance against bacterial blight disease. CRISPR/Cas9 technology through effectors-triggered immunity (ETI) led to resistivity against the *Xanthomonas citri* disease. Promoter *CsLOB1* (*lateral organ boundaries 1*) when mutated retarded the recognizing ability and response of rice to bacterial effector initiating resistance against the pathogen with no phenotypic changes (Jia H et al. 2016). In tomato, the CRISPR/Cas9 technology hasnot only been used against the pathogen *Pseudomonas syringae*to inactivate the downy mildew resistance 6 (DMR6), acting as a negative regulator in the host plant immunity but also helped in spreading the infection (Langner et al. 2018; Zeilmaker et al. 2015). *SlDMR6-1* (tomato orthologue) gene has been knocked out thereby increasing the resistivity of the plant against the pathogen *Phytophthora capsici* without affecting the health of the plant (Thomazella DPDT et al. 2016). Equivalent results were also observed when the DMR6 gene is modulated in *Arabidopsis thaliana* concluding that the level of salicylic acid enhanced in plant immunity leading to increased resistivity against the phytopathogen (Zeilmaker T et al. 2015). Ortigosa A et al. 2019 in his experiment concluded that after mutating the *Jasmonate ZIM-domain-2* ortholog (*SlJAZ2*) gene by CRISPR/Cas9 technology, resistance against the bacterial speck

disease caused by *Pseudomonas syringae* can be observed. EvolvR- gene is a genetically created and modified novel allele by using CRISPR/Cas9 technology in rice that identifies the ligands of *Xanthomonas* strain (Luu et al. 2019). CRISPR/Cas9 mechanism has been used in all most every living plant crop to provide resistance against the bacterial pathogen.

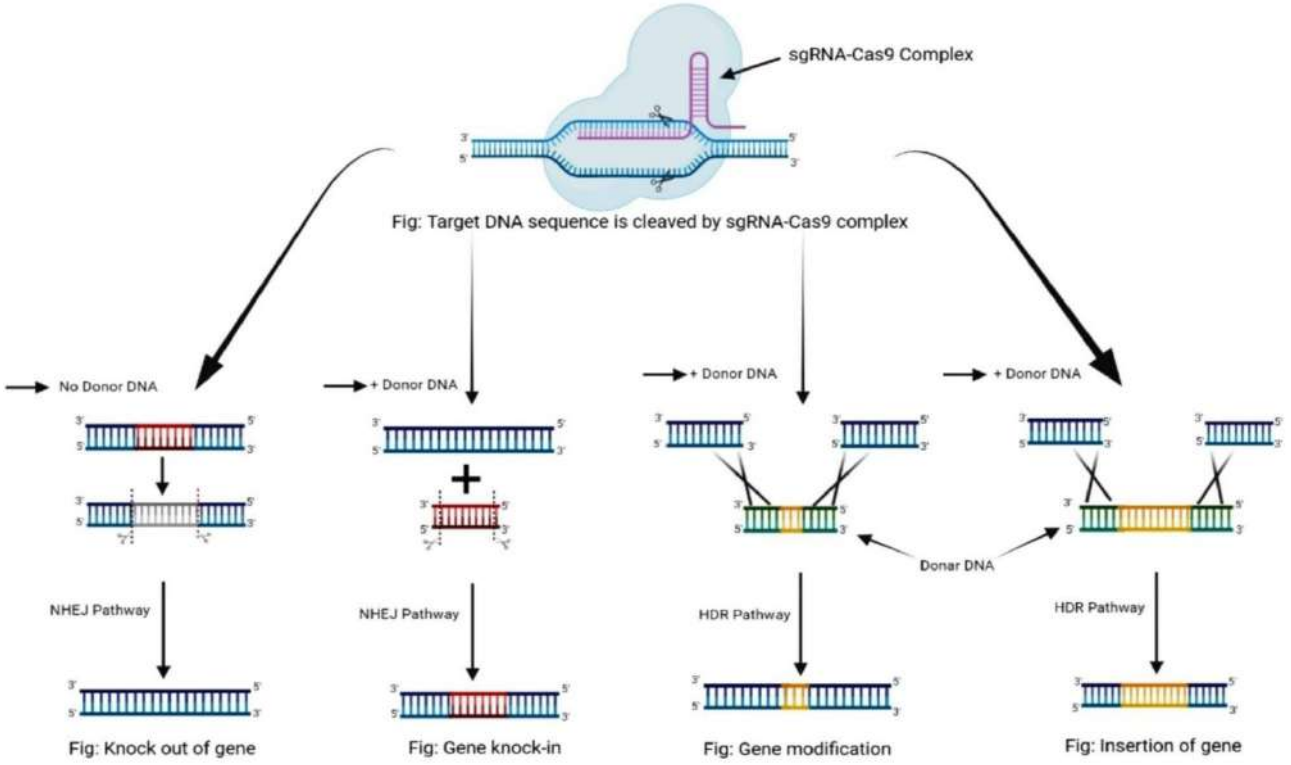

Fig 3: Plasmids of the bacteria contains the gene for cas protein that are inoculated into the cell through transformation process. The cas endonuclease protein binds with sgRNA forming a RNP complex that cleaves both the strand of a DNA. The PAM sequence consisting of 2-6 nucleotides, is present on the non-complementary strand of the DNA i.e., downstream the targeted sequence is present. The PAM will be recognized by the cas9 nuclease. Once the double stranded break is generated, DNA repair mechanism is triggered.

1.4.4 Viral infection:

Virus are obligatory parasitic in nature, and hence have high ability to cause infection in plants leading to huge decline in the crop productivity ratio (Das A et al. 2018). As virus doesn't have its own translational mechanism, so they take over the plant system machinery for their replication. Therefore, resistivity against the virus can only be achieved

either by modifying the host susceptibility gene or by targeting the pathogenicity-related genes (Swati Tyagi et al. 2020).

In 2015, the first report showing resistance against DNA virus was achieved using CRISPR/Cas9 technology inducing resistance against geminivirus like *tomato leaf curl virus* (*TYLCV), bean yellow drawf virus* (*BeYDV*), *Beet severe curly top virus* (*BSCTV),* in model species like *Nicotiana benthamiana* and *Arabidopsis thaliana* (Ali Z et al. 2015; Baltes N et al. 2015, Hirano H et al. 2015). In an experiment Ali et al. (2016), mutated the coding and non coding regions of hypo pathogenic virus like *Cotton leaf curl Kokhran virus* (*CLCuKoV*), *Tomato yellow leaf curl virus* (*TYLCV)* genome, by CRISPR/Cas9 technology where the virus ultimately failed to replicate and thereby inducing resistance in plants. The CRISPR/Cas9 technologies were also used against the *begomoviruses*, which mainly causes leaf curl disease in Cotton plants causing high yield loss. Kis A et al. (2019) proposed that plants could stimulate resistance by editing the viral complex along with its DNA with the multiplex type sgRNA. Banana plant achieved resistance against the *endogenous banana streak virus* (*eBSV*) by inactivating the targeted viral sequence with the help of CRISPR/cas9 method. In initial phases it was believed that the resistance against the RNA virus was difficult because of the incompatibility of Cas9. But later on, Aman R et al. (2018) and Zhang et al., (2018) in their separate experiments have discovered two new types of cas nucleases, viz. FnCas9 derived from *Francisella novicida* and LwaCas13a obtained from *Leptotrichia wadei* having the ability to trace the viral RNA and bind to it. In other host plants like melon, tomato, cucumber, strawberry etc., S genes was found to display resistance against the phytopathogenic virus and hence used as an essential targeted gene.

1.4.5 Fungal Infection:

Due to genetic flexibility the fungal pathogen can easily invade the host plant. Fungi causes many diseases like mildew, smut, rot etc., resulting in severe yield loss (Tyagi S et al. 2018; Dong et al. 2019; Das A et al. 2018). Some phytopathogenic fungi produces mycotoxins and epitomize that even infects the animals as well as the humans (Das A et al. 2018). The fungus secretes some chemicals compounds that degrade the cell wall of the plant and the

pathogen enables to enter the plant cell. The plant too secrets some enzyme like callose, that inhibits the invasion of the phytopathogenic fungi by adding an extra layer of defense (Imam J et al. 2016).

Employing CRISPR/Cas9 technology, *Powdery Mildew Resistance 4* (*PMR4*) gene ortholog *SlPMR4* was targeted to improve the resistivity against *Oidium neolycopersici*, as the PMR4 gene is involved in callose deposition (PRR gene) (Imam J et al. 2016). Due to inversion mutation, the PMR4 gene loses its function and resulting in increase the level of salicylic acid and activates the HR response in host plant. Hence overexpressing PRR, or with impaired S genes provides resistance against pathogen (Swati Tyagi et al. 2020). The *mildew resistance locus O* (*MLO*), a well known host S gene that encodes for a transmembrane protein (PRR), with the help of CRISPR/Cas9 technology was modified in three different plant species viz., wheat, grapevine, and tomato to achieve resistance against the powdery mildew disease caused by the fungal pathogen *Blumeriagaminis f. sp. Tritici* (Wang et al. 2014; Malnoy et al. 2016; Nekrasov et al. 2017). The grapevine gene VvMLO7 when modified by CRISPR/Cas9 technology using ribonucleoprotein (RNP) provided fungal resistance (Malnoy et al. 2016). A non-transgenic plant called the "*Tomalo*" was developed by self-fertilization between a genetically modified gene SlMlo1 in a tomato plant with the help of CRISPR/Cas9 and hence provided resistance against *Oidium neolycopersici* that causes Powdery mildew (Nekrasov et al. 2017). In rice the *Ethylene Response Factor 922 (*OsERF922) and *enhanced disease resistance 1* (*EDR1*) OsSEC3A were improved genetically with the help of genetic engineering by CRISPR/Cas9 technology to develop ethylene signaling and diseases resistance respectively in *Magnaporthe oryzae* against the blast causing fungal pathogen without affecting the typical growth of the crop (Huibers et al. 2013; Wang et al. 2016; Ma et al. 2018). The mutation of Ossec3a gene proved the level of salicylic acid increased along with the up-regulation of pathogenesis, although this modification led to create dwarf plant when compared to the wild variant (Ma J et al., 2018). In wheat, mutation of a susceptible gene *Taedr1* (an ortholog of EDR) through CRISPR/Cas9 technology developed better resistance against the phytopathogen fungi *Erysiphe cichoracearum* providing a broad range of protection against oomycetes, bacteria,

and other pathogens (Zhang et al. 2017). The transcription factor in grapevine VvWRKY52 gene when modulated led to resistance against *Botrytis cinerea*, affecting the phenotype of the plant when compared with the wild type (Wang et al. 2018).

1.5 Conclusion:

The CRISPR/Cas technology along with time has broadened their spectrum of utility. Regardless of its limitation, the genome editing has succeeded in obtaining the desired progeny. The increase of pathogenic invasion and effect due to abiotic stress has increased causing an immense threat to the plants both quantitatively and qualitatively. This technology is more accurate, fast, robust versatile simple and cost friendly than the other genome editing technologies. There capability of gene editing has created a better quality of crop production. By simply knock-in, knockout, replacement, fine-tuning of gene regulation and point mutations at any gene locus, stress resistant plant can be generated with or without using tissue culture.

Although this genome editing technology have not been well explored yet, sometimes interruption in specific gene may lead to other side effects the growth and development of the plant. Therefore extensive studies is the need of the hour in controlling gene progression and editing the precisely to reduce the error as much as possible.

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LATE BLIGHT DISEASE OF POTATO-A COMPREHENSIVE REVIEW

Abstract

Late blight of potato caused by the fungal pathogen *Phytophthora infestans* is a great threat for potato cultivation in all over the world causing a huge economic loss every year worldwide. The disease possesses very serious threat to potato cultivation because of high variability capability of the pathogen against fungicides. In India, A1 mating types has almost been displaced by more virulent A2 strain in temperate highland. To overcome the threat of the disease and to minimize the yield losses it necessary to investigate more about the disease symptoms and managing production in an eco-friendly way. New fungicides with different mode of action need of the hour discover as indiscriminate use of metalaxyl based fungicides has led to the development of metalaxyl resistance globally. This is an exhaustive review on the life cycle and infection pattern of the fungi in conjunction with its disease management and forecasting system.

1. Introduction :

After wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.) potato is the most important vegetable crop grown all over the world (Ewing, 1997). It originated in the hills of Andes and Bolivia in South America. Later it was spread to England and European countries in 16th century (Lal et al., 2018; Swiecz, 1995). The Portuguese introduced it to Asia, particularly in India in the 17th century (Lal et al, 2016).

Considering the nutritional content, potato contains fiber (upto 3%), ascorbic acid (up to 42 mg / 100 g), potassium (up to 693.8 mg / 100 g), anti oxident, phenol, amino acid, vitamins, mineral, some beneficial and harmful bio active components and less amount of protein (0.84-4.2 %) (Swiecz, 1995).

Late blight of potato is a fungal disease caused by an Oomycete or water mould, a microorganism *Phytophthora infestans* which not only has a serious effect on growth, yield and productivity of potato (Fry, 2008) but other solanaceous members too (Chand and Sudeep, 2009; Nowicki et al., 2011). It has been reported that *Phytophthora infestans* causes 27 diseases in over 100 different plant species in Japan (Watanabe, 1998).

Late blight causal organism firstly reported as *Botrytis infestans* in 1845 by C. Montagne. Later it was renamed as *Phytophthora infestans (mont.) de Bary* by a German scientist Anton de Bary (Alexopoulos et al., 1996). This disease can cause 50-70 % potato yield loss under favourable environmental condition (Khair and Haggag, 2007; Haq et al., 2008; Rahman et al., 2008). Late blight disease is held accountable for the great devastation namely The Great Irish Potato Femine in 1840 leading to death of over one million people due to starvation. Another one million migrated to USA and other parts of the world (Zadoks, 2008; Jonathan Cape, 1940) in search of food.

This devastating disease led to direct loss of approximately US dollar 30-100 million potential yields per year in Bolivian Andes (Fernandez–Northcote et al., 2000). Under favourable condition, it has been reported that late blight of potato resulted to 50–70% yield loss in Pakistan in 2007 (Haq et al., 2008). According to Ahmed et al., 2015 it can cause 100% yield loss in Pakistan under epidemic condition. In Indian scenario, 5–90% crop loss occur depending upon environmental condition. In the year 2013-2014, 10-20% yield loss was reported in Uttar Pradesh, Punjab, West Bengal, Karnataka and Uttarakhand (Lal et al., 2016). Thereby having a consequential impact on Indian economy (Tariq et al., 1995).

2. History and occurrence of late blight of potato:

Late blight was first observed in United states in 1843 which gradually propagated to North America (Peterson et al., 1992). In 1845, it appeared in Belgium and later spread to many European countries like France, Switzerland, Ireland, Scotland, Great Britain, sparking off severe damage (Robertson, 1991). Since then various part of Europe got affected every year, causing severe damage to potato production (Sakai, 1961).

Heterothallic fungus *P.infestans* needs two mating types during sexual reproduction. In 1981, Mexico and Switzerland first reported A2 mating type strains. In 1981 it was observed first in Switzerland, outside of Mexico (Hohl and Iselin, 1984). The pathogen later migrated from Mexico to European countries during 1977 (Fry et al., 1993). Potato importation from Egypt resulted in migration of pathogen in England (Shaw et al., 1985).

Subsequently A2 isolate was detected in Russia during 1990s (Vorodev et al., 1991), India (Singh et al., 1994), Pakistan (Ahmed and Mirza, 1995), USA (Deahl et al., 1991), Belarus

(Ivanyuk and Konstantinovich, 1999), Canada (Chycoski and Punja, 1996), Northern Ireland (Cooke et al.,1995), Netherlands (Drenth et al, 1993), France (Gilet, 1996), China (Zhiming et al., 1996), Italy (Cristinzio and Iesta, 1997), Myanmar (Myint, 2002), Hungary (Bakonyi and Ersek, 1997), Ecuador (Oyarzun et al., 1997), Colombia (Vergas et al., 2009) and Sri lanka (Kelaniyangoda, 2011). It have been found in many parts of Asia (Koh et al., 1994; Mosa et al., 1989,1990; Nishimura et al., 1999, Ogoshi et al., 1988), Europe (Malcolmson, 1985; Lebreton and Andrivon, 1998; Tantius et al., 1986; Sehober and Rullich, 1987), Africa (Sedegui et al., 2000). Through genetic analysis, it was predicted that A2 mating types migrated globally through import and export of infected tubers and not generated by mutation nor native to these countries (Goodwin and Drenth, 1997).

Both A1 and A2 mating types were detected in Sweden, Hungary and Estonia and also in Mexico (Widmark et al., 2007; Nagy et al., 2006; Runno et al., 2009; Fernandez – Pavia et al., 2005). Occurrence of both A1 and A2 mating type are lead to production of thick walled oospores which could dangerously survive extreme winter and summer (Medina and Platt, 1999). In Europe and North America, production of oospore was noticed (Gotz, 1990; Shattock et al., 1990; Chycoski and Punja, 1996). Oospores were also found in Japan in an experimental field under artificial condition (Kato et al., 1993). Only A1 mating type was detected in China (Guo et al., 2009; Li et al., 2009) and Southern Germany (Moller et al., 2009). New strain is more aggressive to the old stocks (Fry et al., 1999), So, in most countries new strains displaced the old strains (Spielman et al., 1991).

In India A2 was dominated in temperate hills and A1 type has stabilized in sub tropical plains (Singh et al., 2005; CPRI, 2013). During 1870-1880 the disease was detected first in Nilgiri hills (Butler, 1918). Subsequently it spread in Hooghly district of West Bengal in 1898-1900 (Butler, 1903), Darjeeling in 1883, Khasi hills in 1885, Shimla hills in 1902 (Butler, 1903; Dastur, 1915), Kumaon hill in 1897, Assam and Bihar in 1913 (Dastur, 1917; Basu, 1913; Dey, 1947; Lal, 1949; Woodhouse and Dutt, 1913), Dehradun and Meerut in 1943 (Lal, 1949), Punjab in 1958-1963 (Srilantaiaya, 1962), Mahabaleshwar hills in 1973 (Kadam et al., 1974), Gujrat and Madhya Pradesh in 1968, Rajasthan in 1958 (Dutt, 1979).

In Japan, late blight of potato first occurred in Hokkaido in 1990 (Ideta, 1901) where both A1 and A2 mating types were observed (Ogoshi et al., 1988).

3. Late Blight Disease Cycle :

A potato plant can be infected either by the *Phytophthora infestans* spores originating from infected potato stocks, wild plants, voluntary plants or by the infected tuber. Infected seed tubers put into cold storage are major sources to cause the infection. 0.01% to 3.0% of tuber infection is enough for developing late blight disease in the next cropping season (*Bhattacharyya et al., 1990*). According to Forbes and Landeo (2006) *Phytophthora infestans* is carried out by several Solanaceous plant. 10-16 degree centigrade temperature in night with light rain and 13-16℃ along with high humidity in next day, is the perfect weather for late blight infection and development (Krik , 2009 ; Krik et al., 2013) . 18 to 22℃ temperature and 80 to 100% relative humidity is ideal for spreading of late blight disease (Fry et al., 2001).

Life cycle of *Phytophthora infestans* may last between three to fifteen days. It depends on weather and the level of plant innate resistance. After coming in contact with *Phytophthora infestans* spores, a healthy plant gets infected. This is the start of the life cycle of *Phytophthora infestans*. Brown blotches originate on healthy leaves and stems of newly infected plant. Generally lower plant parts are affected first. Blotches grow fast in high temperature (up to 25℃).

Next step of the cycle is sporangia formation and their germination. It is an important stage in the life cycle of the pathogen. High relative humidity plays an important role in the formation of sporangia. Sporangia can germinate either by direct or by indirect means. Indirect germination i.e. through zoospores needs less time than direct germination. This process is depend on temperature. In low temperature (5 to 6℃), motile zoospores can survive up to 22h, whereas at high temperature (24-25℃) motility reduced by 19 min (Melhus, 1915). The non motile, zoospores cling firmly with leaf surface and start germinating. Germination of zoospores occur at 3 to 28℃ and germ tube elongation occurs rapidly at 21℃ (Crosier, 1934). Sporangia has higher shelf life in presence of high relative humidity (Martin, 1923; Agrios, 2005). In saturated air at 15℃ about 9.8×10^5 sporangia are generated over one leaflet of a susceptible potato cultivar in 12 days(Harrison, 1992). Sporulation and the infection procedure lose velocity in low humidity (Easton, 1982). Sporulation slows down in bright sunshine because related humidity of foliage decreases with increase in extreme sunlight (Harrison and Lowe, 1989).

The fungal spores are dispersed by air currents and rain from diseased plant to healthy plant and disease cycle continues. According to several worker, air current transfers the pathogen *Phytophthora infestans* to the field (Martin et al., 1994; Krik et al., 2013).

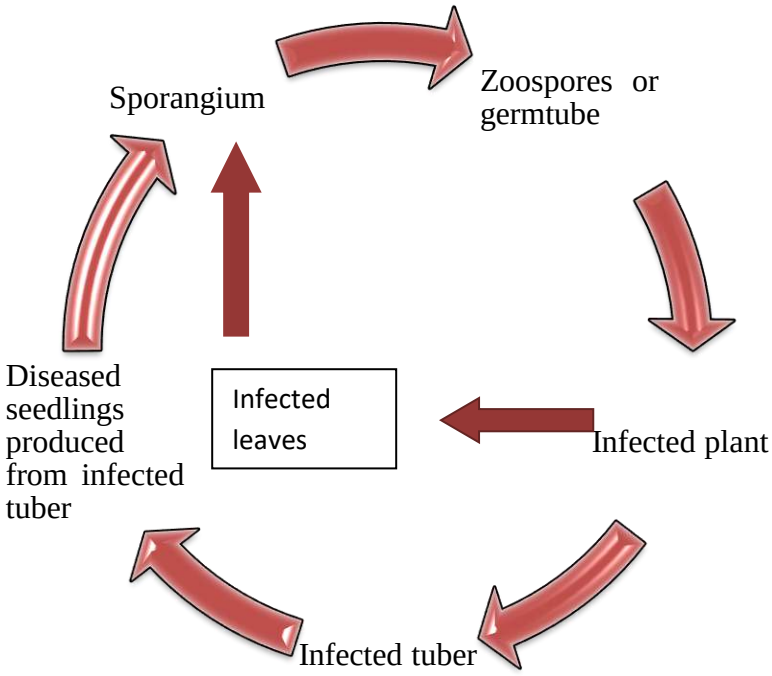

Figure1 –Diagrammatic Representation Disease Cycle of *Phytophthora infestans*

Spores are propagated from diseased plant to healthy one by rain irrigation. Spore spreads its infection gradually from the leaves towards the tuber. The infected tuber when planted in the next season, develops late blight disease and starts a new cycle. Potato tuber may get infected at any time whenever sporangia draw close to the concerned tissue (Shailbala and Kumar, 2017). Absence of high humidity and surrounding film of water causes lose of endurance of air borne sporangia. The rain occurrance has more effect on disease development than total rainfall (Arora et al., 2014). Overhead water spattering can causes severe outbreaks of the disease (Everdingen, 1935). Wind shows two contradictory effect in disease development. In wet weather, wind help in spread of pathogen spore. In contrary, during dry weather, wind causes inhibition of the disease indirectly speeding up the evaporation of surface moisture from leaves and by dehydrating the sporangia (Arora et al., 2014).

Prolong survival of pathogen facilitate the development of disease. The fungus can survive in living tissues, infected seed tubers, infected tubers in cull piles, unharvested and volunteer potatoes left in the ground (Shinners et al., 2009). Fungus remains alive in soil for many years (Fernandez Pavia et al., 2004). If a field get infected with the pathogen, it results rapid increase of disease, and the pathogen completes reproduction cycles in a season (Tsedaley, 2014).

4. Disease Symptoms :

All plant parts especially leaf, stem and tubers get affected by the blight disease.

At first small, pale green, circular irregular shaped water soaked spot (2-10 nm) develop (Lal et al., 2018). These lesions are produced usually on the lower surface of the leaf, near the leaf tips and edges (Robinson et al., 2017).

Under moist weather, these spots enlarge rapidly, produce dark brown or black lesions, turn necrotic and the whole leaf die rapidly (Lal et al., 2018). A large yellow hallow may appear surrounding the lesion (Mohan et al., 1996).

On the lower surface a white mildew appearing area is found. It is an active spore producing zone of late blight pathogen. In warm weather, these lesions become dry and stop sporulating.

Not only the leaves but also stems and petiole get affected by late blight pathogen as well. Pale brown spot produce which encircle the stem as well as the petiole. These affected parts become weak and may deteriorate (Arora et al., 2014). Entire crop shows a black blighted appearance and often produce a distinct odour.

Late blight infected tuber shows rusty brown slightly depressed area on the skin. These lesions extending irregularly into the tuber flesh usually less than ½ inch. Temperature and length of time after initial infection have large effect on extention of rotting in tuber *(*Robinson et al., 2017).

Generally late blight infected tubers are hard but secondary pathogen such as soft rot bacteria pink rot etc get associated, causing rot in field and stores. Tuber with late blight symptoms do not have bad odour (Caceres et al., 2008).

In primary stage the disease remains unnoticed because late blight appears on the lower most leaves of the plant. Slowly it develops in the middle and then spreads to the upper leaves. Then entire crop is destroyed, looking like fire burned field (Lal et al., 2018).

5. Response of potato to the pathogen:

Several molecules obtain from *P. infenstans* have elicitor actively in Potato cells. Potato cell produces race-specific R proteins and pathogen produces Avr factors. After inoculation, interaction between them causes drastic physiological change in plant cell. An oxidative burst that is rapid production of reactive oxygen species (ROS) is the earliest response. It was first reported as earliest response by Doke, 1983. In wide range of plant-microbe interaction, rapid production of ROS is a very common response (Torres et al., 2006). Level of Nitric Oxide is gradually increased in potato cells treated with HWC elicitor (Yamamoto et al., 2003). After penetration of the pathogen, whole cytoplasm and cellular component of the cell immediately translocated towards the site from where the pathogen penetrates. This type of cytoplasmic aggregation was first reported by Tomiyama (1956). This event now became a very common response in plant pathogen interaction (Kobayashi et al., 1992; Takemoto et al., 2003; Takemoto and Hardham, 2005). It induces hypersensitive response like cell death and produced antifungal phytoalexin. After the death of infected cell, phytoalexin starts to accumulate. All of these events resist the growth of pathogen hyphae. Sesquiterpenoid phytoalexin rishitin and the related metabolites are produced by potato (Ishizaka et al., 1969). 3-hydroxy-3-methylglutaryl coenzyme A reductase and a sesquiterpene cyclise vetispiradiene are involved in the production of potato phytoalexins (Stermer and Bostock, 1987; Yoshioka et al., 1999). According to Katou et al., 1999, in HWCs treated potato plants MAPK (mitogen activated protein kinase) cascade is activated. To induced the disease resistance, MAPK cascade act as a central signal transduction pathway. Many reaction including hyper sensitive cell death, ROS production etc are induced bt StMEK2 (Asai et al., 2008), a active form of potato MAPK kinase (Katou et al., 2003). Transgenic potato shows disease resistance by expressing StMEK2dd under the control of the PVS3 promoter (Yamamizo et al., 2006).

6. Management of *P. infestans* using Genetic engineering:

Genetic resistance to *P. infestans*is the best option for potato varieties to protect against late blight. Late blight resistance became a serious concern after Irish Famine. In Mexico, it was noticed that wild *Solanum* species were immune to *Phytophthora* attack. In India, it was found that *S. demissum* and *S. antipoveizii* possesses a fair degree of resistance to pathogen, later they are used as parents for late blight resistance breeding. In 1906 late blight resistance in *Solanumedinense* Berth. (a natural hybrid of *Solanum demissum* Lindl. And *Solanum tuberosum* L.) was first found by R.N. Salaman, 11 R genes (R1-R11) have been identified in S.demissum (Ross, 1986). Recently K.Mohan, a new variety, is reported with field resistance to late blight (Luthra et al., 2017). Development of resistance variety has played an important role in disease management (Bhardwaj et al., 2005, 2007, 2013; Kaushik et al., 2007; Joseph et al., 2007, 2011)

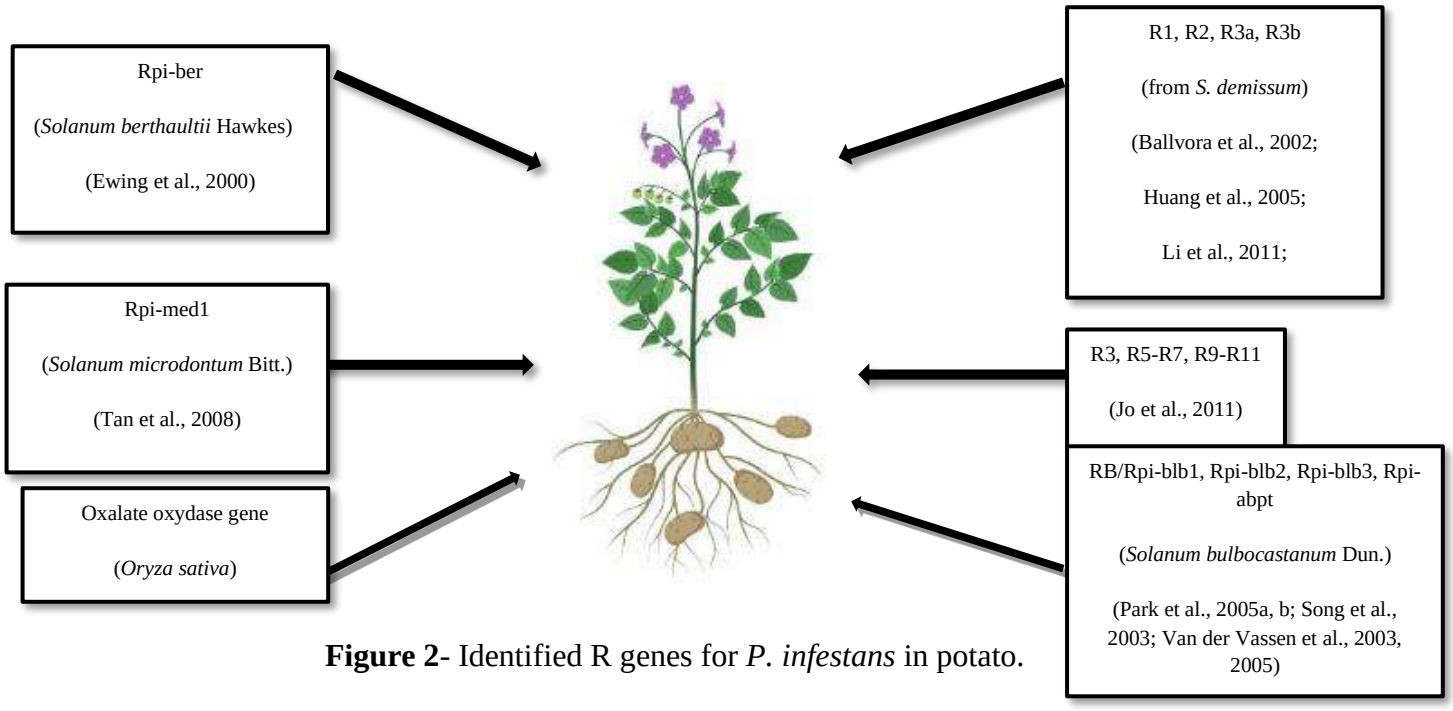

Figure 2- Identified R genes for *P. infestans* in potato.

7. Screening of potato varieties against late blight:

Name of variety	Varietal Response	References
Simply red	MS	Mohsan et al.,2016
FD 71-1	S	Do
FD 77-4	MS	Do
SL 15-10	S	Do
FD 63-1	MS	Do
FD 78-36	MS	Do
FD 76-67	HS	Do
Sante	MS	Do
FD 74-21	HS	Do
FD 35-36	S	Do
SL 5-2	HS	Do
FD 76-18	MR	Do
FD 61-3	S	Do
SL 9-4	MR	Do
FD 73-73	R	Do
FD 78-51	MS	Do
SL 15-11	MS	Do
SL 14-15	HS	Do

FD 35-32	MS	Do
FD 76-67	MS	Do
SH 704	HS	Do
FD 74-8	HS	Do
FD 74-4	HS	Do
FD 74-51	S	Do
FD 78-51	S	Do
FD 78-76	S	Do
FD 75-21	HS	Do
N-34	S	Do
FD 76-18	S	Do
FD 73-75	MR	Do
NARC 39012-96	R	Do
FD 69-2	R	Do
FD 69-25	MR	Do
FD 74-19	MR	Do
FD 78-10	S	Do
FD 78-104	S	Do
FD 73-77	S	Do
FD 78-3	S	Do

NARC 39-457-21	MS	Do
FD 35-36	HS	Do
Berber	HS	Hansen et al.,2005
Bintje	S	Do
Latona	S	Do
Red Scarlet	S	Do
Asterix	MS	Do
Sava	MS	Do
Folva	S	Do
Van Gogh	MS	Do
Maret	R	Do
Var Kollane	R	Do
Piret	R	Do
saturna	R	Do
Vivaldi	R	Do
Ants	R	Do
Oleva	HR	Do
Danva	HR	Do
Anti	HR	Do
Ando	HR	Do

Sarme	HR	Do
Kuras	HR	Do
S.albornozii	S	Karki et al.,2018
S.agrimoniifolium	MS	Do
S.berthaultii	MR	Do
S.bulbocastanum	R	Do
S.chacoense	S	Do
S.cardiophyllum	MR	Do
S.demissum	R	Do
S.microdontum	S	Do
S.okadae	R	Do
S.polyadenium	R	Do
S.pinnatisectum	MS	Do
S.schenckii	R	Do
S.stoloniferum	R	Do
S.verrucosum	R	Do
S.stipuloideum	R	Do
S.venturii	R	Do
Michoacan	HR	Gopal and Singh , 2003
CFK 69-1	HR	Do

MS 82.60	HR	Do
CEW 69.1	HR	Do
3053-18	R	Do
AGG 69.1	R	Do
AND 69.1	R	Do
CFJ 69.1	R	Do
I 931	MR	Do
Luke	MR	Do
ARX 69.1	MR	Do
Yana	MR	Do
F-7	S	Do
P-6	S	Do
Seseni	S	Do
CFQ 69.1	S	Do
Palma	HS	Do
Rila	HS	Do
TS-2	HS	Do
V-3	HS	Do

Table 1: Response of different potato varieties against late blight disease

(S – Susceptible , HS – Highly Susceptible , MS – Moderately susceptible, R – Resistant, HR – Highly Resistant , MR – Moderately resistant)

8. Management of *P. infestans* with chemical fungicides :

Several management strategies are utilised by farmers to prevent the late blight disease among which the farmers prefer chemical management most.

Trade name	Active Ingredient	Formulation	Mode of Action	References
Bordeaux mixture	CuSo4	Copper sulphate, Hydrated lime, Water	Copper ion of the mixture affects the pathogen spore's enzyme to prevent germination.	Liu et al., 2017
Metalaxyl (Phenylamide group with FARC 4)	Mefenoxam	R and S enantiomers of N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alaninate	It penetrates into the fungal cell and inhibits growth of mycelium and formation of spore and haustria and affects DNA synthesis.	Gisi et al., 1996
Mefenoxam	Metalaxyl M	Metalaxyl M	It inhibits RNA polymerase 1 to inhibit sporulation and mycelia growth inside host tissue.	Davidse et al., 1983; Bhat et al., 2009
Maneb / Manzate /Mancozeb /Penncozeb	Ethylene(bis)dithiocarbamate	Ethylene(bis)dithiocarbamate	Disturb several biochemical process in fungal cell by interfering with sulphydryl group in mitochondria and cytoplasm.	Housenger et al., 2015; Durkin , 2015; Richard, 2015
Curzate	Cymoxanil	Cymoxanil , Mancozeb	Inhibits sporangium and germination of zoospore.	Evenhuis et al., 1996
Acrobat MZ	Dimethomorph, Mancozeb	Dimethomorph, Mancozeb	Inhibits sterol synthesis and break cell wall to kill the pathogen.	Lal et al., 2018
Bravo	Chlorothalonil	Chlorothalonil	Deactive Glutathione and suppressed growth of pathogen.	Tillman et al., 1973

Tatto C	Chlorothalonil	Chlorothalonil, Propamocarb HCl	Suppressed growth of pathogen	Lal et al., 2018
Master	Metalaxyl	Metalaxyl, Mancozeb , Ofurace	Provide double protection	Lal et al., 2018
Fenamidone	Fenamidone	Fenamidone	It affacts cytochrome bc1 in Mitochondria complex iii of *P.infestans*	Bardsley et al., 2002
Cymoxanil	Cymoxanil	Acetone, methanol, hexane , tolune, acetonitrile, ethyl acetate.	It can penetrate the crop leaf and improved usefulness of other fungicides.	Thind et al., 2002; Rodriguez et al., 1999
Dimethomorph	Dimethomorph	Dimethomorph	Break the cell wall of the pathogen.	Lal et al., 2015; Rani et al., 2007; Lal et al., 2017
Victory 72 WP	Metalaxyl , Mancozeb	Mancozeb, Metalaxyl, Ridomil gold	It penetrates into the fungal cell and inhibits growth of mycelium and formation of spore and haustria .	Amin et al., 2013
Ridomil Gold	Mefenoxam and mancozeb	Mefenoxam and mancozeb	Acts as a contact fungicide on the surface of diseased tissue and inhibits germination of spore.	Lal et al., 2015
Dithane M 45	Mancozeb	Mancozeb	It inactivates the sulfhydryl groups of amino acid of fungal cells to stop lipid metabolism respiration and production of ATP.	Lal et al., 2015

Initium	Ametoctradin	Ametoctradin	It affacts cyt bc1 in the electron transport chain to inhibit ATP synthesis in the fungal cells.	Merk et al., 2011
Propamocarb	Propamocarb	Propamocarb	Fungicide with protective action.	Lal et al., 2018
Q0I compounds	Fenamidone	Fenamidone	Oomycete specific fungicide, block electron transfer in cytochrome and prevent ATP formation in pathogen.	Koller et al., 1998
Dithio-carbamates	Dithiocarbamates	Dithiocarbamates, anhydrous oil, alkenyl glycerine ether ethoxylate, anioic surfactant.	Inhibits sulfhydryl enzyme system in fungi.	Daayf and Platt, 2002; Samoucha and Cohen, 1988; Saville et al., 2015
Revus	Mandipropamid	Mandipropamid	Inhibit cellulose synthesis	Blum et al., 2010; Gisi et al.,2007; Daayf and Platt, 2002; Samoucha and Cohen , 1988; Saville et al., 2015

Abound TM 2.08F/ Amister TM/ Heritage TM/ Quadris TM	azoxystrobin	strobilurins	Inhibit fungal respiration	Vincelli, 2002; Daayf and Platt, 2002; Samoucha and Cohen , 1988; Saville et al., 2015
Frowncide, Shirlan, Omega etc.	Fluazinam	Fluazinum	Disrupt energy production in fungi	Guo et al., 1991
Quintal, Fuji one , ergon, monceren	Cyazofamid	cyazofamid	Used as protectant fungicide, inhibits all stage of life cycle of pathogen	Cooke et al., 2011

Table 2: Several fungicides with their mode of action

9. Late blight disease forecasting systems :

Environmental factors like temperature, humidity etc have high effect on late blight pathogen for causing the disease. So, various concepts on disease forecasting models have been developed. By studying those models we can predict about the severity of the disease and can take consequetive precaution accordingly. There are many concepts for predicting the disease worldwide including 'DUTCH RULES' , Beaumont's periods (Beaumont et al., 1947) , Irish rules , moving day concept (Hyre et al., 1954), Severity Value accumulation (Wallins et al., 1962), negative prognosis (Ullrich et al., 1966), mathematical models etc. By using night temperature, dew period, cloudiness, rainfall, Van Everdingen first forecasted potato late blight, known as Dutch rules, in 1926 in Holland. Though it was found quite satisfactory but in some cases it was found that disease appears without fulfilling dutch rules. By modifying this, Beaumont proposed 'Beaumont rules' for UK condition (Beaumont, 1947). This rule is depend on RH period and temperature for two consecutive days. This concept also can't predict the disease in all region. After that, 'Moving days concept' was proposed by Hyre (1954). Depend on temperature and relative humidity Wallin (1962) developed 'Severity value' concept. It is a worldwide accepted concept and many USA growers used it. Growers of Washington state, USA used Mathematical model proposed by Johnson et al., 1996. Ullrich and Schrodter develop 'negative prognosis' depending on temperature, rainfall, relative humidity in 1966. This concept was used by the growers of Germany and Europe. Excess application of Fungicide can be reduced by using those models and by calculating the risk of outbreak of disease throughout the crop season.

After advancement of more powerful computers, farmers now have the option to use many forecasting system like BLITECAST, ProPhy, Phytopro, SIMCAST, PROGEB, Web-Blight, NegFry, PhytoPRE+2000, Plant Plus, China Blight, Bio-PhytoPre etc. In different region of world.

Hyre and Wallin proposed BLITECAST for forecasting the disease in USA and Europe (Krause et al., 1975). To predict the disease in Netherlands ProPhy was developed (Schepers, 1995). According to Forrer et al., 1993 PhytoPRE was developed in Switzerland. In Denmark, NegFry model was proposed, which is based on negative prognosis (Ullrich and Schrodter,1966) and on the method of Fry et al.,1983. According to Gtsche (1993) PROGEB model was developed in Germany. Danish Institute of Agricultural Sciences (DIAS) collaborating with Danish

Agricultural Advisory Centre (DAAC) developed Web-blight in 1996 (Jensen et al., 1996). As a decision support system Plant-Plus was developed in Netherlands in 1994 (Hadders, 1997). According to Goeminne et al., 1997, Guntz-Divoux model was validated in Belgium and France. Phyto PRE+2002, an improved version of PhytoPRE, was implemented in Switzerland (Cao et al., 1966). A decision support system Bio-Phyto PRE was developed by Agroscope FAL Reckenholz for organic potato farming (Musa-steenblock and Forrer, 2005).

Two disease forecasting models, BLITECAST and SIMCAST have been linked with GIS (Geographical Information System) by International Potato Centre. It helps us to estimate severity of late blight worldwide. Depending on 13 kinds of weather data a new forecasting model Blight-SVR was developed and it can provide 64.3% accurate. Prediction for first appearance of late blight of potato (YH G et al., 2016).

Depending on 12 years rainfall data on Indian forecasting model has been developed on the concept of Hyre's and Cook's moving graph (Chaudhury et al., 1959). For Shimla, Shillong and Ootacamund, another forecasting model has been developed on the basis of daily data of weather condition (temperature, rainfall, RH) (Bhattacharyya et al., 1982). Fermers of UP utilised a computer based forecasting model 'JHULSACAST' for both rainy and non rainy condition to detect the first appearance of late blight in their region (Singh et al., 2000). This model provide data based on a wireless network and it has been found to be pretty accurate than the other models (Winsteland Wallin model, Ullrich, Fry etc.) in Uttar Pradesh region (Jagyasi et al., 2015). Some models which are developed for Punjab (Arora et al., 2012), Tarai of Uttarakhand (Pundhir et al., 2015), West Bengal (Chakraborty et al., 2015) are produced modifying JHULSACAST model. In India ICAR-CPRI developed a decision support system which include three models- 1. Decision rules to predict the pathogen depending on weather data (temperature, relative humidity, rainfall) 2. Rules for using fungicides 3. Decision rules for preventing yield loss. Recently a web based model INDO-BLIGHTCAST has been developed depending on JHULSACAST which can be implemented across the country without any calibration (Singh et al., 2016). With the help of disease forecasting model not only we can predict the initial appearance of the disease but also we can manage the schedule of spraying fungicides.

10. Conclusion :

Late blight of potato caused by *Phytopthora infestans* is the most dreaded disease because of huge loss in production. Pathogen is continuously acclimatizing to host and new environment as well as against fungicide. There is need of advanced genetic study to categorize the pathogen and to understand the characteristics of each categories. Keeping in mind all of these changes in pathogen population, development of a universally applicable disease forecasting model is the need of this hour. There is a necessity to stay focussed towards the path of biological control rather than extensive use of fungicides. Last but not the least, there is a requirement of a proper farmer friendly disease management strategy.

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ABBREVIATION:

35SCaMV	:	Promoter of 35S RNA in Cauliflower Mosaic Virus
AA	:	Ascorbic acid
ABA	:	Abscisic acid
AMF	:	Arbuscular Mycorrhizal Fungi
APX	:	Ascorbate Peroxidase
ATHK1	:	Histidine Kinase 1
ATPase	:	Adenosine triphosphatase
AVP	:	Vacoular H^+- pyrophosphatase gene
BADH	:	Betaine Aldehyde Dehydrogenase
CAT	:	Catalase
CIPK	:	CBL interacting protein kinase
COR	:	Cold Responsive
DHAR	:	Dehydroascorbate reductase
DNA	:	Deoxy Ribonucleic Acid
DREB	:	Dehydration Responsive Element Binding Protein
ERF	:	Ethylene responsive Factor
FAO	:	Food and Agricultural Organisation
GA_3	:	Gibberellic Acid
GR	:	Glutathione reductase
GSH	:	Glutathione
GSSG	:	Glutathione disulfide
GST	:	Glutathione-S-Transferase
HKT	:	High- Affinity Potassium Transporter
HSP	:	Heat shock protein

IAA	:	Indole Acetic Acid
IRE1	:	Inositol requiring enzyme 1
LEA	:	Late Embryogenesis Abundance
MDHAR	:	Monodehydroascorbate Reductase
Mha	:	Mega hectare
MKK	:	MPK kinase
mM	:	Milli Molar
MPK	:	Mitogen activated protein kinase
MV	:	Methyl Violagen
NAC	:	NAM (no apical meristem)-ATAF1/2- CUC2(cup shaped cotyledon)
NaCl	:	Sodium Chloride
NADP	:	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	:	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NHX	:	Sodium/ Hydrogen Antiporter
PCD	:	Programmed Cell Death
PDH	:	Pyruvate Dehydrogenase
PQ pool	:	Plastoquinone pool
ProT	:	Proline Transport
PS II	:	Photo System II
PUFA	:	Polyunsaturated Fatty Acids
RNA	:	Ribonucleic Acid
ROS	:	Reactive Oxygen Species
SOD	:	Superoxide Dismutase
SOS	:	Salt Overly Sensitive
ZIP	:	Leucine Zipper

INTRODUCTION:

Stress is any unusual condition that tries to disrupt the usual growth and development of an organism altering its metabolic processes. All organisms deal with various kinds of stresses in its lifetime for survival. Plants deal with all biotic and abiotic stresses with maximum efficiency in order to survive and flourish. Stress is often the condition that prevents the plant to gain its maximum genetic potential. In its lifecycle, a plant faces several kinds of environmental stresses, of which salt stress is the most frequent. Salinity is one of the major environmental factors limiting crop production. Over 6% of the world's total land area is affected by salinity and an additional 1.5 million hectares of irrigated land each year pass the salt stress threshold due to salinization (Munns and Tester, 2008). Plants use different ways to cope with it. Salinity stress disrupts all the physiological actions and metabolic reactions from germination to development. It is projected that about 900 Mha land is affected due to salt which possess a serious threat to agricultural productivity (Munns 2002; Flowers and Yeo, 1995). In our attempt to understand the salt stress effects on plants we will look upon the salt stress effects on plants and how plants deal with the salt stress.

SALINITY STRESS:

Stress is general deals with all factors that prevent the plants to get its maximum efficacy. Stress can be broadly classified into biotic and abiotic stress.

Biotic stress deals with every biotic interaction that takes place in a plant's vicinity. In its lifecycle a plant comes in contact with several organisms. All these interactions can be beneficial or harmful to the plant. From mutualism, symbiosis, to parasites attack that leads to plant destruction all comes under the same roof of biotic interactions and subsequently biotic stress.

Abiotic stress, on the other hand mostly includes unusual environmental conditions that disrupts the common metabolic processes. It could be heavy metals accumulation, high salt concentration as well as other different environmental factors that affect plant's optimum genetic functions. Several metabolic processes that are inter-regulated often behave abnormally leading to formation of different toxic compounds in response to several stress conditions. As every species and every physiological processes and biochemical components of every plant species varies in one way or other, reflecting on stress effects is not at all an easy task. It requires discussion of every possible aspect that is affected by stress.

The major environmental factor that currently reduces plant productivity is salinity (Majeed et el., 2010). According to FAO, 2007 report, 900 million hectares of land (approximately 20% of the total agricultural land) are affected by salt and it often increases due to climate change (Suzuki et al., 2016; Shabala, 2013). Salinity stress has deleterious effects on plant growth and development at both the physiological and biochemical level, including limited water uptake, reduced water content in plant tissues, mineral nutrient imbalance and specific ion effects (Ashraf and Harris, 2004; Marschner, 1995).

Salinity stress refers to accumulation of salt at a very high concentration. Earth is a predominantly salty planet, with most of its water containing about 3% NaCl and this concentration has rendered the lands very salty (Nawaz et al., 2010). Under non saline conditions, the cytosol of higher plant cells contains about 100mM K^+ and less than 10mM Na^+. In saline conditions the concentration of Na^+ increases to a much larger extent and the ion becomes cytotoxic. Naturally occurring salinization is primarily caused by capillary water level elevation and subsequent evaporation of saline groundwater (Nawaz et al., 2010). The accumulation of toxic salts in the leaf apoplasm leads to dehydration and turgor loss, and eventually death of leaf cells and tissues (Marschner, 1995). As a result of these changes, the activities of various enzymes and plant metabolism are effected (Lacerda et al., 2003).

EFFECTS OF SALT STRESS: A HOLISTIC APPROACH

Immediate effect of salinity stress is the reduction of water potential (chemical potential or free energy associated with water), a key regulating factor that controls every action associated with water i.e. osmosis and diffusion. Salt stress enhances accumulation of NaCl in chloroplasts of higher plants, affects growth rate, and is often associated with decrease in photosynthetic electron activities (Kirst, 1989). In higher plants, salt stress inhibits photosystem (PS)-II activity (Kao et al., 2003; Parida et al., 2003).

The reduced water potential (owing to excessive salinity) leads to reduced cell and leaf expansion. It eventually leads to immediate osmotic effects, reduces all cellular and metabolic activities including stomatal opening-closure, photosynthetic inhibition, leaf abscission, altered carbon portioning, cavitation, membrane and protein destabilization and most importantly reactive oxygen species (ROS) production and eventually cell death.

Here we try to take an overall look on how each stage of plant growth and development is altered, modified, regulated and disrupted by salinity stress.

1.1 SALINITY STRESS ON SEED GERMINATION:

Seed germination is the very first and the most crucial stage for a plant's development. Dynamics of germination depends on genetic predispositions and health status, but it is strongly influenced by environmental conditions, including soil water availability (Gutterman, 1993). Plants are most sensitive to salinity during their germination and emergence, when the toxic effects of salts are very direct and strong (Abdelly et al., 2006). Various scientific reports noticed that maximum percentage of seed germination attain under distilled water condition and the rate declined with increased media salinity (Ratnakar and Rai, 2013; Hong et al., 2010; Pena and Hughes, 2007). Several authors have reported that salinity stress affected seed germination either by decreasing the rate of water uptake (osmotic effect) or facilitating the intake of ions. It may change by certain enzymatic or hormonal activities within the seeds (Huang and Redman, 1995; Dubey and Rani, 1990). Germination of seeds represents often the critical step in the establishment of crop canopy, and determines successful agricultural production. Salt tolerance can therefore be evaluated by the precocity of germination (Rastogi et al., 2018).

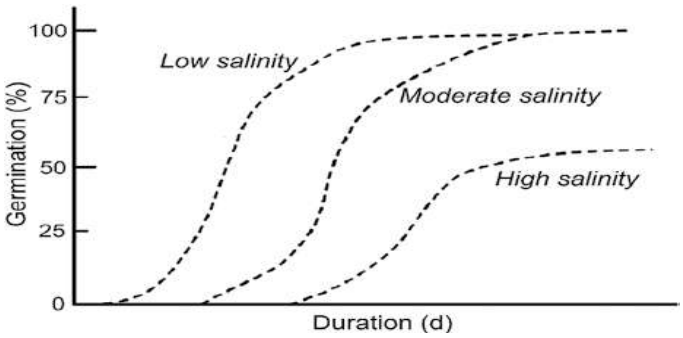

Fig.1: Relationship between rate of germination and time after sowing at different salinity levels (Modified from Ibrahim, 2016)

1.2 SALINITY STRESS IN SEEDLING GROWTH:

The action of salinity on growth is a dual effect of imbalanced nutritional value of essential ions and high uptake of toxic ions by the plant (Munns, 2002). It is connected with stress inducing low osmotic potential of soil solution (Munns and Tester, 2008). It is well acknowledged that mineral nutrients' action regulate several cellular enzymatic and metabolic processes. Increased salinity and ion accumulation reduces the mineral uptake and thus mineral nutrition is disturbed. It is observed that at much higher concentration of salt the effects were far more severe leading to reduction in rate of photosynthesis, resulting in less carbohydrate accumulation, thereby leading to stunted growth of seedlings.

1.3 SALINITY STRESS ON MINERAL NUTRITION:

Increased salinity alters the intake of Na^+ ions, thereby disturbs the uptake of other cations (K^+, Ca^{2+}) (Haouala et al., 2007). K^+ receptor channels show significant affinity towards Na^+. NaCl in excess indirectly interferes with accumulation of K^+. As the balance among the ions is disturbed, the whole metabolic system is disrupted.

1.4 SALINITY STRESS ON PHOTOSYNTHESIS

Salinity stress affects all the cellular metabolic processes to some extent. Photosynthesis is no exception. It has been reported that salt stress makes plants more sensitive to photoinhibition (PSII), causes osmotic stress and leads to a higher generation of ROS (Parida and Das, 2005). It is often observed that salinity does not affect all the photosynthetic organisms at same manner or extent. Several genera of different families have shown varied results of photosynthesis in salinity stress. In *Chlamydomonas reinhardtii*, a microscopic algae as well as in different varieties of chick pea, severe salt stress shows an inhibition of electron flow between PQ pool and P700 (Cruz et al., 2001)

REACTIVE OXYGEN SPECIES:

Any oxygen derived molecule whose oxygen atom has at least one unpaired electron in its orbital is known as reactive oxygen species (ROS). It is an integral part of any biological system as it is often formed as an intermediate or by products of one or other metabolic processes formed in various cellular organelles like peroxisome, mitochondria and chloroplast. ROS are controlled by the scavenging action of several antioxidants. In every biological system, the antioxidants and the ROS are maintained in equilibrium by their regulated activity. Under stress conditions, this balance is often hampered leading to rise of excessive ROS disrupting the equilibrium of the organism metabolic activity. ROS react to molecules like DNA, RNA, lipids, carbohydrates and proteins. Photosynthesizing organisms are always at risk of oxidative damage due to abundance of photosensitizers and poly-unsaturated fatty acids (PUFA) in chloroplast envelope. Generation of ROS from oxygen molecule involves a multi-step process led by energy transfer between electron states.

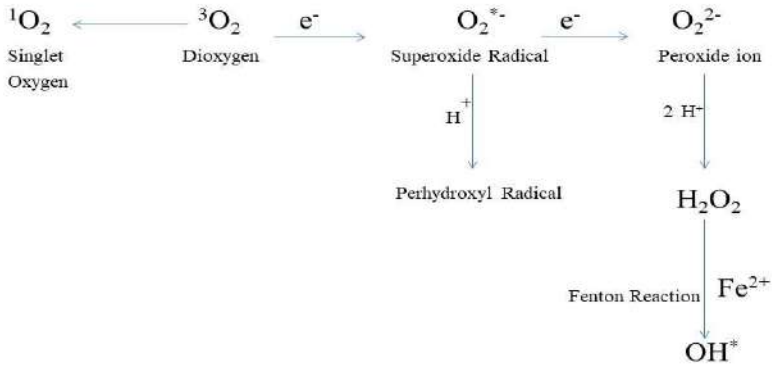

Fig.2: Generation of ROS by energy transfer(Gill and Tuteja, 2010)

Some of the ROS and the ways by which plants deal with them are as follows:

1. Super Oxide Radicals (O_2^{*-}) :

   Formation of superoxide radicals mostly occurs by the partial reduction of oxygen molecules or energy transfer in the thylakoid membrane-bound primary electron acceptor of PSI as well during aerobic respiration. At the terminal stage of respiration, cytochrome c oxidase and other oxidases react with O_2, $4e^-$ are transferred and water molecule is released. In certain cases, if the O_2 molecule reacts with any other components of ETS only one e^- is transferred and superoxide radical is formed (Puntarulo et al., 1998). The generation of superoxide molecules lead to the formation of other ROS molecules and in turn cause lipid peroxidation in cellular and organelle membranes.

Scarpeci et al., 2008 studied the methyl viologen (MV, O_2- propagator in the light) induced generation of O_2 in *Arabidopsis thaliana* chloroplasts during active photosynthesis and suggested that O_2- generated in photosynthetically active chloroplasts leads to the activation of genes involved in signalling pathways. In *Amaranthus* sp, under salinity, superoxide radicals were detoxified by SOD and the antioxidant Amarathine thereby lowering lipid oxygenation (Gambarova and Gins, 2008).

2. Singlet Oxygen ($^1O_2$) :

Formation of singlet oxygen ($^1O_2$) is not related to electron transfer in oxygen molecule. Singlet oxygen often forms due to insufficient energy dissipation during photosynthesis leading to the formation of chloroplast triplet state. The chloroplast triplet state reacts with $^3O_2$ and produces very reactive $^1O_2$. Salinity stress causes stomatal closure; in turn intercellular CO_2 concentration of chloroplast is reduced and as a result of which $^1O_2$ is formed. $^1O_2$, an oxidizing agent for a wide range of biological molecules can react with proteins, pigments, nucleic acids and lipids, and is thought to be the most important species responsible for light induced loss of PS II activity and cell death (Wagner et al., 2004; Trebst et al., 2008). Certain bacteria that generate singlet oxygen on exposure to light and the singlet oxygen participate in oxidation of protein, lipid, and nucleic acids. *Arabidopsis* leaf tissues with optimum exposure of singlet oxygen show about 80% of total lipid peroxidation non-enzymatically and *Arabidopsis* mutants that favour formation of singlet oxygen gives rise to photooxidative stress, increased lipid oxidation and ultimately cell death (Mueller et al., 2008). Singlet oxygen is suggested to not act as toxin but as a primary signal that activates several stress-response pathways (Pryzybyla et al., 2003). As an antimicrobial secondary metabolite phytoalexins are formed on the onset of pathogenesis. The occurrence of phenalenonechromophores in phytoalexins suggests that these plants respond to pathogen-attacks by biosynthesizing $^1O_2$ photosensitizers (Zhang et al., 2005).

3. Hydrogen peroxide (H_2O_2):

The reduction of O_2^{*-} produces H_2O_2. Excessive H_2O_2 can inactivate enzymes by oxidizing their thiol groups. H_2O_2 plays a dual role in plants: at low concentrations, it acts as a signal molecule involved in acclamatory signalling triggering tolerance to various biotic and abiotic stresses and, at high concentrations, leads to programmed cell death (PCD) (Zhang et al., 2005). H_2O_2 acts as a key regulator in a broad range of physiological processes, such as senescence (Lin et al., 2005), photorespiration, photosynthesis, stomatal movement, cell cycle and growth and development. It can also act as secondary messenger. In a study targeted to understand antioxidant defence, citrus leaves were pre-treated with H_2O_2 or Sodium nitroprusside and it was

noted that H_2O_2 and Sodium nitroprusside increased the activities of SOD, catalase and various other enzymes (Tanoua et al., 2009).

4. Hydroxyl Radicals (OH^*):
   It is the most reactive ROS of all. In presence of transition metals like Fe, O_2 and H_2O_2 react to form hydroxyl radicals by the Fenton reaction. OH^* can potentially react with all biological molecules like DNA, proteins, lipids and almost any constituent of cells (Bhattacharjee, 2005). There is no such natural enzyme to remove hydroxyl radicals. As a result often excessive synthesis of hydroxyl radicals leads to cell damage and eventually cell death.

Formation of ROS is a very frequent phenomenon in every plant's life cycle as ROS is formed as part of several environmental factors. From extreme temperature to salinity stress, drought to nutrient deficiency, pollutants to water scarcity all have played direct or indirect role in excessive formation of O_2^{*-}, H_2O_2, OH^*, $^1O_2$. That's why ROS scavenging machinery of a plant is an essential component for existence.

1.5 SALINITY STRESS ON NITROGEN METABOLISM :

Soil salinity not only affects crop production and agricultural sustainability, but also interrferes with nitrogen metabolism. Most legume species have been found to be either sensitive or moderately tolerant to salinity as they depend on symbiotic nitrogen fixation for their growth (Chalk et al., 2010; Aydi et al., 2008; Drevon et al., 2001). Salinity effects nodule establishment and nodule growth (Abdelmoumen and El-Idrissi,2009). Nitrogenase enzyme gets affected leading to inhibition in nitrogen-fixing activity (Bolanos et al., 2006; Tejera et al., 2006). Salt mediated reduction of nodule respiration decreases in leghaemoglobin production (Dulormne et al., 2010; Lopez and Lluch 2008). Different cultivars and genotypes of some legume species show varied extent of nitrogen fixation.

It is evident from the discussion that salinity is the most significant abiotic stress faced by plants and crops. To ensure food security for the whole world dealing with salinity requires immediate action.

Severely affected areas of India are pointed on the map.

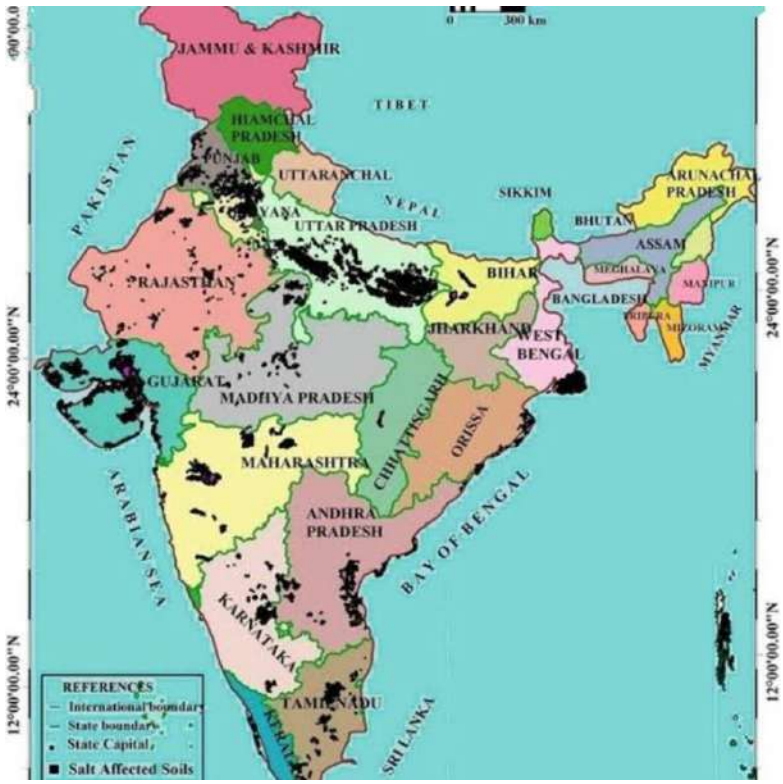

Fig.3: Distribution of salt-effected soils in India (Based on map prepared by NRSA Hyderabad, CSSRI Karnal, and NBBS and LUP, Nagpur, 1997)

There are several ways how a plant senses and regulates salinity stress. Some of them are discussed.

STRESS SENSING MECHANISM: From the afore-said discussions it is evident that plants are affected to varying degrees and extent in all the species. The effects considered can be genetic, physical, biochemical, metabolic even epigenetic. Plants do have a specific stress sensing mechanism in order to mitigate stress effects following various approaches. Briefly the stress sensing mechanism in plants can be of 5 types (Taiz and Zeiger, 2014).

- **Physical sensing:**
  Physical sensing includes the mechanical effects on cells on the onset of any environmental stress. It includes the most common physical processes like diffusion or osmosis. For example, plasma membrane of cells contract when there is stress related to scarcity of water.
- **Biophysical Sensing:**
  It includes alteration of biomolecules in presence of heat stress or any other physical processes. For example, in higher temperatures the protein structures are distorted and denatured and the enzyme activity is altered.
- **Metabolic sensing:**

There are so many metabolic processes taking place in the cellular system simultaneously resulting in several by products formation. Alteration in metabolic pathways results in by-products accumulation ultimately leading to stopping of metabolic processes. Thus extent to which these by-products are formed often works as an indicator of the stress.

- **Biochemical sensing:**
  In this, particular chemical compounds, molecules or ions take part in identifying stress through the detection of changes in ion homeostatis.
- **Epigenetic sensing:**
  In case of epigenetic sensing, the genetic components such as DNA, RNA and the transcription and translation processes are altered due to stress factors. Stress can even cause the chromatin modifications in organisms leading to various epigenetic changes.

All these stress sensors help to identify stress before-hand for better signalling response in order to deal with stress factors. Some of early stress sensing mechanisms that are part of signalling responses include- Calcium ion (Ca^{2+}) channels, kinase, plasma membrane histidine kinase (ATHK1), endoplasmic reticulum trans-membrane sensor inositol-requiring enzyme (IRE1) .

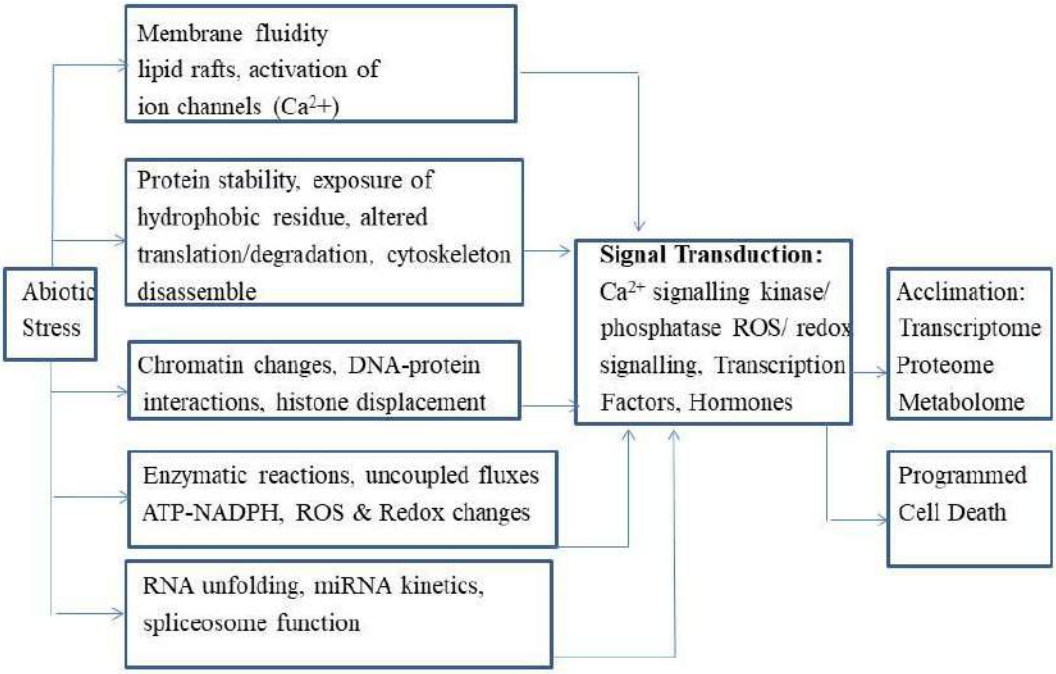

Fig.4: Early events in the sensing of abiotic stress by plants and the signal transduction and acclimation pathways activated by these events (Mittler et al., 2014)

The stress specific signals that emerge from these pathways, in turn, activate or suppress various networks that may allow growth and reproduction of to continue under stress or enable the plant to survive the stress until favourable conditions reoccur.

SALT STRESS REGULATION:

We have looked into the broad effects of salinity stress on every plant in its lifetime. It is thus evident there are certain mechanisms that regulate all these stress responses and keep the plants healthy. Plants have very intricate line of inter regulatory pathways that exist inherently. And on the other hand in commercially produced crops dealing with salinity stress is mostly done using various biotechnological processes.

SALINITY STRESS AND ADAPTATION:

1.1 GROWTH REGULATORS ON DEALING WITH SALINITY STRESS:

Plant hormones are a crucial part of development. They not only regulate the physiological processes of plants but also act in response to stress. Plant hormones are active members of the signal compounds involved in the induction of plant stress responses (Pedranzani et al., 2003). Abiotic stresses result in both altered levels of phytohormones and decreased plant growth (Morgan, 1990).

- **Gibberellic Acid:**

GA_3 has been reported to be helpful in enhancing wheat and rice growth under saline conditions (Parasher and Varna, 1988; Prakash and Prathapasenan, 1990). Under saline conditions, seed germination has been improved by application of GA_3 resulting in improved growth and grain yield. Lipid peroxidation owing to ROS accumulation is also reduced in presence of gibberellic acid (Choudhuri, 1988).

- **Abscisic Acid:**
  Increase in ABA accumulation salt stressed *Brassica* (He and Cramer, 1996) and *Phaseolus* (Montero et al., 1998) provide evidences for the production of ABA under salinity stress. Salt stress stimulated synthesis in roots and xylem transport of ABA was well correlated to stomatal cloosure (Kaya et al., 2009). When roots are exposed to the salt, ABA in roots stimulates ion accumulation in vacuoles of barley roots, which may be necessary for adaptation to saline conditions (Jeschke et al., 1997). Jae-Ung and Youngsook, 2001 reported that ABA induces rapid depolymerisation of cortical actin filaments, thus there is slower formation of a new type of actin that is randomly oriented throughout the cell. This change in actin organisation is suggested to be basic in signalling pathways involved in stomatal closing responses to ABA.

- **Cytokinins:**
  A reduction of cytokinin supply from the root alters gene expression in the shoot and thereby elicits appropriate responses to ameliorate the effects of stress (Hare et al., 1998). Kinetin is capable of breaking stress-induced dormancy during germination of tomato, barley and cotton seeds (Bozcuk, 1981). Kinetin acts as a direct free radical scavenger or it may involve in antioxidative mechanism related to the protection of purine breakdown (Chakrabarti and Mukherji, 2003).
  Jasmonate and Triazoles also contribute to salinity stress adaptations.

1.2 NITROGEN METABOLISM ON DEALING WITH SALINITY STRESS:

In nitrogen fixers, it is observed that with increase in salinity, new organic compounds (Trehalose) contribute in osmotic adjustments. Trehalose (α-D-glucopyranosyl-1, 1-a-D-glucopyranoside) is a non-reducing disaccharide that has been found to play an important role as an abiotic stress protectant in a wide variety of organisms (Elbein et al., 2003). Symbiotic nitrogen fixing bacteria *Rhizobium* have the capacity to synthesize trehalose and its accumulation has been detected in bacteroids and in nodules and correlated to the maintenance of efficient nitrogen fixation and whole plant tolerance under drought and salinity stress (Farias-Rodriguez et al., 1998; Zacarias et al., 2004).

1.3 ROS SCAVENGING ENZYMES ON DEALING WITH SALINITY STRESS:

The antioxidants are the most important way to deal with the ROS accumulation. The components of antioxidant defense are both enzymatic and non-enzymatic. Enzymatic antioxidants include SOD, CAT, APX, MDHAR, DHAR and GR and non-enzymatic antioxidants are GSH, AA, carotenoids and tocopherols (Gill et al.,; Mittler et al., 2004). SOD simultaneously oxidizes and reduces superoxide anion to produce H_2O_2 and O_2. APX catalyzes destruction of H_2O_2 using ascorbic acid as a reducing agent. CAT catalyzes the detoxification of H_2O_2 into H_2O and O_2. Glutathione peroxidise (GPx) catalyzes the detoxification of H_2O_2 using Glutathione as reducing agent. As formation of ROS is an inevitable phenomenon in plant system, the continuous cycle of ROS formation and antioxidant formation takes place.

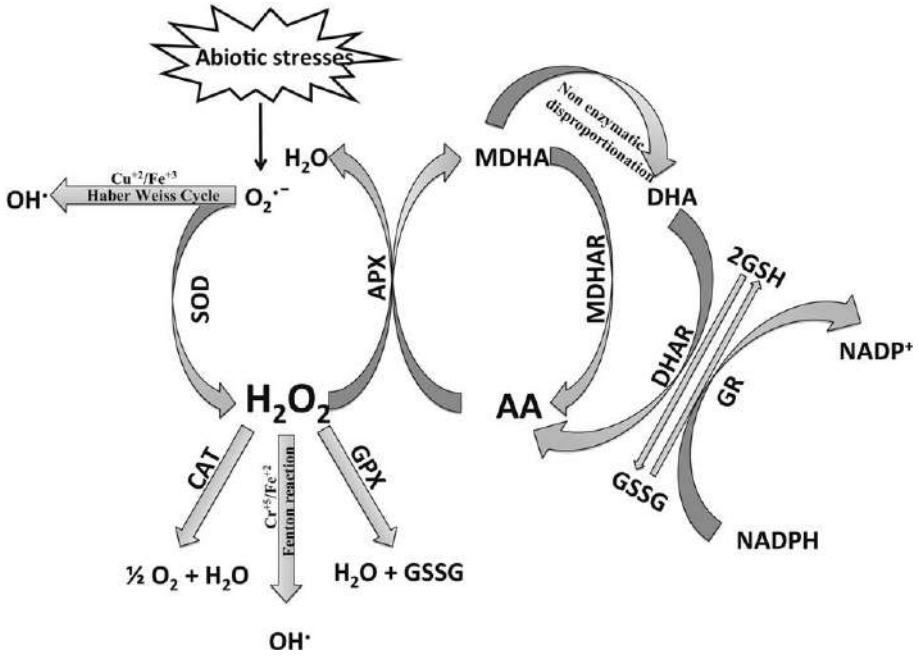

Fig.5: ROS and antioxidants defence mechanism.(Gill and Tuteja, 2010)

1.4 ARBUSCULAR MYCORRHIZAL FUNGI IN DEALING WITH SALINITY STRESS:

AMF are widely distributed in saline land of terrestrial ecosystem (Yamato et al., 2008). The symbiotic association improves water and nutrient uptake of the host plant and protects the plant from various biotic and abiotic stresses (Zuccarini and Okurowska, 2008; Gupta et al, 2000). The positive cumulative effects like nutrient uptake, photosynthetic, biochemical and physiological performance on plant growth due to mycorrhizal inoculation can mitigate salt tolerance (Borde et al., 2011). Various plants have demonstrated that mycorrhizal fungi help the plant under salinity stress. *Piper*

nigrum shows enhanced chlorophyll content (Kohler et el., 2009); *Citrus sinesis* shows uptake of K^+, Ca^{2+} (Wu et al., 2010); *Glycine max* shows increase CAT activity (Ghorbanli et al., 2004); *Acacia nilotica* shows increased K^+ intake (Giri and Mukerji, 2004). AMF play a key role in regulation of ion and membrane transport proteins that control the ion homeostasis of the host plants. The K^+: Na^+ ratio is maintained by the accumulation of P. Hammer et al., 2011 reported that AMF can selectively take up elements such as K^+ and Ca^{2+}, which act as osmotic equivalents by avoiding uptake of toxic Na^+, thereby lowering Na^+ concentration in plant cell under salinity stress condition.

1.5 MOLECULAR CHAPERONS ON DEALING WITH SALINITY STRESS:

Molecular chaperones physically interact with other proteins to facilitate protein folding, reduce misfolding and stabilize tertiary structure. Heat Shock proteins (HSP), (type of molecular chaperone), are synthesized in response to salinity stress.

There are several proteins and many other molecular structures involved in dealing with salinity stress. But emerging levels of salinity requires new anthropogenic approaches. The halophytes hold a way to deal with salinity stress as they grow and thrive in saline conditions.

LEARNING FROM HALOPHYTES IN DEALING WITH SALINITY:

Halophytes are the plants that survive under extreme salt stress conditions. Halophytes are well adapted to thrive under high salinity by using two strategies, salt tolerance, and salt avoidance (Mishra and Tanna, 2017). Halophytes basically follow three mechanisms of salt tolerance; reduction of the Na^+ influx, compartmentalization, and excretion of Na^+ (Flowers and Colmer, 2015; 2008). Salt avoidance is done by shedding, secretion and sometimes succulence. The salt tolerance mechanism of halophytes is a result of collective coordinately regulated events. It includes scavenging ROS, to regulating detoxifying signal transduction pathways.

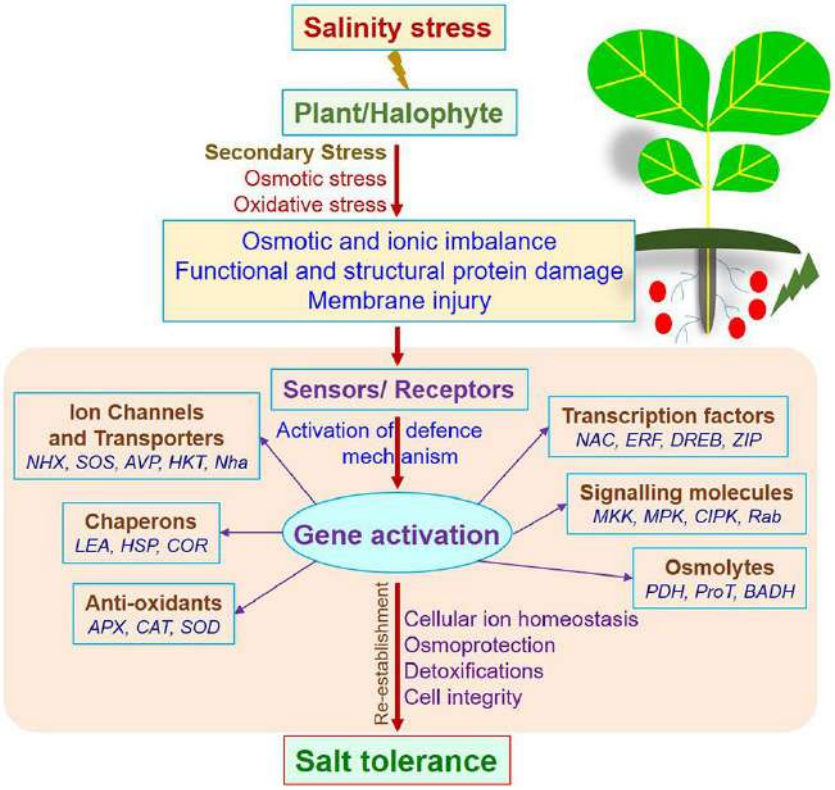

Fig.6: Generalized schematic representation of salt tolerance in plants (Mishra and Tanna, 2017)

Halophytes are traditionally salt tolerant while glycophytes are salt sensitive. But often it is seen that the plants believed to be glycophyte show salt tolerance whilst halophytes, believed to be salt tolerant is actually sensitive to salt concentrations. Different genomic and transcriptomic efforts have been made to isolate salt responsive genes from some halophytes followed by their functional validation through transgenic approaches. The overexpression of several halophytic genes, under the control of a non-specific 35S CaMV promoter, have been claimed to enhance abiotic stress tolerance in glycophytic plants (Mishra and Tanna, 2017). A number of crops have been transformed with halophytic genes for the improvement of salt tolerance. Most of the genes encode for Na^+/H^+ antiporters (vacuolar or plasma membrane), vacuolar pyrophosphatase, potassium transporters, ion channels, antioxidants, ROS scavengers, and proteins that involve in protective function and signal transduction (Mishra and Tanna, 2017). *Thellungiella salsuginea* t exhibit high tolerance to salt and drought, can be considered as a potential model for abiotic stress tolerance studies (Bartels and Dinakar, 2013). The genome sequence of *T. salsuginea* provides evidence about the genetic basis of abiotic stress defence mechanisms, and comparative genomics identified this plant as a gene resource for cation transporters, abscisic acid signalling genes, and other upregulated genes that show a response to stressful environments (Wu et al., 2012).

A comparative analysis of salt tolerance in glycophyte and halophyte helps us to get to a clearer picture. A number of antiporters isolated from both glycophytes and halophytes were

functionally characterized (Sreeshan et al., 2014; Kronzucker and Britto, 2011). The overexpression of glycophytic transporters encoding genes (NHX, SOS, HKT, ATPase, etc.), under the control of nonspecific CaMV35S promoter, showed tolerance in the range of 150-250 mM NaCl (Volkov, 2015; Sreeshan et al., 2014; Kronzucker and Britto, 2011). In several previous studies, the effects of overexpression of halophytic genes were commonly observed under salt stress treatments, however, negligible differences were observed between wild type plants and the transgenic lines under unstressed conditions (Tiwari et al., 2015; Volkov, 2015; Joshi et al., 2012; Jha et al., 2011). It is evident that any one particular model of halophyte is not enough to deal with the vastness of salinity stress but it sure is a promising way to look at for dealing the emerging heights of salinity stress.

SALINITY AND CLIMATE CHANGE……. An outlook towards future…

Climate change is an evolving phenomenon and it is inevitable. Climate change has a severe impact on every organism in the planet. Climate change basically predicts a future of increased average global temperature, shift in overall rainfall, evaporation extremities and extreme local climatic conditions. The continuous carbon sequestering is disrupting due to the changing temperature, pH. The increased CO_2 concentration interferes with the salinity stress of the land. Secondary salinization is a prevalent phenomenon of today's world. Secondary salinization is caused by human interference and agricultural effects etc. According to FAO, 1994 the irrigated water on cultivation lands is not absorbed by plants fully. About 45% is absorbed by the plants and the rest is stored in the land. After successive years, this accumulated water leads to percolation beyond the root zone eventually leading to the rise of water table and if there is salt accumulation in this water it may lead to the death of all the crops. Substituting flood irrigation with drip irrigation can control both salinity and water usage.

Salinity, being a very common phenomenon cannot be totally avoided but it must be taken care of in order to have a thriving agriculture. Need of agricultural methods' modification is very crucial. With the rise in carbon dioxide and temperature, the process is even more difficult. So salinity should be taken care of for larger good in agricultural sector (Mishra and Tanna, 2017).Salinity stress requires immediate action to ensure both food security and a healthy planet.

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DISSERTATION

SINGLE CELL OMICS: A PRAGMATIC WAY TO CAPTURE AND STUDY THE DIFFERENT CELLULAR PROCESS AT THE CELLULAR LEVEL

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SINGLE CELL OMICS: A PRAGMATIC WAY TO CAPTURE AND STUDY THE DIFFERENT CELLULAR PROCESS AT THE CELLULAR LEVEL

1. Abstract

Cells are the fundamental unit of life. Cellular processes are strongly impacted by individual cells in an organism that are variable in nature. It is therefore important to study the phenotype and behaviour of individual cells in the complex biological system. To understand the complex cellular interactions in plants it is required to gain insights about cell lineage and functional cell type, whilst the field of single cell plant genomics is in its infancy. The cellular functional diversity can be understood by cell phenotypes (epigenomics, transcriptomics, proteomics, and metabolomics) that exhibit dramatic heterogeneity between and within different cell types. In the form of somatic genetic variation, cell genotypes can also display heterogeneity throughout an organism- most notably in the emergence and evolution of tumours. The aspects of cell identity that have enabled a revolution in the study of multicellular system and revealed by the recent technical advances in single cell isolation and the development of omics approaches. In this review, we discuss about those technologies that are available to resolve the genomes, epigenomes, transcriptomes, proteomes, and metabolomes of single cells from a wide variety of living systems.

2. Introduction

The cell is the smallest structural unit of an organism that is capable of functioning independently, consisting of cytoplasm, one nucleus and various cell organelles, surrounded by semipermeable cell membrane. In this world every living being is composed of communities of individual cells. Cells are associated with living organisms in various aspects like providing structural support to the organism, providing the genetic information, supplying the required energy to perform different metabolic functions. Thus, in order to analyze the behaviors of the organs and organisms at single cell level it is necessary to inspect the activities of these communities of cells.

Omics is the comprehensive approach for the collective characterization and quantification of pools of biological molecules that forms structures, function and dynamics of any organism. Cells show considerable heterogeneity within and between different types and indicates functional diversity between cells. Single cell-omics is a relatively new technology to detect, quantify and characterize genes at single cell level. As well as it helps to determine the interaction between single cell.

Studying a single cell includes isolation of the cell community the enabling analysis of the cell's unique molecular identity. Flowcytometry helps to perform the analyses of small panels of proteins/markers in individual single cells. Study of functional and molecular profiling of heterogenous cell population is performed by fluoresce-activated cell sorting (FACS) (Mincarelli et al.,2018).

Several technologies have been developed for studying the activities of an isolated single cell like Genomics, Transcriptomics, Proteomics, metabolomics. The field of genomics and proteomics are very closely related. Genomics is the study of the entire set of genes in a cell where proteomics is this study of all the proteins produced by a cell. Genomics include a combination of recombinant DNA, DNA sequencing method and bioinformatics to sequence and analyse the structure and functions of genes.

Transcriptomic techniques involve the study of transcriptomes, which is all the RNA transcripts produced by a cell. Every information of an organism is recorded in the form of DNA in its genome and expressed through transcription. Here mRNA serve as a transient intermediary molecule in the information network, in contrast non coding RNA perform additional diverse function. A transcriptome captures a snapshot in time of the total transcripts present in a cell. To provide a broad account of which cellular processes are active and which are dormant a technology has been discovered that is known as transcriptomics. In molecular biology there lies a major challenge to understand how the same genome can give rise to different cell types and how gene expression is regulated.

A proteome is the complete set of proteins expressed by any organism. The proteome is an expression of an organism's genome. The cells sense and response to virtually all extrinsic and intrinsic stimuli to the protein and post translational modification. By a detailed analysis of a cell's proteome, we can give very detailed definition of cell types ad steps possible by molecular means, but such observations are impossible because of the limitation of current approaches (Mincarelli et al.,2018). For the detection of proteins from single cell western

blotting approaches have been developed (Sinkala and Herr,2015; Kang et al.,2016; Kang et al.,2014; Hughes et al.,2014; Duncombe et al.,2016).

Full collection of all low molecular weight metabolite that are produced by a cell could be a key indicator of cell state-reflecting the precise metabolic activity and condition within the cell is defined as metabolomes. Metabolomics is the large-scale study of small molecules, commonly known as metabolites. Collectively these small molecules and their interaction with in a biological system are known as the metabolomics.

Single cell-omics has various kinds of applications. In biomedical researches and basic biology which includes single cell analysis in stem cell biology, in cancer, in reproductive medicine, in plant researches and in microbial community. Single cell omics and its technologies helps us to understand the complexity of various disease mainly cancer. Each cell of our body has its own unique structure which allows the reestablishment of cell lineage trees with very high precisions that can predict the existence of this small population of stem cells. This information is required for cancer researches (Macaulay et al.,2014; Shapiro et al.,2013). For understanding the intricate cellular interplay involved in immune response that require single cell resolution, especially with rare antigen specific T or B cell, single cell approaches have been utilized (Shalek et al.,2013; Georgiou et al.,2014). Now a days, the researchers have started to mix single cell genomics with proteomics to address the often-arriving questions on various areas of medical sciences such as cancer, neuroscience, stem cell biology, developmental biology and infectious disease. Heterogeneity in cell populations, knowledge of complex cellular studies will escalate interest among researchers to diagnose and treat disease with ease.

3. Single Cell Isolation Methods in Plants

For analyzing the cell's unique molecular identity, it is necessary to study the single cell and therefore we need to effectively isolate them. There are different methods discovered for cell isolation. The manual isolation of cells using micropipette or micromanipulation is very time-consuming technique and it does not permit broad studies of cellular heterogeneity. However, this technique is only performed for the biopsy of the daughter cell from a single cell division (Macaulay et al.,2015). Analyzing of small panels of protein / marker in individual single cell is performed by using flowcytometry. For the functional and molecular profiling of

heterogenous cell population, the fluorescent activated cell sorting (FACS) based isolation has been performed.

Single cell isolation requires to isolate the cell of interest which is not a trivial task (Gawad et al., 2016). For isolation of single cell, the development and standardization of best practices is still under process (Eberwine et al.,2014). The first isolation step includes the maceration or the removal of cell wall, allowing the exposure of individual cell within a suspension (Gawad et al., 2016). Plant cells usually have a rigid cell wall which can complicate isolation technique as compared to animal cells (Efroni et al.,2016). For isolation of single cell from potato leaves and apple, enzymatic hydrolysis was used to indicate that pectinase is a crucial enzyme for cell isolation. However long time enzymolysis of a cell may damage the activity and completeness of the cell. Later many studies improved this method for, instance, to obtain protoplast from wheat leaf cellular digestion technique was used (Jia et al.,2016).

Several approaches are used for single cell isolation after obtaining a suspension, among which micromanipulation (Shapiro et al.,2013) serial dilution (Ham& R.G.,1965), fluorescence activated cell sorting (FACS) (Spangrude et al.,1988), optical tweezers (Landry et al.,2013) are important. For isolating a single cell in a single well, serial dilution method is the simplest approach as because, cells are serially diluted to approximately one cell per microliter. However, this approach has rarely been used in recent single cell study because it has very low accuracy of serial dilution. For isolating single cell such as early embryo, micromanipulation technique is exploited as it is a very simple and cheap method (Wang et al.,2017). However, micromanipulation technique has high misidentification rate and it is very low conventional and time-consuming technique (Shapiro et al.,2013; Wang et al.,2017). For isolating individual cells based in size, granularity, fluorescence of cells using FACS technique is commonly used (Lindstrom et al.,2010). Companies like BD Biosciences (San Jose, CA, USA) and Beckman Coulter (Brea, CA, USA) has made FACS commercially available (Navin et al.,2011). FACS may yield low abundance cell sub population as because it requires large number of cells in suspension (thousands of cells). Additionally, the cell might be damaged during FACS due to rapid flow (Shapiro et al.,2013). Optical tweezer is an alternative technique that uses a highly focused major beam to capture cell (Shapiro et al.,2013). Optical tweezers can isolate cells in succession or a cell array inside a microfluidic device with the assistance of imaging-based selection (Saliba et al.,2014).

Techniques such as laser microdissection (LMD), and laser microdissection pressure catapulting (LMPC), and laser captured microdissection (LCM) (Emmert-Buck et al.,1996) are used to extract single cell on *in-situ* based cellular morphology. It is used in addition to suspension-based isolation method (Wang et al.,2017; Misra et al.,2014). However, low throughput, additional splicing of cell during sectioning, UV damage to nuclei, and contamination from neighboring cells are some of the major drawbacks that has to be overcome (Wang et al.,2017; Navin et al.,2014). Another commonly used single cell isolation method is magnetic activated cell sorting method (MACS). It is a technique that is column based and can isolate cell using antibody, enzyme, or lectins to bind specific cell surface proteins (Hu et al.,2016). However, the separation magnet, the column, the antibody is cost very high and the specific sensitivity to positively and negatively charged cell population makes it uses far more limited than FACS (Hu et al.,2016).

More recently, microfluidic technology has been discovered that has shown to be a parallel, accurate, high throughput, and sensitive single cell isolation technique (Whitesides &G.M,2006). However, while using this commercial microfluidic platform (Chen et al.,2016) costly proprietary reagents are needed to complete the isolation technique. The microfluid technology limiting the applicability for cell samples with varying size because microfluidic platform requires uniform cell cycle (Wang et al.,2015). Currently for isolating the animal cell microfluidic is only being used, but is expected that in the near future it will be applied in plant cells.

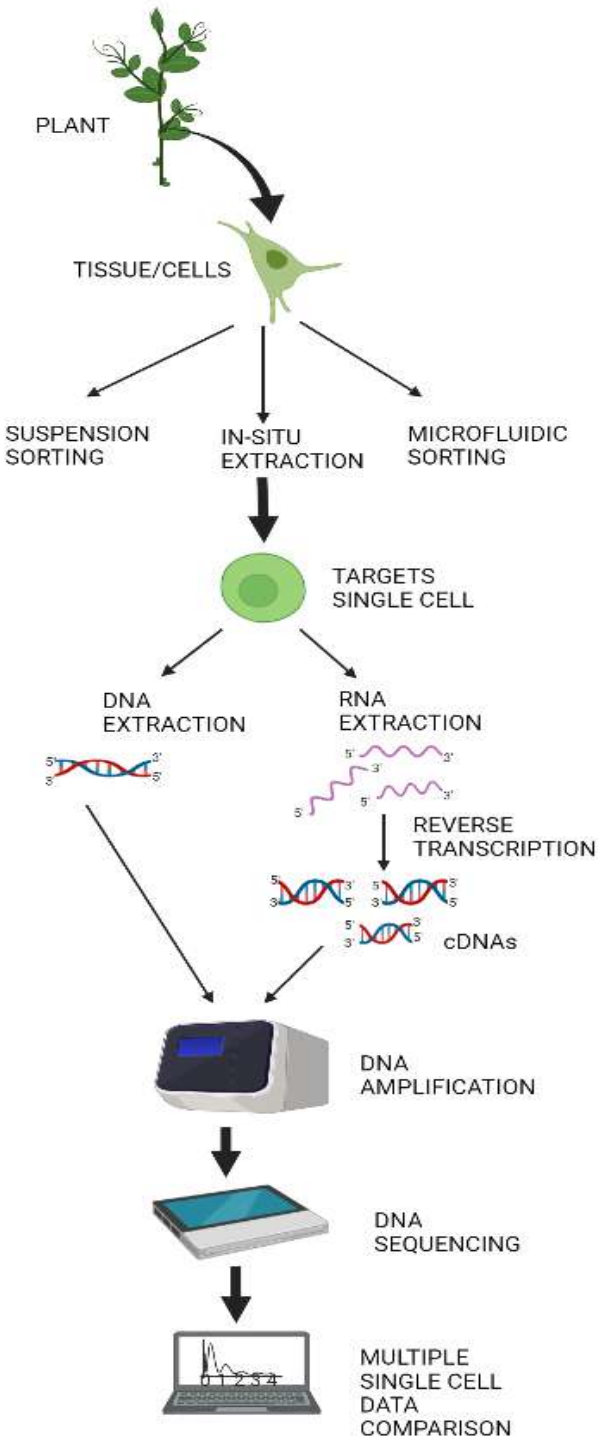

FIGURE 1: An outline of plant single cell isolation and its genome analysis. First targeted cells are isolated in a suspension, extracted mechanically in-situ, or sorted by microfluidics. DNA or RNA is extracted. RNA is reverse transcribed to single stranded or double stranded cDNA. Next, DNA or cDNA are amplified. Libraries are prepared for genomic DNA or cDNA and next generation sequencing is performed. Bioinformatics analysis is performed for comparing single cell sequences and finding functional variants between cells.

Approach	Isolation techniques	Accuracy	Cell material requirement	Challenges	References
Suspension	Serial dilution	Very low accuracy	High	These techniques are very time consuming, contain low accuracy and requires large number of cells due to high mis identification rate and that may affect the yield of cell sub population and the cells may damage.	Ham,1965; Shapiro et al.,2013; Scangrude et al.,1988
	Micromanipulation	Moderate accuracy	Low		
	Fluorescence-activated cell sorting	High accuracy	High		
In-situ	Laser microdissection (LND)	Moderate	High	The cells can be sliced accidentally, the cell nuclei can be damaged by UV rays and contamination may occur from neighbouring cells.	Emnert-Buck et al.,1996
	Laser microdissection and pressure catapulty (LMPC)	Moderate	High		
	Laser captured micro dissection (LCM)	Moderate	High		
Microfluidics	microfluidics	High	Moderate to high	This technique costs very high and need all uniform cell sizes.	Whitesides, 2006

Table 1. Comparison of various single-cell isolation techniques.

4. Single Cell Omics Technologies

Recent technological advances have provided unprecedented opportunities to analyze the complex activities a functional cell ranging from genomics, transcriptomics, proteomics and metabolomics.

4.1. Single Cell Genomics in Plants

The genome of an organism is immortal. The genome is relatively stable throughout the life and remains the same independent of any cell types with exceptions of post-meiotic germ cells, vertebrate immune cells, and in pathological conditions such as cancer (Mincarelli et al.,2018).

Genomic heterogeneity between the individual cells of the same organism can be occur at different levels, including single nucleotide variants (SNVs), indels, inter and intra chromosomal fusions, copy number variants (CNVs) and aneuploidies of whole chromosomes as well as insertion sites of viruses and mobile elements. Therefore, each of these phenomena can be studied specifically with higher resolution by sequencing the genome of a single cell instead of sequencing a massive DNA specimen.

The process of single cell DNA is more adverse than single cell RNA sequencing. It is because of the much-required error prone nature of the DNA amplification step (Yuan et al.,2018). A single mammalian cell consists of less than 10 picogram (pg) of DNA (Hu et al.,2016), and plant cells may have 0.1pg to 120 pg. of DNA, including flowering plants with a low modal weight of 0.6 picograms of DNA (Gregory &T.R.,2005). Single cell DNA sequencing requires DNA amplification because of the fact that sequencing generally requires over 200 nanograms of DNA and low input protocol needs 500 picogram to 0 nanograms of DNA. Several challenges are there in amplifying DNA such as allelic dropout, nonuniform coverage, and false positive mutations (Wang et al.,2015). These technical challenges affect the DNA sequencing products and hinder downstream analyses which overall complicates the discovery of real biological variations (Yuan et al.,2018). To get over these problems, several approaches has been proposed for DNA amplification.

PCR based methods like linker-adapter PCR (LA-PCR) (Sugimoto et al.,2011), primer extension preamplification PCR (PEP-PCR) (Birnbaum & K.D,2016), interspersed repetitive sequence PCR (IRS-PCR) (McCarthy et al.,1995) and degenerate oligonucleotide PCR (DO-PCR) (Hossain et al.,2015) were primarily considered for single cell DNA (scDNA) amplification. These approaches have several drawbacks like the low genome coverage

(~10%), limited production, several biases in amplification, and allelic dropout (Wang et al.,2015). Later, multiple displacement amplification (MDA) (Dean et al.,2002) was discovered and extensively used in DNA amplification. The function of MDA is simple, and it generates a higher genome coverage (>90%) with lower false positive rate (~10^7) (Wang et al.,2015). There is another method named multiple annealing and looping based amplification cycle (MALBAC) (Zong et al.,2012)) which increase the uniformity of coverage and lowers the allelic dropout. As a result, the allelic dropout using MALBAC is reduced to ~1% leading to amplification of approximately 93% genome coverage (Zong et al.,2012). However further improvement is needed on the high false positive rates of MALBAC. MALBAC is specifically self for CNV and single nucleotide variant (SNV) detection. There is another amplification method named microwell displacement amplification system (MIDAS) (Gole et al.,2013). It uses a massive parallel polymerase cloning method for reducing amplification bias and reduce nonuniform coverage (Yalcin et al.,2016). This method lowers down reaction volume up to1000-fold, compared to MDA.

4.2. Single Cell Transcriptomics in Plants

Previous studies have provided insight into the distribution of gene expression levels across cells using population sample. However, it is difficult to quantify gene expression in individual cell because of the bulk cells used in RNA sequencing. Single cell RNA sequencing can shade light on variability on gene expressions across cells and this is proved through studies. Whole transcriptome amplification (WTA) is required, as because the RNA material in a single cell is insufficient for single cell RNA sequencing. WTA is less challenging because of the presence of multiple transcript copies that reduces the dropout rate, as compared to whole genome amplification (WGA). Numerous technologies have been developed to improve WTA, in recent years. The throughput, sensitivity, accuracy, and precision have improved through WTA method, although the challenges of additional noise and amplification bias remains present (Kalisky et al.,2017).

mRNA must be reverse transcribed into cDNA before WTA, to characterize the transcriptome of a single cell. To analyse expression from single cell cDNA microarray were applied. In prior to the use of next generation sequencing (NGS). This method could miss many rare but key transcripts however, this method was less sensitive (Brady et al.,1993). In 2010 Tang et al. (Tang et al.,2010) improved the WTA method and used NGS to detect genes and splice junction in one cell for overcoming the limitations of microarray. In their method, before

PCR amplification oligo deoxy thymine primer (DT) with anchor sequences were used for mRNA reverse transcription. Mainly due to the limited length of the cDNAs this method could generate 3‘end mRNA bias (Liang et al.,2014). A WTA method named SMART-seq was developed to alleviate this situation (Zhu et al.,2001). By using Moloney murine leukaemia virus (MNLG) to perform reverse transcription, and SMART-seq generated and amplified full length cDNA from single cell. However, the low sensitivity of SMART-seq prompted development of the improved SMART-sequence to approach (Picelli et al.,2013). Researchers can detect gene expression differences in multiple samples at the exchange of a strong 5‘end bias that enabled by SMART-seq2.

Several *in vitro* transcription (IVT) methods were discovered in recent time that include cell expression by linear amplification sequencing (Cel-seq) (Hashimshoni et al.,2012). IVT reduces amplification bias, compared to exponential amplification method such as PCR, called linear amplification and is the main benefit of IVT (Shapiro et al.,2013). However, the bias towards the 3‘end makes it difficult to control, which prevents the detection of the full spectrum of transcript variants (Shapiro et al.,2013). Unit molecular identifier (UMIs) are used in single cell WTA, to mitigate this bias (Islam et al.,2014). UMIs can be prevented for quantitative single cell RNA (scRNA) sequencing with absolute molecule count. Droplet based RNA-ser technologies have been discovered in recent time that include the commercial chromium system platform (10x genomics, Pleasanton, TA, US). Droplet based RNA-seq technology can help to differentiate the cell of origin of each mRNA molecule that help to study single cell in complex tissues. The analysis of thousands of different cells in parallel generates low level of noise (Wang et al.,2017).

Techniques	Platform	Number of cells	Description of the methods	Applications	Typical number of sequencing reads per cell	References
Smart-seq/smart-seq2	Microwell plate/ tube/ fluidigm C1 platform	100s - 1000s	It is a PCR based full length transcript amplification, templates can be switched and can be applied to cells or nuclei	Alternative splicing can be analysed by this process, the number of transcripts can be counted and allelic expression may be studied	500000-4000000	Picelli et al.,2013 Picelli et al.,2014
CEL-seq/CEL-seq2	Microwell plate/tube	100s-1000s	3 times transcript amplification method and this is based in-vitro	Numerical analysis of transcripts	100000-1000000	Hashimsoni et al.,2012 Hashimsoni et al.,2016
STRT	Microwell plate / tube	100s-1000s	The template can be switched and it is a PCR based full length transcript amplification, followed by 5‘selection	Numerical analysis of transcripts	100000-1000000	Islam et al.,2011; Hochgerner et al.,2017
Sci-RNA	Combinatorial indexing	1000s-10000s	Combinatorial indexing technique is a technique in which during sauce strand synthesis and during PCR of 3‘sequencing tag the transcripts are first indexed	Numerical analysis of transcripts	20000-200000	Cao et al.,2017
Droplet-based approaches	Microfluidic platform: Drop-seq InDrop Commercial platforms: 10x genomics Chromium Dolomite Nadia	1000s-10000s	Individual droplets are formed when the cells are partitioned and during reverse transcription cDNA molecules are uniquely barcoded	Numerical analysis of transcripts	20000-200000	Macosko et al.,2015; Klein et al.,2015
Nanowell approaches	Custom nanowell chip: SeqWell Commercial platfroms: Nanogrid (ICell8) BD Rhapsody	1000s-10000s	The cells are partitioned into individual wells of a custom build nanowell chip and during reverse transcription cDNA molecules are unique	Numerical analysis of transcripts		Gierahn et al.,2017

Table 2. Various methods for transcriptomic analysis in single cell.

4.3. Single Cell Proteomics in Plants

The functional identity of a cell largely depends on its proteome. The study of proteome is done through proteins and post translational modification that the cell can sense and respond accordingly to all extrinsic and intrinsic stimuli through virtual system. The most detailed definition of cell type and state is possible by molecular means and this can be achieved by a comprehensive overview of a cell's proteome. The sensitivity required for proteome wide screening although cannot provide either antibody or mass spectrometric base detection or quantification of proteins. The potential of proteomic analysis is beginning to show the advances in antibody levelling and detections, microfluidics, and recently sensitive mass spectrometry approaches, for validation of single cell RNA-seq results an important tool is used that is the protein level detection.

For detection of protein from single cell western blotting approaches has been developed (Sinkala et al.,2015; Kang et al.,2016; Kang et al.,2014., Hughes et al.,2014; Dunkombe et al.,2016). This approach may offer a unique specificity which additionally allows protein isoform and modification detection. This approach affect enable capture lighting and electrophoresis of ~3000 individual cell in parallel, uses micropatterned polyacrylamide array, that is followed by cross linking to immobilize the protein by using primary and secondary antibody. Detection of ten protein in the same single cell was demonstrated by stripping and reprobing the gel. To enable parallel analyses of protein expression in the nucleus and cytoplasm of the same cell, advances in this method of subcellular fractionation of single cell (Yamauchi &Herr,2017).

For several decades multiplex analysis of single cell protein expression has been performed by FACS analysis. FACS analysis includes the process of detection of immunofluorescent~~ly~~ tagged protein in thousands of single cells, analysing up to 50 parameters in parallel with the help of modern high parameter instruments. Due to the potential of overlapping between fluorescent spectra and these high parameter applications are likely to represent the higher limit of the capability of FACS which is however technically challenging. There is a technique known as CyTOF approach which is a variant of FACS, in which the antibodies are labelled with heavy metal ion tags rather than fluorophore. The abundance of metal ions labelling the cell is read out by using a technique that is known as time-of-flight mass spectrometry (Palma & Bodenmiller,2015). High levels of multiplexing (greater than 40 proteins in parallel) are

possible because these instruments having over 100 of nonoverlapping detection channel. In fixed and permeabilised cell including phosphorylation event, both FACS and CyTOF have potential to measure extracellular and intracellular parameter. The Abseq method has been shown DNA barcoded antibody in parallel with a custom microfluidic platform that can be used to detect surface protein abundance in single cell with high yield and that has a theoretically unlimited capacity for multiplexing (Shahi et al.,2017).

Antibody based method always relies on the specificity and availability of the antibody. Due to the limitations on the sensitivity of the mass spectrometry techniques, unbiased proteomic analysis of single cell is very challenging process. The method in which paramagnetic beads are used to enrich proteins or peptides from low input samples, including single human oocytes, generating input material for liquid chromatography coupled to tandem mass spectrometry (IC-MSMS) is known as phase enhanced sample preparation (Sp3) method (Virant-Klun et al.,2016; Hughes et al.,2014).

For high multiplexed measurements of proteins from single cells there is another class of microfluidic techniques. Quantification up to 40 nucleus, and secreted protein across thousands of single cells can be concurrently enabled by a technique that is known as microchip-based proteomics analysis which a sensitivity of as low as a few100 protein copies per cell (Yang et al.,2016). These tools allow measurement of secreted protein from viable cells and offer control over the cell's micro environment before analysis in contrast to the CyTOF that allows function of screens to be performed (Wei et al.,2013).

4.4. Single Cell Metabolomics in Plants

The full collection of all low molecular weight metabolites that are produced by a cell could be a key indicator of cell state reflecting the precise metabolic activity and condition within the cell. Largely due to the diversity and rapid dynamics of the system that lacks tagging and/or amplification approaches for small molecules metabolome is challenging to measure at the single cell level (Zenobi, 2013). The measurement of metabolites in real time in single cell has been made possible by the advancement of optical tools (such as genetically encoded optical nanosensored) together with improved expression system and *in vivo* imaging (Zenobi,2013; Barros et al.,2013).

The empowerment of single cell mass spectrometry has provided an advancement to the metabolomic investigation to the size of individual cells and subcellular structure. Metabolite's quantification has been performed individually on isolated neuron by using single cell capillary electrophoresis coupled to the electrospray ionization time-of-flight MS (Nemes et al.,2013). Application of microarrays for mass spectrometry platform and analytical validation of a single cell metabolite helps to monitor cellular responses upon environmental and genetic perturbation (Ibanez et al.,2013).

Further technical advances will lead to more complete coverage of metabolome from the significance of cell metabolism in the definition of cell state and function resulting in accurate and fast metabolite identification and non-destructive measurement of single cell.

5. Application of Single-Cell Omics in Plants

Specific cell types such as root hair (Kwasniewski et al.,2010; Lan et al.,2013; Libault et al.,2010) trichomes (Hulskamp, M,2004) and cotton fibres (Haigler et al.,2009) served as early single cell type models due to their easy isolation methods. These single cell type models increase the way of our understanding in cellular processes and differentiation of plant roots shoot epidermal hairs and cell wall, compared to bulk cell study. For example, gene expression profile during secondary wall cellulose synthesis in cotton fibre resembled sclerenchyma cells (Haigher et al.,2009; Betancur et al.,2010), despite being morphologically recognised as leaf trichome. Another example is transcriptomes of root hairs single cell isolated from soybean only contains 25% of the transcription factors in whole root transcriptome studies (Libault et al.,2010).

Differentiated somatic plant cells can be provoked to from embryos in culture (Nabors, M.W,2004), as the plant cells show high developmental flexibility. Whether plant cell fate regulation is a lineage dependent mechanism remains still unclear, as in animals (Kragl et al.,2009), or based on cell relative position (Kidner et al.,2000) or a mixed of both (Yu et al.,2017). For mapping of individual cell stage from initial to differentiated, single cell analysis can be used. In animal and human studies (Woodworth et al.,2017) protocols for single cell lineage tracing were established and could be adopted for using plant analysis. By replaying the patterns of embryogenesis *Arabidopsis* root showed that multiple cell type could rapidly reconstitute stem cells (Efroni et al.,2016) which could be analysed by recent single cell studies. For the identification of critical genes in regeneration of single cell, transcriptomics can be applied, which can further be trapped as marker for developmental studies.

In both disease resistance and trait improvement for crop breeding, stress tolerance of plants has always been of great interest. For understanding stress signalling in plant, bulk extraction of tissue material is required in *Arabidopsis* (Coolen et al.,2016; Rasmussen et al.,2013; Zeller et al.,2009). It is also used for detection of markers such as nucleotide polymorphism in soybean flowering (Xia et al.,2012) and copy number variations (CNVs) in rice grain size (Wang et al.,2015). Novel insights into stress adaption in plants, particularly for modelling gene regulatory network has been highly benefitted due to advancement of single cell sequencing. Though the interactions between hormone signalling pathways are poorly understood, plant hormones are the key mediators of the stress response (Nguyen et al.,2016). The interaction between hormones directly manipulates tissue formation and patterning using single cell information (Efroni et al.,2016). The technology can be directly applied to signalling network in stress responses dissecting the conflicting evidence of ethylene during high salinity stress at different developmental stages (Tao et al.,2015), moreover ethylene-jasmonate-abscisic acid exchange (Cheng et al.,2009; Ghassemian et al.,2000; Song et al.,2014). Novel regulatory processes can also be detected by single cell analysis. The identification of new rhizobial infectious genes and novel processes in *Medicago* root hairs which was previously undetected in bulk-cell whole -root studies is a good example to it (Breakspear et al.,2014). Evidences of regulation of stress response were also (Staiger et al.,2013) studied in isoforms of resistance gene regulation against tobacco mosaic virus (Dinesh & Baker, 2000) and temperature induced stress in *Arabidopsis* results in alternative splicing (Filichkin et al.,2010). As gene isoforms are found to be allocated to different cell types (Laval et al.,2002), prior to development and stimuli, single cell analysis can mark and track alternative transcripts.

Single cell analysis application has the potential to locate unknown cell types through deconvolution of heterogenous cell population by identifying biological variations between adjacent cell state. The development of single cell analysis is helps to collect physiologically-based markers and provide~~s~~ a foundation of cell type marking in future.

5.1. Applications of Single-Cell Technology in Plant Research

The single cell technology has a number of exciting potential applications, although the examples of plant single-cell analysis are relatively uncommon. Genotyping, developmental studies and cell typing using single cell approaches with animal and human samples are ~~all~~ highly relevant. These approaches are extensively used for the analysis of biomolecule

synthesis and interactions (Efroni et al.,2017). Due to the presence of a cell wall, and lack of suitable protocols for rapid tissue dissociation, single cell analysis is a major challenge. Without enzymatic treatment few cell types in multicellular plants were readily dissociated. The removal of the cell wall through enzymatic or mechanical cell wall digestion creates stress on the cell protoplasts thereby negative repercussions of gene expression is very common. Studies of cell identity in *Arabidopsis thaliana* have been successfully performed and single cell transcriptome amplification approaches are compatible with protoplast amplification (Efroni et al.,2015).

In pollen typing, single cell genome sequencing has immediate and highly beneficial application, in basic molecular genetics and agricultural breeding. Chromatids recombine during the meiotic cycle that results in genetic differences in the daughter cell. The frequency of segregation of different alleles in pollen grains determines the genetic diversity and distribution of beneficial traits (e.g., crop yield) to the offspring plants. Currently, studies of plant population genomics are required in large numbers within offspring plants using low-throughput cytological assessment of the pollen grains and conventional breeding. These plants often have long generational time, as for example, wheat can take up to 9 months to mature in the field, making the process slow and costly.

To haplotype the parental chromosomal contribution and understand factors regulating the frequency of crossing over sequencing the genomes of single pollen grains may be a possible way out. Pollen typing is highly efficient than FACS, where only one plant is needed. Quantitative- trait loci (QTL) association or mapping usually requires thousands of replicates (Chen et al.,2008). In order to assess the number and location of recombination sites along the length of each chromosome Drtudied that the majority of the sites are located at the distal ends despite the “peri-centromeric” regions in barley pollen (Dreissig et al.,2017). To link the whole genome sequencing with other “omics” data such as those from the transcriptome, proteome single cell multi omics approaches are used, and these approaches may further enable researchers to understand the biology of plant meiosis and pollen formation.

5.2. Data Repository for Plant Cells

Protocols used in whole genome amplification (WGA) and whole transcriptome amplification (WTA) at the moment are diverse, which gradually evolved in the study of single cell (Chen et al.,2016). The data extraction and compilation algorithms are developed and both

are different from each other. The amount of genome and transcriptome data generated possess a potential challenge for data storage and sharing and this can be determined by single cell studies. Data repositories are required and should be able to categorise each data format and make data reusable, comparable and sharable. It efficiently documents each single cell experiment. Proper data management and noble algorithm are needed to achieve this, that ensure users track experimental parameters and allow upload and download plant single cell data.

Data repository such as National Centre for Biotechnology Information (NCBI), provide a good data storage and management software. NCBI, in spite of providing a similar service for single cell sequencing data it has missed the importance and demand for experimental meta data such as molecular information. Comprehensive data repository for single cell are expected in the near future. The established sequence format FASTQ or the alignment map format are similar to standardise experimental data format and that are also needed to make the study of single cell more robust.

6. Conclusion

Over the last 5 years, the single cell analysis methods help in identifying single cells at cellular level. It gives novel solutions to study the cell which play important roles in system behaviour, tissue development, regeneration, and repair. Currently, implementation at large scale multi-centre studies of whole organism biology such as Human cell atlas (Regev et al.,2017), and detailed studies on distinct biological cell types and states, these techniques are more specific, thus becoming routine tool in cellular genomics. Continued application and adaptation of these techniques will draw further impact on these methods in plants and microbial researches.

The methods described in this review has limitations too, particularly in selecting the analyte of interest specifically needed in base level events (SNVs or individual base modification). Advances in molecular biology and microfluidics may solve some of these issues and computational approaches are being applied for finding of missing data (Lin et al.,2017). Techniques for high-throughput single cell splice variant analysis will gradually evolve and be further integrated with genomic, epigenomic and proteomic data of a single cell with the increase in sequencing capacity both in terms of yield and read length. Although there are few challenges in single cell preparation, DNA/RNA amplification, DNA sequencing, and

bioinformatics analysis can conglomerate for rapid evolution of single-cell technologies. It is expected that this technology will play an important role in feeding the world by generating high-yielding and stress-tolerant elite cultivars.

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Physiological and biochemical changes in plants in response to arsenic stress:

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Abbreviations:

ADP	=	adenosine di-phosphate
APX	=	ascorbate peroxidase
AsIII & AsV	=	Arsenite & arsenate
ATP	=	adenosine triphosphate
BDP	=	Bengal delta plains
CAT	=	Catalase
DMA	=	dimethylarsine
ER	=	endoplasmic reticulum
ETS	=	electron transport system
FR	=	free radical
GADPH	=	gleceraldehyde-3-phosphate dehydrogenase
GOGAT	=	glutamate synthase
GR	=	glutathione reductase
GS	=	glutamine synthetase
GSH	=	Glutathione
MMA	=	Monomethylarsine
NADP+	=	nicotinamide adenine dinucleotide phosphate
NO	=	nitric oxide
NTP	=	nodulin 26-like intrinsic protein
Pi	=	inorganic phosphate
PCs	=	phytochelatins
PDH	=	pyruvate dehydrogenase
PHT	=	phosphate transporters
ROS	=	reactive oxygen species
RuBisCO	=	ribulose-1,5-bisphosphate carboxylase/oxygenase
SOD	=	superoxide dismutase
TMA	=	Trimethylarsine

Abstract:

Living organisms are exposed to various biotic and abiotic stresses. These stress factors affect organisms at various levels. The growth, development and reproduction are inhibited by the stress factors. Among various stress factors, metal stress is one of the greatest issues nowadays. Plants and animals exposed to various heavy metals show inhibition in their normal physiological activities.

The contamination of arsenic (As) in soil and its toxic affect to plants and animals is one the burning topics at present situation. India, Bangladesh and few parts of China are the most affected countries due to arsenic.As may contaminate either naturally (volcanic eruption, flood etc.) or by human interruptions. In West Bengal, the major Ascontamination occurs through natural processes, such as, flooding in rice field. Through soil they can enter the plant body, as well as, the food chains and harm various organisms. As may enter the plant body in inorganic forms or organic forms. Inorganic forms are generally more toxic than the organic forms.

Within plant body, As affect various biochemical and physiological activities. As can alter photosynthesis, decrease glucose yield, affect respiration and causes lipid peroxidation. It alsoadversely affectsvarious enzymes, proteins, cell membrane, as well as generates Reactive Oxygen Species (ROS) at molecular level.

Plant system has developed several mechanisms to decrease the toxicity of As. These detoxifying processes help to copearsenic stress condition as well as decrease the arsenic accumulation within the plant body, thus checking its entry into the food chain.

This review paper is primarily focused on the contamination of arsenic in soil, its uptake by the plant body, various toxic effects due to arsenic in plants and also the different arsenic detoxifying processes in plants.

Introduction:

Organisms face a lot of chemical and physical stresses during their growth and development. Those stress factors may vary in time and geographical regions. Development of organisms depends on their internal factors as well as external factors. External factors may include light intensity and quality, pH of soil, water, air flow, salinity, presence of metals in soil etc. Fluctuations of these environmental factors affect plants and animals creating abiotic stress conditions. As a result, organisms change their normal physiological activities. On the other hand, biotic stress may include attack by pathogens, such as – fungi, bacteria etc.

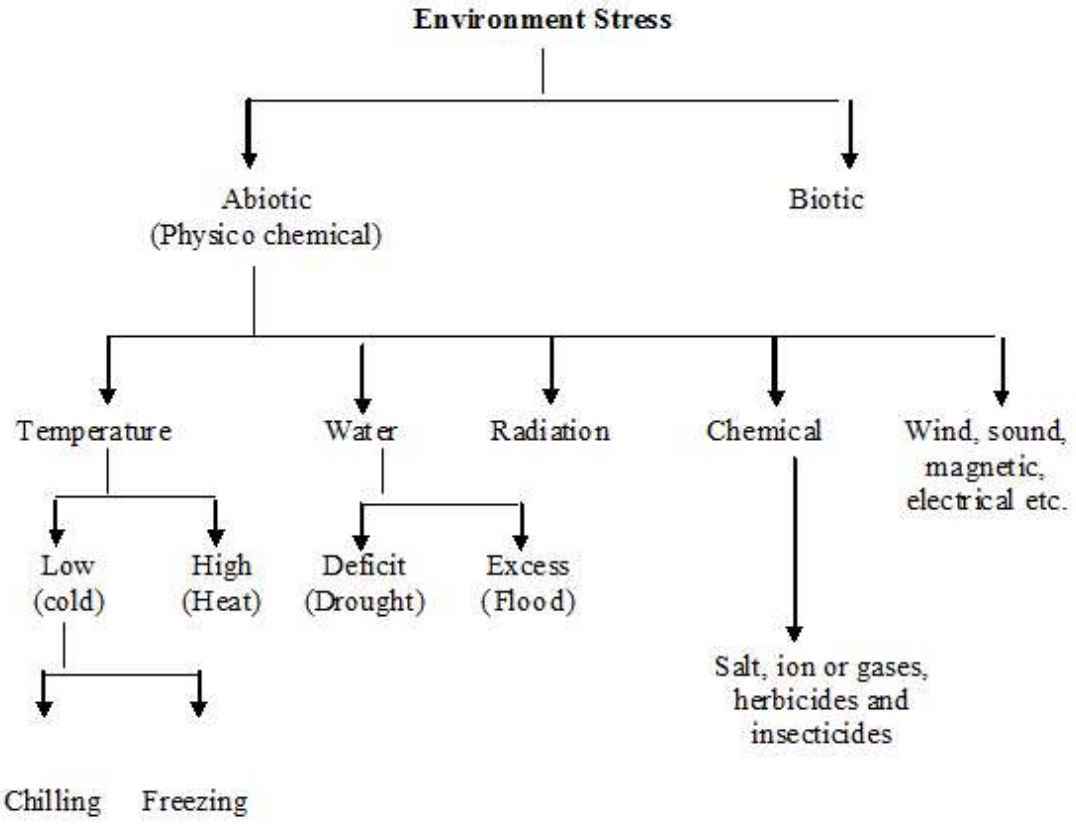

Fig1: Types of stresses (source: Ghosh and Mukherjee, 2017)

Plants can generate various activities through which they can overcome stress conditions by changing their morphology and physiology in response to a particular environment.

Sometimes plants do not require any genetic modifications to response those stresses. These kinds of responses are referred to as phenotypic plasticity. (Taiz and Zeiger, 2010)

Metal stress in Plants:

Amongst all abiotic stresses,heavy metal stress is one of the most dangerous. Most plants exposed to high concentrations of heavy metals,show inhibited growth and reproduction. Sensitive plants also exhibit visible symptoms, such as chlorosis, necrosis etc. in response to metal toxicity. Some plants can accumulate heavy metals such as arsenic (As), lead (Pb), nickel (Ni), cobalt (Co), iron (Fe), chromium (Cr) etc. within their body. Heavy metals may have severe effects on crop productivity. (Gill, 2014).

Theheavy metals mainly react with the active sites of biomolecules, such as some proteins or enzymes which are needed for plant metabolic activities. This causes deactivation of such biomolecules affecting the normal plant physiological activity. Heavy metals influence the production of Reactive Oxygen Species (ROS) and Free Radicals (FR) within plant body by metal mediated inhibition machinery. Excess production of ROS and FR within the plant cell may cause oxidative stress in plants. It happens when the detoxification of such elements can't compete with the production of those harmful compounds. Heavy metal tolerant plants are able to avoid the occurrence of heavy metals in cellular compartment, thus avoiding the generation of oxidative stress. (Dietz et al.,1999)

Arsenic is a highly toxic metals for plants as well as for animals including human. As is included into non-essential element for plants and As-toxicity has become a global issue due to its increasing concentration in water and soil.

Characteristics of Arsenic:

Arsenic is ubiquitous in most of the environments. In soil, it mainly exists in two forms – arsenite (AsIII) and arsenate (AsV). Arsenate is dominant under oxidized condition. The forms of arsenic are very much similar to phosphorus (P).

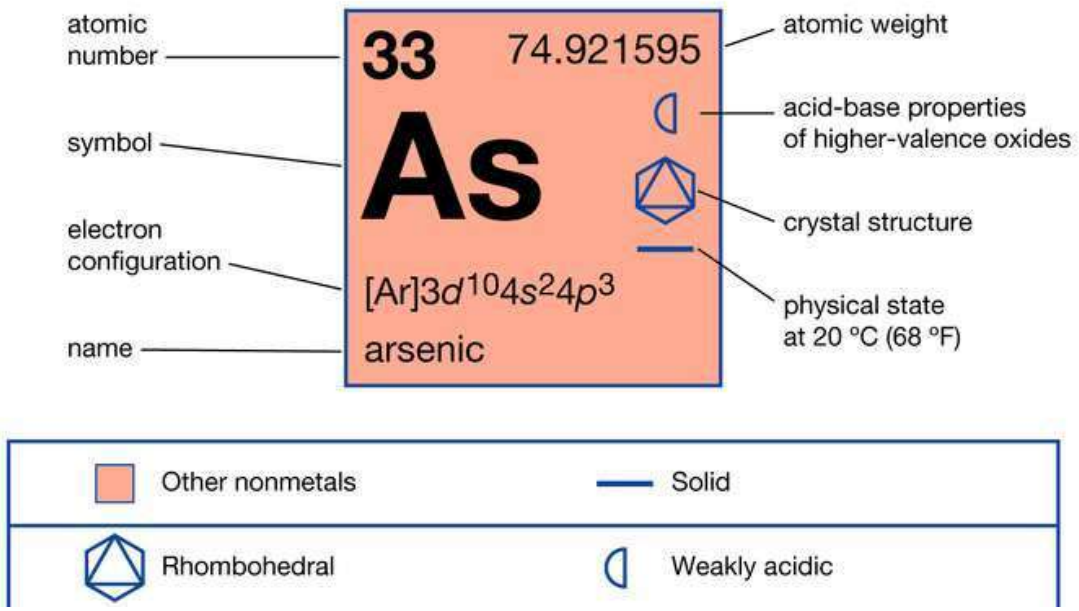

Fig2: Encyclopædia Britannica, Inc.

Arsenic toxicity:

As contaminates drinking water and can enter the food chain, causing various symptoms such as skin lesions, cancers etc. It has been reported that in some areas of India and Bangladesh, the arsenic concentration in ground water has been increasing for several years. Rice field is the major area of arsenic toxicity. Though there are several physical and chemicals strategies to protect that of crop plants from arsenic toxicity. Arsenic hyperaccumulating ferns or some aquatic plants can also be used against arsenic poisoning in crop fields (Dixit et al.,2015).

Arsenic contamination in water and soil:

Many districts in West Bengal and some parts of Bangladesh are currently facing issues with crop productivity due to arsenic contamination in crop fields.Arsenic contaminates ground water and soil either by natural processes or human activity. Naturally arsenic may be introduced to the environment by natural emissions. Anthropologically arsenic may contaminate due to fossil fuel combustion or mining processes.

Fe(III) present in the surface of Bengal Delta Plains (BDP) sediments adsorb Fe(II) due to iron oxide reduction, resulting in the reduction of As(V) to labile As(III) which may contaminate ground water and eventually contaminates with the soil.(Shrivastava et al.,2015).

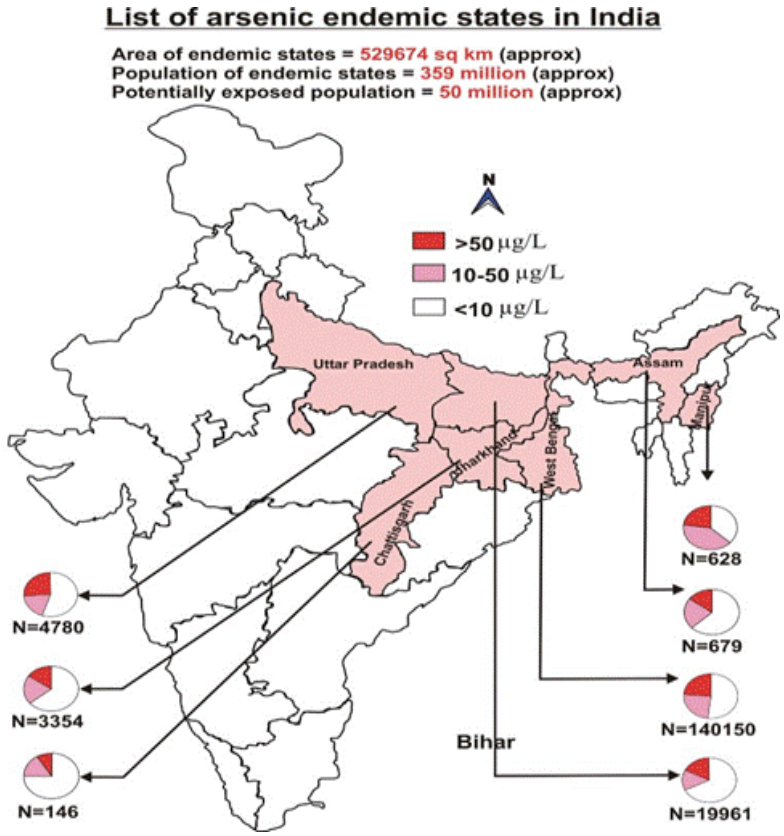

Fig3:Arsenic affected areas in differentstates in India(Ghosh et al., 2010)

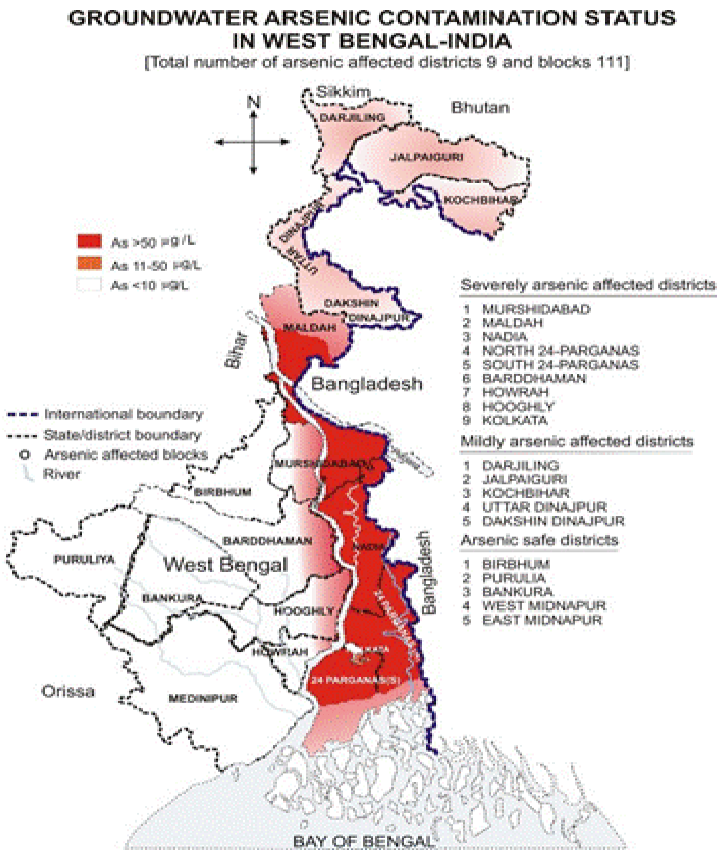

Fig4: Arsenic affected areas in different districts of West Bengal (Ghosh et al., 2010)

Forms of Arsenic:

As is present in the soil in organic and inorganic forms. Organic forms of arsenic are present in living organisms due to arsenic consumption. Inorganic forms of arsenic enter the food chain and get methylated to form less toxic organic forms, such as – monomethylarsine (MMA), dimethylarsine (DMA), trimethylarsine (TMA). Among which MMA and DMA are more toxic than TMA.

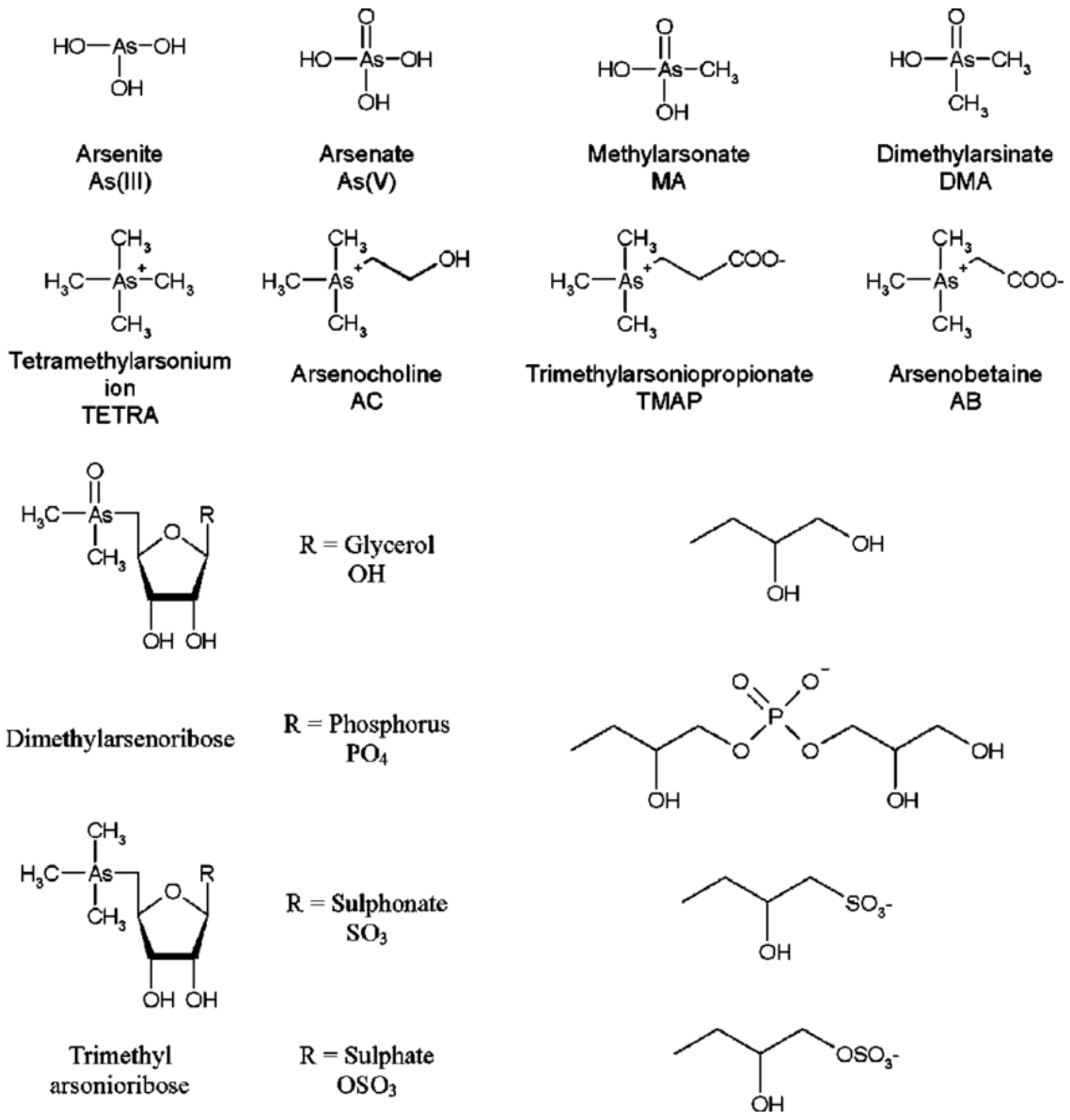

Fig5:different forms of arsenic (Foster, 2008)

On the other hand, inorganic forms of arsenic, mainly in forms of minerals are more toxic than the organic form and also dominant in soil. There are more than 300 inorganic forms of arsenic including arsenates, arsenides, arsenites, metal alloys etc. Among which As(V) and As(III) forms are most abundant in soil. As(V) is less toxic then As(III) and present in soil in immobile form. But As(III) form is more toxic and can enter the living cells.

Arsine is arsenic containing very toxic gas which is produced in highly reducing environment. In low pH arsine is formed in marshy or swampy soil along with MMA, DMA and TMA (Shrivastava et al.,2015).

Table 1. Major mineral forms of Arsenic (Shrivastava et al., 2015):

Mineral group	Mineral name	Formula
Oxide of arsenite	Arsenolite	As_2O_3
	Claudetite	As_2O_3
Oxide of arsenate	Arsenic pentoxide	As_2O_5
Fe-arsenate	Arseniosiderite	$Ca_2Fe_3O_2(AsO_4)_3 \cdot 3H_2O$
	Parasymplesite	$Fe_3(AsO_4)_2 \cdot 8H_2O$
	Pharmacosiderite	$K[Fe_4(OH)_4(AsO_4)_3] \cdot 6.5H_2O$
	Scorodite	$FeAsO_4 \cdot 2H_2O$
	Symplesite	$Fe_3(AsO_4)_2 \cdot 8H_2O$
	Yukonite	$Ca_7Fe_{12}(AsO_4)_{10}(OH)_{20} \cdot 15H_2O$
Fe sulfoarsenates	Beudantite	$PbFe_3(AsO_4)(SO_4)(OH)_6$
	Tooeleite	$Fe_6(AsO_4)_4(SO_4)(OH)_4 \cdot 4H_2O$
	Zýkaite	$Fe_4(AsO_4)_3(SO_4)(OH) \cdot 15H_2O$
Ca-Mg arsenates	Hörnesite	$Mg_3(AsO_4)_2 \cdot 8H_2O$
	Pharmacolite	$Ca(HAsO_4) \cdot 2H_2O$
Other metal arsenates	Annabergite	$Ni_3(AsO_4)_2 \cdot 8H_2O$
	Erythrite	$Co_3(AsO_4)_2 \cdot 8H_2O$
	Köttigite	$Zn_3(AsO_4)_2 \cdot 8H_2O$
	Mimetite	$Pb_5(AsO_4)_3Cl$

Translocation of Arsenic in Plants:

According to Gulz et al.,2005, a very low concentration of arsenic in plants (less than 1.0mg per Kg dry weight of plants) may not have much negative effects on plants. Basically, arsenic uptake by plants, mainly depends on the concentration gradient between the source and sink. Initially plants accumulate arsenic in their roots (after uptake from soil), and finally transport it to the shoot. As(V) is analogous to Phosphate (P), and mainly enters through phosphate channels. There are several protein co-transporters that are related to Pi transport in plants. Some of them are high affinity Pi co-transporters and others are low affinity Pi co-transporters. An example of one the important Pi/As(V)co-transporters is Phosphate Transporters (PHT). PHT are mainly unidirectional co-transporters. Deficiency of Pi or presence of As(V) in soil leads to the transport of arsenic into the plants via PHT1 protein.

Beside PHT, there are another protein transporters which is related to As(III) uptake. These proteins are nodulin-26-like intrinsic proteins (NTPs). These are bi-directional transporters. So that the As(III) can transport in both directions from soil to plant cell or vice-versa depending on the As(III) concentration between the source and sink.It has been also reported that, sometimes As(III) moves through silicon transporters in Si deficiency conditions (Abbas et al.,2018).

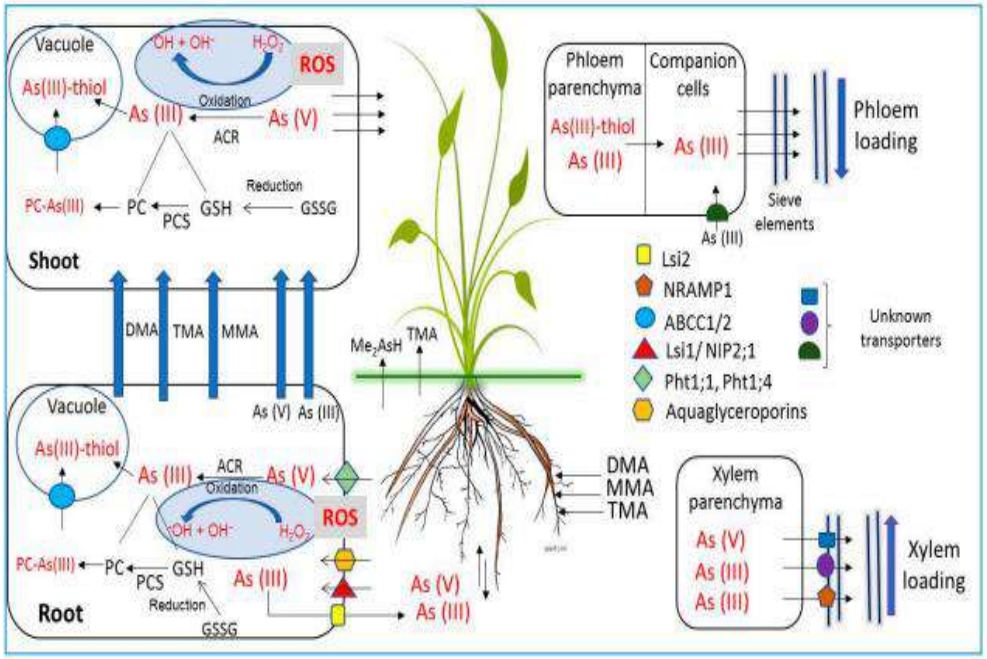

Fig6: uptake and translocation of Arsenic in plants (Abbas et al.,2018)

Physiological and biochemical effects of Arsenic in Plants:

Arsenic contamination in soil poses serious threats to the plants. Various physiological and biochemical abnormality in plant due to arsenic toxicity has been reported.

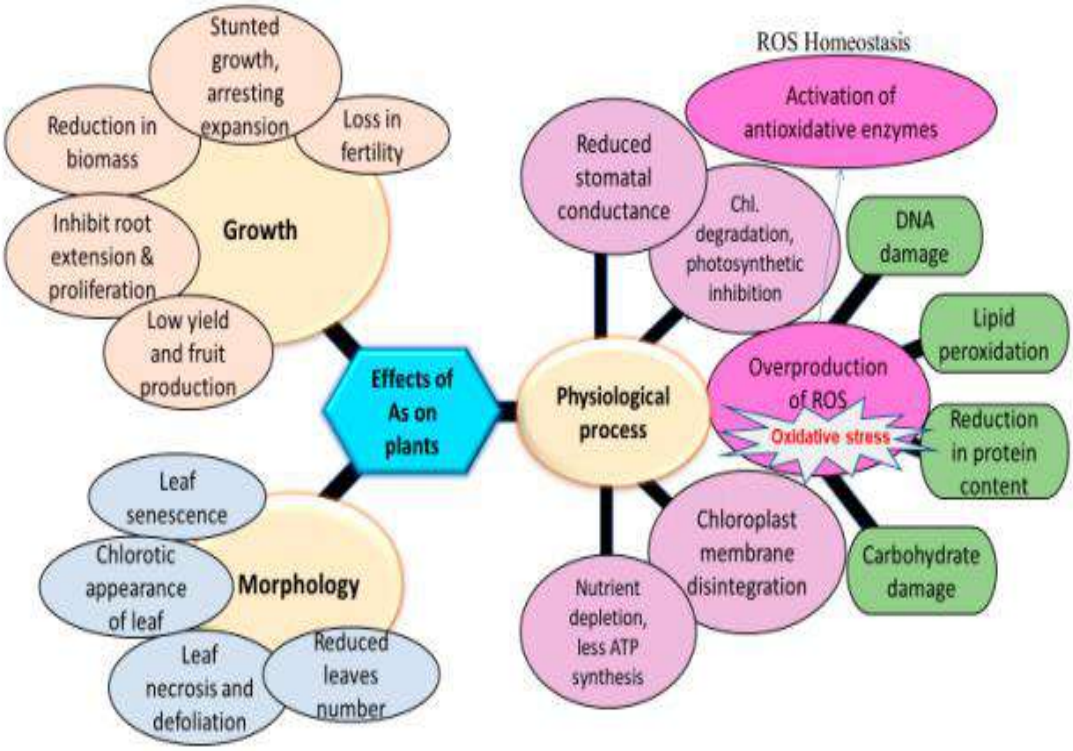

Fig7: physiological and biochemical effect of Arsenic in plants (Abbas et al., 2018)

- **Effect of Arsenic in Photosynthesis:**Arsenic pollution in soil has gained an attention due to industrial activity. It competes with phosphate ions and enters plants through phosphate transporters.It has been reported that the light harvesting complex in photosynthesis can be affected due to arsenic toxicity.Photosystem II, thereby chlorophyll a is affected more than photosystem I. The reduction in chlorophyll content has also been reported due to the decreased activity of the enzymes involved in chlorophyll biosynthesis. As arsenic level increases in plants, it also causes injuries in the chloroplast-membrane.High level of arsenic disrupts membrane integrity, resulting in ROS generation which disrupts the structures of several proteins and enzymes, involved in photosynthesis, making them functionally disabled. Ahsan et al., 2010 reported down-regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) enzyme complex's large subunit under arsenic stress (Srivastava et al., 2012). Arsenic toxicity negatively affects gas exchange and

fluorescence mechanisms during photosynthesis. In some plants, such as, *Zea mays*, *Trifolium pratense* L. and *Lactuca sativa*, high arsenic concentration affects the biosynthesis pathways resulting in the wilting and decolouration of leaves, decrease in leaf and root growth and decrease in biomass accumulation. (Srivastava et al., 2012; Stoeva et al., 2003). During photorespiration, AsIII inhibits the activity oflipoamide dehydrogenase (catalyses the transfer of an electron from dihydrolipoamide co-factor to NAD^+) (Finnegan et al., 2012).

- **Effect of Arsenic in ATP synthesis:** AsV accumulation, being analogous to Pi, can affect various Pi dependent metabolic pathways. When AsV comes in contact with the cells of plant root, a competition takes place between inorganic phosphate (Pi) and AsV for uptake. Thus, arsenic toxic is lower under high phosphate concentration in media. Once within the plant body, AsV moves across the mitochondrial inner membrane and plastid's inner membrane via Pi transporters and dicarboxylate carriers. One of the most important Pi dependent metabolic pathway is ATP synthesis. As(V) can compete with Pi, forming As(V)-ADP complex, that lowering ATP synthesis during photophosphorylation and oxidative phosphorylation. The Km and Vmax of the enzymes are quite similar for both AsV and Pi indicating that the enzymes for ATP synthesis, are able to recognize AsV and Pi equally. In some cases, mitochondrial enzymes are also can react with both As(V) and Pi equally. In this way As(V) can also disrupt these enzymes negatively causing ATP decrease in cells. (Finnegan et al., 2012).

- **Effect of Arsenic in Carbohydrate metabolism:** The effect of arsenic in sugar metabolism is largely unknown. The proteins associated with carbon metabolism are largely unaffected under arsenic stress. But there are some changes in the abundance of proteins or enzymes associated with glycolysis and citric acid cycle. Under arsenic stress condition, the interconversions of sugars are affected. Arsenic stress causes the

high accumulation of soluble sugars.Furthermore, there is inhibition of the activity of starch degrading enzymes.Normally starch degrades into glucose, maltose and malto-oligosaccharides. Arsenic decreases liberation of maltose. This particular reaction requires ATP, but in this case AsV substitutes for Pi affecting the whole reaction. Glucose-1-AsV is produced and immediately hydrolysed to glucose. Ultimately this leads to shortage in energetic yield for glycolysis.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an important enzyme in both glycolysis and photosynthesis. GAPDH protein can utilize both Pi and AsV as its substrate. Under high AsV concentration GAPDH acts as AsV-reductase. As a result, 3-arsenoglycerate rapidly hydrolyses, uncoupling the GAPDH reaction. In this way ATP yield is lowered in high As concentrations. Similar effects occursin pyruvate and 2-oxoglutarate metabolism. Major enzymes, malate dehydrogenase and ATP synthase as well as FAD subunit lose their activity owing to AsV toxicity.
AsV inhibits the enter of pyruvate in the citric acid cycle converting the wholeprocess tofermentation. In many AsVaffected plants, there is increased amount of transcription of alcohol dehydrogenase. These all cases have a negative impact on glycolysis with decrease in the net yield in ATP. (Finnegan et al., 2012)

- **Effect of Arsenic in Nitrogen metabolism:** Arsenic toxicity negativelyeffects biological nitrogen fixation through symbiotic association. In AsIII contaminated soil, the degree of nitrogen fixation normally decreases owing to the disruption of legume plant root system, such as, root necrosis or root hair damage due to arsenic toxicity. As a result, the infections by the *Rhizobium*bacteria as well as number of root nodules decrease. AsIII exposer also inhibits the transcriptions of nodule genes (Lafuente et al.,2010). Thus,the net amount of biological nitrogen fixation is affected.

  AsV disrupts the nitrogen assimilation in plants. Non-legume plants generally uptake nitrogen in form of nitrate or ammonium ions. Within plant body the ammonium ion is immediately assimilated into amino acids by the combined effect of two enzymes, named glutamine synthetase (GS) and glutamate synthase (GOGAT). AsV directly

disrupts the transporter of ammonium ions and also affect the transcription of that particular transporter gene. As a result, the whole process of nitrogen assimilation gets disrupted. Later amino acid biosynthesis is also affected. The abundance of proteins in plant readily decreases following protein degradation (Finnegan et al., 2012).

- **Arsenic induced Oxidative stress:** Arsenic is known to be highly toxic metalloid. Inorganic arsenic can generate oxidative stress in plant body by directly producing Reactive Oxygen Species (ROS) or indirectly by inhibiting the antioxidant enzymes, such as, superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR), through binding with the thiol groups of the enzymes. Oxidative stress is a dynamic condition of a cell, when these antioxidants become unable to overcome the increasing levels of oxidants. ROS is mainly produced due to the conversion of AsV to AsIII within plant body.

  ROS are chemically very reactive, oxygen containing compounds having impaired electron at their valence shell (also known as free radicals). In mitrochondria and in plastids, the electron transport chain (ETS) is the main pathway where ROS can be generated. But also produced in endoplasmic reticulum, peroxisome, cell membrane, cell wall, where electron transport process takes place. ROS causes imbalance in cellular homeostasis. On the other hand, ROS also act as secondary messenger and they create abnormality in normal cellular physiology.

  Some studies also have shown that, Dimethylarsine reacts with oxygen, producing DMA radicals and superoxide anions increasing the levels of ROS in plant body. High level of Monomethyle arsenous acid causes the production of ROS mainly in mitochondira and on the other hand, exposure to Dimethyle arsenous acid causes the production of ROS mainly in other organelles. These ROS have negative effect in DNA and RNA structures. AsIII mediated ROS also affect the enzymes or proteins which are rich in cysteine effecting the -SH groups causing structural changes in enzymes and proteins.

Table: Types of reactive oxygen species (ROS) with sites of their origin on the subcellular and biochemical level other than chloroplasts and mitochondria (Kostecka-Gugała and Latowski, 2018)

Organelle	Type of ROS	Process of ROS production
Peroxisomes	$O_2^{\bullet-}$	Fatty acid chain β-oxidation
	H_2O_2	Glycolate oxidase reaction during photorespiration
		Enzymatic reaction of flavin oxidases
		Dismutation of $O_2^{\bullet-}$ by catalase
		Xanthine and hypoxanthine oxidation to uric acid in peroxisome matrix
		Reactions in peroxisomal membranes
Endoplasmic reticulum	$O_2^{\bullet-}$	NAD(P)H-dependent electron transport involving cyt P_{450}; an organic substrate (RH) reacts with cyt P_{450} and then is reduced by a flavoprotein to the radical intermediate (cyt P_{450} R^-). In the reaction with oxygen, it forms cyt P_{450} ROO^- which can be reduced by cyt *b* or occasionally release $O_2^{\bullet-}$ during decomposition
Plasma membranes	$O_2^{\bullet-}$	Electron transfer from NAD(P)H to oxygen, forming $O_2^{\bullet-}$ by NAD(P)H oxidase (NOX). This enzyme is proposed to play a major role in the production of ROS in plants under stress conditions. NOX is also considered a key factor in the immune system of plants
Cell wall	H_2O_2	NADH oxidation catalyzed by a cell wall peroxidase (NADH–POD)
		Reduction of diamines or polyamines catalyzed by diamine oxidases
		Oxidation of oxalates by oxalate oxidase

ROS inhibits the interaction of lipoic acid (cofactor) with pyruvate dehydrogenase (PDH) as well as synthesis of acetyl-CoA, that participates in many physiological and biochemical pathways. Decrease in acetyl-CoA synthesis leads to the decrease of zeaxanthin synthesis, an important xanthophyll compound having photoprotective function. Excessive ROS production causes oxidative damages of carotenoids (the

accessory pigments) leading to. damage of chlorophyll molecules. Endoplasmic reticulum (ER) is an important site of ROS production. Various processes, such as, transcriptionand protein folding also occur at ER and are affected by ROS action (Kostecka-Gugała and Latowski,2018).

Strategies to overcome Arsenic stress:

Arsenic pollution is one of the most potent problems, which is growing day by day.There are several plant species, which are able to accumulate and tolerate high levels of arsenic in their body. Some plant species have evolved certain physiological adaptation through with they can tolerate high concentration of arsenic, and are known as arsenic hyperaccumulators or tolerants. Arsenic hyperaccumulator or tolerant plants can be used in a new technique, known as Phytoremediation.

Phytoremediation: It's a very cost-effective, eco-friendly green approach to clean up the high concentration of arsenic from soil and ground water. This technology uses certain plant species that can accumulate and tolerate high levels of arsenic without showing any toxicity.

The entry of arsenic by roots depends on the soil type. Arsenic can enter the plant body in two forms- AsV and AsIII. After entering the root of the plantbody, one of two mechanisms may occur. In arsenic tolerant or non-hyperaccumulating plant, volatilization of arsenic occurs. This reduces the translocation of arsenic from root to shoot. This mechanism involves the production of less toxic organic forms of arsenic, such as, AsIII-glutathione/phytochelatin complexes in the root cells and transported to the vacuoles, preventing the translocation of arsenic to the shoot.

A second type of mechanism may occur in arsenic hyperaccumulating plants. In those plant species, AsV is reduced to AsIII in root cells. This AsIII is the predominant form of arsenic that can translocate with xylem sap from root to shoot. The high level of capacity to load a large amount of arsenic is an important feature of arsenic hyperaccumulating plants. There are also several transporter proteins that are to

translocate arsenic from root to shoot. The reduction of AsV to AsIII is catalysed by an enzyme, named Arsenic Reductase (AR). It has been suggested that the activity of AR is much higher in arsenic hyperaccumulators than arsenic sensitive plants. In arsenic hyperaccumulator plants, AsIII forms complexes with glutathione (GSH) and phytochelatins (PCs) and enters the vacuoles in shoot. AsIII mainly attach with the thiol groups of GSH and PCs during arsenic detoxification. Glutathiones are the precursors of PCs. Phytochelatins play an important role in arsenic detoxification. Phytochelatins are metal-binding thiols of low molecular weight, consisting of amino acids- glutamate, cysteine and glycine. These are very effective in arsenic hyperaccumulating plants (Souri et al., 2017).

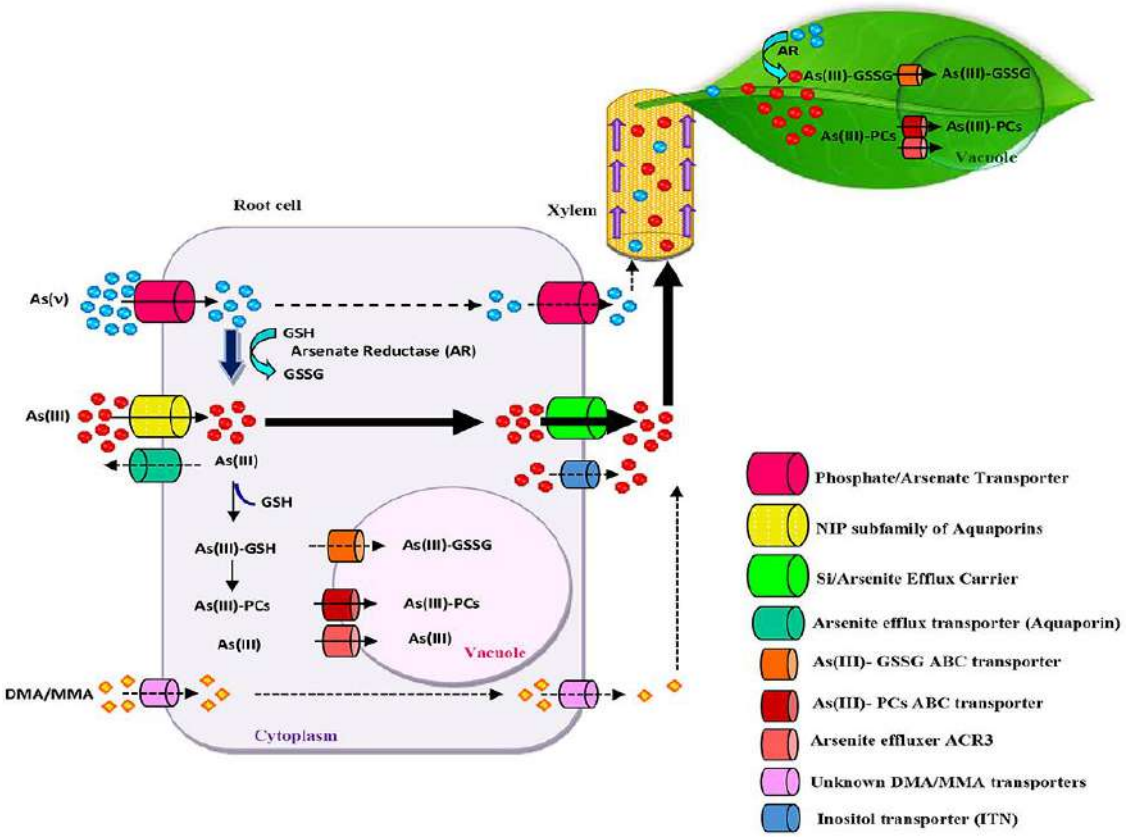

Fig8: Overview of Arsenic (As) uptake, transport, translocation, and detoxification in plants. (Souri et al., 2017).

Antioxidant enzymes: Interconversion of AsV to AsIII within plant body causes oxidative damages by producing ROS. Meanwhile to overcome arsenic toxicity, the activity of some antioxidant enzymes, such as, SOD, CAT, GR, and APX increases. These enzymes help in balancing the free radicals of ROS. Generally, the antioxidant enzymes act as electron donor and react with ROS to form non-toxic end products.

Among all, SOD, a type of metalloenzyme, lies in the front line of defence mechanism of arsenic induced ROS poisoning. SOD mainly reacts with superoxide radicals, converting them into oxygen and hydrogen peroxide. SOD is mainly located in the cell organelles which are connected with generating ROS. There are three forms of SOD, found in plants- Cu/Zn-SOD, Mn-SOD, and Fe-SOD. All forms are encoded by nucleolar genome. The Mn-SOD is mainly localized in mitochondria, Fe-SOD in chloroplast, and Cu/Zn-SOD is mainly localized in cytosol, chloroplast, peroxisome, and mitochondria.

CAT helps in scavenging the hydrogen peroxide level in peroxisome, producing water without any electron donor under arsenic stress condition. The activity of CAT depends on the strength of the stress condition. Many reports suggest the presence of CAT in mitochondria, chloroplast as well as in cytoplasm also.

GR is an enzyme that helps to maintain the GSH/GSSG ratio by NADPH-dependant reduction of oxidised glutathione (GSH). GR has a very conserved di-sulfide bond between Cys76 and Cys81. Oxidative damage enhances the activity of GR by breaking that particular bond. Soon GR reduces GSH level by converting them into PCs that can fight against arsenic toxicity.

APX also plays an important role in detoxification of hydrogen peroxide. This enzyme uses two molecules of ascorbate and reduces hydrogen peroxide, producing water molecule. Studies have suggested that APX has greater activity under arsenic stress (Tripathi and Tripathi, 2019; Abbas et al.,2018).

Non-enzymatic antioxidants (Ascorbate, Polyamines and Phenols): Ascorbate and dehydroascorbate are low molecular weight anti-oxidants that helps to scavenge the free radicals of ROS. Polyamines are a type of organic compounds that have more than two amino groups. They are also found to protect the plant against harmful effect of ROS

during arsenic stress. Phenolic molecules also show their ability to protect the plant during arsenic stress. (Tripathi and Tripathi, 2019).

Amino acids: Various essential and non-essential are found to protect the plants during arsenic stress. Amino acids (the building blocks of proteins) act as chelating agent of various metals, including arsenic. The major essential amino acid includes valine, leucine, methionine and alanine and the major non-essential amino acid includes proline, histidine, cysteine, glutamic acid that have protective role against arsenic stress in plantbody. Cysteine is one of the important amino acids that help the plant to tolerate arsenic stress. It's the major compound that is used in the synthesis of GSHs and PCs which later helps in arsenic detoxification.

Proline is an important amino acid that can act as osmo-protectant during stress condition. It helps to maintain the structures of DNA, cell membrane, various proteins. It can maintain the minimum hydration level of cell membrane, required for its normal activity. Proline can also scavenge singlet oxygen and hydroxyl molecules during arsenic mediated ROS production. It is also involved in the synthesis of PCs and helps in the increase in chlorophyll quantity. Various studies have suggested the over accumulation of proline during arsenic stress within plant body for osmotic adjustment of cell. Proline can reduce the arsenic toxicity through various way –

- It can reduce the arsenic uptake by changing the cell wall's structure.
- It can increase the activity of several anti-oxidants that can scavenge the free radicals.
- Proline directly quenches ROS compounds.
- It also acts as signalling molecule and change the expression of several genes to protect the plant against arsenic toxicity (Tripathi and Tripathi, 2019; Abbas et al.,2018).

Carbohydrates: Arsenic toxicity inhibits the activity of starch degrading enzymes, such as, alpha and beta amylase. Due to this the amount of non-reducing sugar (sucrose) in the plant body decreases. During this stress, two enzymes- acid invertase and sucrose synthase play an important role. Both of the enzymes are able to degrade sucrose and

produce glucose and fructose. As a result, an increase in hexose level in plants occurs (Tripathi and Tripathi, 2019).

Nitric oxide: Nitric oxide (NO) is referred to as reactive nitrogen species. It has both harmful and useful effect depending on their level and localization within the plant body. Nitric oxide is involved in various physiological effects during normal and stressed condition. NO is a gaseous free radical and also acts as signalling molecule.In cell, NO can act in various ways-

- It can act as signalling molecule and can change various gene expressions under stressed conditions.
- NO also activates some anti-oxidant enzymes that are involved in arsenic detoxification.
- NO inhibits lipid peroxidation under arsenic stress condition.
- It can react with superoxide radicals and form peroxynitrite, which is less toxic than hydrogen peroxide.
- NO also reacts with -OH radicals and form nitrous acid to protect the plant body against the harmful effect of -OH radical(Tripathi and Tripathi, 2019; Abbas et al.,2018).

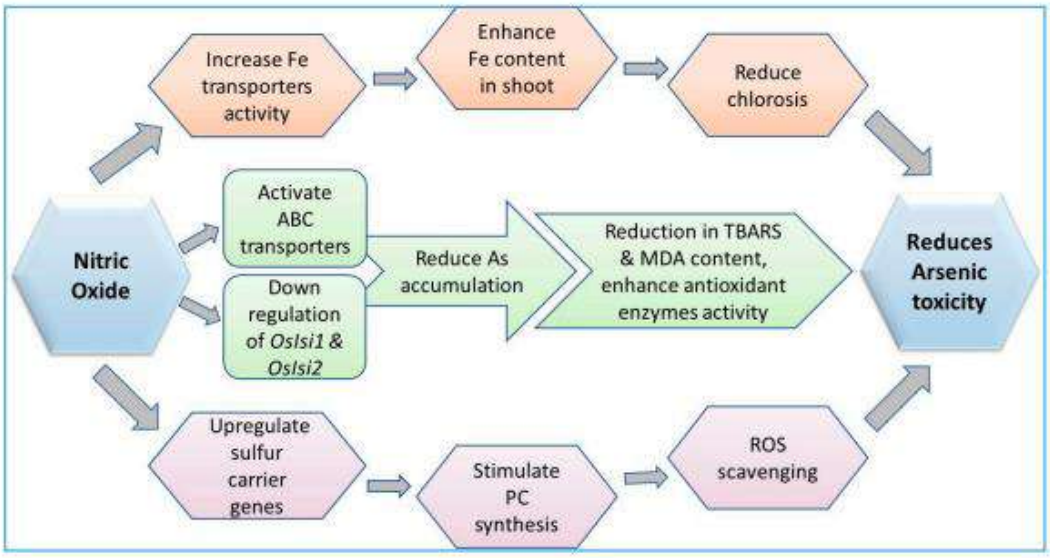

Fig9: role of NO against arsenic stress (Abbas et al., 2018)

Growth hormones: Various phytohormones show their protective role against arsenic stress. Auxin, Cytokinin, Gibberellin, Abscisic acid, Ethylene, Brassinosteroids, Strigolactone, Jasmonates are the major plant hormones.

There are several auxin transporters in plant cell, such as PIN1, PIN2, AUX1 proteins. The mutants of these proteins show arsenic sensitivity than the wild types. Thus, inhibitors of auxin transporters cause decrease in arsenic tolerance in wild type plants. Studies have shown that treatment with indole-3-acetic acid increases arsenic tolerance in mutants.

Brassinosteroidsare also known to have protective role against arsenic stress. Studies suggested possibly Br24 and Br28 inhibits arsenic accumulation, thus protect the cell from the damages caused by arsenic (Tripathi and Tripathi, 2019).

Salicylic acid: Salicylic acid is an important phenolic plant growth regulator that can act as signalling molecule and participates in various physiological processes, such as, photosynthesis, flowering, nitrate metabolism, ethylene production etc. Salicylic acid also has a protective role against abiotic metal stress, such as arsenic stress. Studies have suggested that salicylic acid enhances the production of NO in rice plants. NO is involved in arsenic detoxification. Salicylic acid can remove various oxidative damages caused by ROS. As salicylic acid can enhance the activities of various anti-oxidants, such as, SOD, APX and provide them tolerance against arsenic. Pre-treatment with salicylic acid also lowers the uptake of arsenic. It also helps in the increase in proline concentration in cells. Studies found that salicylic acid increases the photosynthetic pigment synthesis, thus it enhances the photosynthesis process. It also involved in the inhibiting the lipid peroxidation due to arsenic stress. Thus, salicylic acid is a major compound that acts in various way to protect the plant from arsenictoxicity (Tripathi and Tripathi, 2019; Abbas et al., 2018)

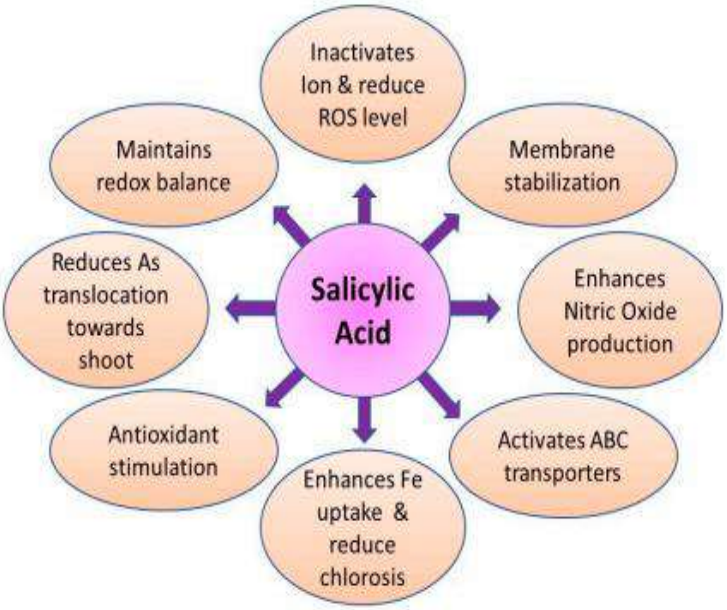

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Fig10: role of salicylic acid against arsenic stress (Abbas et al., 2018)

Thus, in conclusion, it can be stated thatthe different forms of arsenic can affect the plants in various way. From atmosphere, arsenic enters the plant body and act at molecular and biochemical levels. Arsenic inhibits the growth, development and reproduction of plants. On the other hand, arsenic enters the food chains and also causes diseases in animals, as well as in humans.

The various arsenic detoxification processes that have generated by the plants which are exposed to arsenic stress. These detoxifying processes may not stop the uptake of arsenic from soil, but can reduce its harmful effect. These detoxification processes also reduce the concentration of arsenic within the plant body. So that, a lower amount of arsenic can enter the food chain, thereby lowering arsenic related diseases in animals and humans.

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