TWO-YEAR
POST GRADUATE DEGREE PROGRAMME (CBCS)
IN
BOTANY

SEMESTER - IV

Course: BOTDSE T403.2

Molecular Genetics, Advanced Cell Biology, Molecular Breeding & Plant Tissue Culture
(COURSE – II)

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI – 741235, WEST BENGAL

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Director's Messeage

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distane Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personal involved in the process to overcome the challenges inherent to curriculam design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distane Learning (DODL) University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC-DEB Regulations, 2020 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of SLMs, making them useful to the lesrners, besides avoiding errors as far as practicable. Further, suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from **Professor (Dr.) Amalendu Bhunia**, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticisms to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every Members of PGBOS (DODL), University of Kalyani, Heartfelt thanks is also due to the Course Writers- faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learners friendly, flexible text that meets curriculum requirements of the Post Graduate Programme through distance mode.

All the Self Learning Materials are self-writing and collected from e-Book, journals and websites.

Director
Directorate of Open & Distance Learning
University of Kalyani

SYLLABUS

COURSE – BOTDSE T403.2

Molecular Genetics, Advanced Cell Biology, Molecular Breeding & Plant Tissue Culture

(Course – II)

(Full Marks – 80)

Course	Group	Details Contents Structure		Study hour
BOTDSE T403.2	**Molecular Genetics, Advanced Cell Biology, Molecular Breeding & Plant Tissue Culture**	**Unit 1. Genetic Systems and Breeding Methods – I**	1. Genetic Systems and Breeding Methods: Selection and breeding strategies for self pollinated plants.	1
		Unit 2. Genetic Systems and Breeding Methods – II	2. Genetic Systems and Breeding Methods: Selection and breeding strategies for cross-pollinated and clonally propagated plants.	1
		Unit 3. Continuous Variation and its Significance	3. Continuous Variation and its Significance: Qualitative traits and discrete variation, Quantitative trait and continuous variation, Polygenes and polygenic inheritance.	1
		Unit 4. Self-Incompatibility – I	4. Self-Incompatibility: Basic concept, Genetic basis of self-incompatibility.	1
		Unit 5. Self-Incompatibility – II	5. Self-Incompatibility: Molecular basis of self-incompatibility, Methods to overcome self-incompatibility in plants.	1
		Unit 6. Male Sterility – I	6. Male Sterility: Overview; Types of male sterility; Mechanisms, Transgenic male sterility.	1
		Unit 7. Male Sterility – II	7. Male Sterility: Overview; Maintenance of male sterile line, Induction of male sterility, utilization in crop improvement.	1
		Unit 8. Heterosis – I	8. Heterosis: Concept, Types of heterosis, genetic basis of heterosis.	1
		Unit 9: Heterosis – II	9. Heterosis: Molecular basis of heterosis and inbreeding, utilization in crop improvement.	1

Course	Group	Details Contents Structure		Study hour
BOTDSE T403.2	**Molecular Genetics, Advanced Cell Biology, Molecular Breeding & Plant Tissue Culture**	**Unit 10. Molecular Markers – I**	10. Molecular Markers: Development of molecular markers; trends and progress, RFLP, PCR based.	1
		Unit 11. Molecular Markers – II	11. Molecular Markers: Single locus and multi-locus markers, NGS based markers; Applications in crop improvement. .	1
		Unit 12. Genetic Maps – I	12. Genetic Maps: Construction of linkage maps, high-density maps.	1
		Unit 13. Genetic Maps – II	13. Genetic Maps: QTL mapping.	1
		Unit 14. Genetic Maps – III	14. Genetic Maps: association mapping, integration of genetic maps with physical maps/chromosomes.	1
		Unit 15. Molecular Breeding – I	15. Molecular Breeding: Gene tagging, Marker Assisted Selection (MAS).	1
		Unit 16. Molecular Breeding – II	16. Molecular Breeding: Bulk Segregation Analysis (BSA), genomic selection, genome-wide association study (GWAS).	1
		Unit 17. Mutation Breeding – I	17. Mutation Breeding: Utility and accomplishment of induced mutations. Management of M1 generations.	1
		Unit 18: Mutation Breeding – II	18. Mutation Breeding: Management of M2 generations, Factors influencing the mutation spectrum and the quality of mutants.	1
		Unit 19. Breeding for Disease Resistance – I	19. Breeding for Disease Resistance: Pathogenicity vs. Virulence, Physiological races and differential hosts.	1

Course	Group	Details Contents Structure		Study hour
BOTDSE T403.2	Molecular Genetics, Advanced Cell Biology, Molecular Breeding & Plant Tissue Culture	**Unit 20. Breeding for Disease Resistance – II**	20. Breeding for Disease Resistance: Models for plant pathogen recognition, Flor's hypothesis, Vertical and Horizontal resistance.	1
		Unit 21. Back Cross Method of Breeding	21. Back Cross Method of Breeding: Significance and limitations; multiline concept.	1
		Unit 22. Heritability	22. Heritability: Understanding, Components of phenotypic variance, Broad-sense and narrow sense heritability.	1
		Unit 23. Design of Experiments	23. Design of Experiments: general principles of field trails, randomized blocks, latin square, split plot designs, layout of breeding experiment.	1
		Unit 24. Bioreactors	24. Bioreactors: Concept; Types of bioreactors- batch, continuous, multistage and immobilized cell bioreactors; Application in plant tissue culture.	1
		Unit 25. Organogenesis	25. Organogenesis: Developmental sequences, Mechanism of action of plant hormones, Control of in vitro organogenesis by cyclin-dependent kinase activity.	1
		Unit 26. Somatic Embryogenesis	26. Somatic Embryogenesis: Gene expression and signal transduction during embryogeneis-Role of SERK and LEC genes, Brassinosteroid (BR) signaling, Artificial seeds.	1
		Unit 27: Somatic Hybridization	27. Somatic Hybridization: Protoplast isolation technique, protoplast fusion, selection of hybrid cells- Homokaryons, Heterokaryons, Symmetric and asymmetric hybrids, fate of plasmagenes, Cybrids.	1

Course	Group	Details Contents Structure		Study hour
BOTDSE T403.2	**Molecular Genetics, Advanced Cell Biology, Molecular Breeding & Plant Tissue Culture**	**Unit 28. *In vitro* Genetic Variation**	28. *In vitro* Genetic Variation: Somaclonal and gametoclonal variation, Isolation and characterization of somaclones, Molecular basis of somaclonal variation, Advantages of somaclonal variation over induced mutations, Applications in crop improvement, *In vitro* mutagenesis and mutant selection.	1
		Unit 29. Micropropagation – I	29. Micropropagation: Overview, Stages of micropropagation, Advantages and limitations, Horticultural Uses, Production of virus-free plants, Molecular and immunological techniques of plant virus detection.	1
		Unit 30. Micropropagation – II	30. Micropropagation: Genetic assessment by RAPD, RFLP, ISSR and SSR markers.	1

Content

COURSE – BOTDSE T403.2

Molecular Genetics, Advanced Cell Biology, Molecular Breeding & Plant Tissue Culture (Course – II)

Hard Core Theory Special Paper **Credits = 8**

Content Structure

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1. Introduction
2. Course Objective
3. Genetic Systems and Breeding Methods: Selection and breeding strategies for self pollinated, cross-pollinated and clonally propagated plants.
4. Continuous Variation and its Significance: Qualitative traits and discrete variation, Quantitative trait and continuous variation, Polygenes and polygenic inheritance.
5. Self-Incompatibility: Basic concept, Genetic and molecular basis of self-incompatibility, Methods to overcome self-incompatibility in plants.
6. Male Sterility: Overview; Types of male sterility; Mechanisms, Maintenance of male sterile line, Transgenic male sterility, Induction of male sterility, utilization in crop improvement.
7. Heterosis: Concept, Types of heterosis, genetic and molecular basis of heterosis and inbreeding, utilization in crop improvement.
8. Molecular Markers: Development of molecular markers; trends and progress, RFLP, PCR based, single locus and multi-locus markers, NGS based markers; Applications in crop improvement.
9. Genetic Maps: Construction of linkage maps, high-density maps, QTL mapping, association mapping, integration of genetic maps with physical maps/chromosomes.

10. Molecular Breeding: Gene tagging, Marker Assisted Selection (MAS), Bulk Segregation Analysis (BSA), genomic selection, genome-wide association study (GWAS).
11. Mutation Breeding: Utility and accomplishment of induced mutations. Management of M1 and M2generations, Factors influencing the mutation spectrum and the quality of mutants.
12. Breeding for Disease Resistance: Pathogenicity vs. Virulence, Physiological races and differential hosts, Models for plant pathogen recognition, Flor's hypothesis, Vertical and Horizontal resistance.
13. Back Cross Method of Breeding: Significance and limitations; multiline concept.
14. Heritability: Understanding, Components of phenotypic variance, Broad-sense and narrow sense heritability.
15. Design of Experiments: general principles of field trails, randomized blocks, latin square, split plot designs, layout of breeding experiment.
16. Bioreactors: Concept; Types of bioreactors- batch, continuous, multistage and immobilized call bioreactors; Application in plant tissue culture.
17. Organogenesis: Developmental sequences, Mechanism of action of plant hormones, Control of in vitro organogenesis by cyclin-dependent kinase activity.
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19. Somatic Hybridization: Protoplast isolation technique, protoplast fusion, selection of hybrid cells- Homokaryons, Heterokaryons, Symmetric and asymmetric hybrids, fate of plasmagenes, Cybrids.
20. *In vitro* Genetic Variation: Somaclonal and gametoclonal variation, Isolation and characterization of somaclones, Molecular basis of somaclonal variation, Advantages of somaclonal variation over induced mutations, Applications in crop improvement, In vitro mutagenesis and mutant selection.
21. Micropropagation: Overview, Stages of micropropagation, Advantages and limitations, Horticultural Uses, Production of virus-free plants, Molecular and

immunological techniques of plant virus detection, Genetic assessment by RAPD, RFLP, ISSR and SSR markers.

22. Let's sum up
23. Suggested Readings
24. Assignments

1. Introduction

This relates with plant molecular breeding and plant tissue culture. Plant breeding is an art and science, which tells us ways and means to change the genetic architecture of plants so as to attain a particular objective. Plant breeding can be accomplished through many different techniques ranging from simply selecting plants with desirable characteristics for propagation, to more complex molecular techniques. Here, plants with higher qualities are selected by and crossed to obtain plants with desired quality. This results in a plant population with improved and desired traits. Plant breeding has been practiced for thousands of years, since near the beginning of human civilization. It is now practiced worldwide by individuals such as gardeners and farmers, or by professional plant breeders employed by organizations such as government institutions, universities, crop-specific industry associations or research centers. International development agencies believe that breeding new crops is important for ensuring food security by developing new varieties that are higher-yielding, resistant to pests and diseases, drought resistant or regionally adapted to different environments and growing conditions. From this course, you will get the detail knowledge about various breeding approaches and biostatistical experiments relating to estimation and hypothesis testing. Molecular breeding is the application of molecular biology tools, often in plant breeding and animal breeding. In the broad sense, molecular breeding can be defined as the use of genetic manipulation performed at the level of DNA to improve traits of interest in plants and animals, and it may also include genetic engineering or gene manipulation, molecular marker-assisted selection, and genomic selection. More often, however, molecular breeding implies molecular marker-assisted breeding (MAB) and is defined as the application of molecular biotechnologies, specifically molecular markers, in combination with linkage maps and genomics, to alter and improve plant or animal traits on the basis of genotypic assays. Plant tissue culture is a basic, fundamental science from the branch of plant biotechnology that helps in understanding the growth and development of plants at the cellular level. Tissue culture

is defined as growing or culturing of desired cells, tissues, or organs on a designed sterile synthetic medium under controlled conditions of temperature, light, and humidity. This is typically facilitated via use of a liquid, semi-solid, or solid growth medium, such as broth or agar. It also requires more attention. It can be done only in genetic labs with various chemicals. Tissue culture is the growth of tissues or cells in an artificial medium separate from the parent organism. Tissue culture commonly refers to the culture of animal cells and tissues, with the more specific term plant tissue culture being used for plants. This technique is also called micropropagation.

2. Course Objectives

At the end of the course the learners will be able to:

- To know how to improve the characteristics of plants so that they become more desirable agronomically and economically; how to improve the yield of "economic produce on economic part"
- To know various achievements about crossing, mutation breeding
- Details explanation about Mass Selection Breeding, QTL analysis
- Gathering knowledge about disease resistance of plant, heritability, experimental designs, bioreactors.
- To acquire knowledge and abilities to apply the biotechnological tools necessary in the development of products and services involving biological organisms or components, from genetic engineering techniques to the use of bioreactors and development of bioprocesses, along with applications to healthcare.
- To assess the growth parameters in the different types of cultures which plays an important role in evaluating the growth kinetics of plant cell and tissue culture.
- To learn about the production and propagation of genetically homogeneous, disease-free plant material.
- To understand the growth measurements of most common *in vitro* systems, i.e., callus and cell suspension culture, micropropagation protocol.

3. Genetic Systems and Breeding Methods: Selection and breeding strategies for self-pollinated, cross-pollinated and clonally propagated plants.

Selection is essentially based on the phenotype of plants. Consequently the effectiveness of selection primarily depends upon the degree to which the phenotypes of plants reflect their genotype. Selection is basic to any crop improvement. Isolation of desirable plant types from the population is known as selection. It is one of the two fundamental steps of any breeding programme viz., **1. Creation of variation** and **2. Selection.**

There are two agencies involved in carrying out selection: one is Nature itself (Natural selection) and the other is man artificial selection. Though both may complement each other in some cases, they are mostly opposite in direction since their aims are different under the two conditions (nature and domestication). The effectiveness of selection primarily depends upon the degree to which phenotype reflects the genotype. Before domestication, crop species were subjected to natural selection. The basic for natural selection was adaptation to the prevailing environment. After domestication man has knowingly or unknowingly practiced some selection. Thus, crop species under domestication were exposed to both natural and artificial selection i.e. selection by man. For a long period, natural selection played an important role than selection by man. But in modern plant breeding methods natural selection is of little importance and artificial selection plays an important role. Basic Principles of Selection: Notwithstanding the highly complex genetic situation imposed by linkage and epistasis, there are just three basic principles of selection (Walker, 1969):

1. Selection operates on existing variability: The main function of the selection exercise is to discriminate between individuals. This is possible only when sufficient variation is present in the material subjected to selection pressure. Thus, selection acts on the existing variation it cannot create new variation.

Two methods of selection are commonly practiced in breeding of self pollinated crops

i) Pure Line Selection ii) Mass Selection.

Mass Selection

Large number of plants having similar phenotype are selected and their seeds are mixed together to constitute a new variety. Thus, the population obtained from selected plants will be more uniform than the original population. However, they are genotypically different.

Steps:

First year: From the base population select phenotypically similar plants which may be 200 - 2000. Harvest the selected plants as a bulk.

Second year: The bulk seed is divided into smaller lots and grown in preliminary yield trial along with control variety. Dissimilar phenotypes are rejected. High yielding plots are selected.

Third to sixth year: The variety is evaluated in coordinated yield trials at several locations. It is evaluated in an initial evaluation (IET) trial for one year. If found superior it is promoted to main yield trials for 2 or 3 years.

Seventh year: If the variety is proved superior in main yield trials it is multiplied and released after giving a suitable name.

Merits of mass selection

1. Since large number of plants is selected, the variety developed has wider adaptability as compared to variety developed through Pureline selection.
2. Often extensive yield trials are not necessary. This reduces the time and cost for developing new variety.
3. Mass selection retail considerable genetic variability in the new variety. Therefore, another round of mass selection after few years would be effective in improving variety further.

Demerits of mass selection

1. The varieties developed through mass selection show variation and are not as uniform as Pureline varieties. Therefore, such varieties are less liked than pureline varieties.

2. Improvement through mass selection is less than that could be achieved through pureline selection, because some progenies will be poorer in performance as compared to the best progeny/pureline present in new variety.

3. In the absence of progeny test it is difficult to find out whether selected plants are homozygous as there may be some degree of cross pollination in self-pollinated crops, some of the selected plants may be heterozygous.

4. Varieties developed through mass selection are more difficult to identify than pureline varieties in seed certification programmes.

5. Mass selection utilizes variability already present in a variety or population, and it can not generate variability.

Pureline selection

Pureline selection has been the most commonly used method of improvement of self-pollinated crops. Almost all the present day varieties of self-pollinated crops are purelines. Pureline selection has several applications in improvement of self-pollinated crops. It is used to improve:

1. Local varieties

2. Old pureline varieties and,

3. Introduced varieties

Procedure of Pureline selection

First year:

- An old variety or landrace is used for Pureline selection. Population they selected for pureline selection is homozygous. Single plant is selected and harvested separately superior plants must be selected from the mixed population. About 1000-2000 plants are selected depending on the available resources.

Second year:

- The individual progenies are grown separately with proper spacing the top 15-20 progenies are selected and they are bulked. Poor, defective, weak and segregating progenies are discarded. Selection should be based on simply inherited character like plant type, Plant height, grain type, flowering and maturity duration disease resistance this process may be repeated

Third year:

- Seed of the individual plant progenies are not enough to conduct a replication trail. So, they are grown in unreplicated trial with check. Here yield of progenies are taken as a criteria for selection.

Fourth year:

- Replicated yield trials are conducted using the best available check variety. This may be repeated for 2-3 year. All the observations are recorded

Fifth to Eighth year:

- Promising strains are evaluated at several locations along with strains or check. The best progeny / strain is released as a new variety and its seed multiplication in initiated for distributed to the farmer.

Advantages:

- ✓ Maximum possible improvement over original variety.
- ✓ Pureline varieties are extremely uniform and therefore, more preferred by farmers and consumers
- ✓ Due to uniformity, the variety is easily identified in seed certification programmes.

Disadvantages:

- ✓ This method can isolate only superior genotypes, it cannot create new genotypes. And not applicable in cross pollinated crop.
- ✓ Poor adaptability due to narrow genetic base vulnerable for new diseases and pests.
- ✓ Pureline selection requires more time, space and expensive yield trials than mass selection.

- ✓ Improvement is dependent on genetic variation present in the original population.
- ✓ The breeder has to devote more time to pureline selection than mass selection.

Comparison between pureline and mass selection

	Pureline selection	Mass selection
1	The new variety is a pureline	The new variety is a mixture of purelines.
2	The new variety is highly uniform. In fact, the variation within a pureline variety is purely environmental.	The variety has genetic variation of quantitative characters, although it is relatively uniform in general appearance.
3	The selected plants are subjected to progeny test.	Progeny test is generally not carried out.
4	The variety is generally the best pureline present in the original population. The pureline selection brings about the greatest improvement over the original variety.	The variety is inferior to the best pureline because most of the purelines included in it will be inferior to the best pureline.
5	Generally, a pureline variety is expected to have narrower adaptation and lower stability in performance than a mixture of purelines.	Usually the variety has a wider adaptation and greater stability than a pureline variety.
6	The plants are selected for the desirability. It is not necessary they should have a similar phenotype.	The selected plants have to be similar in phenotype since their seeds are mixed to make up the new variety.
7	It is more demanding because careful progeny tests and yield trials have to be conducted.	If a large number of plants are selected, expensive yield trials are not necessary. Thus, it is less demanding on the breeder.

Hybridization

- ❖ Natural variability in self-pollinated population is exhausted during selection, for further improvements new genetic variability has to be created by crossing two different pure lines. Hybridization means the mating or crossing of two plants or lines of dissimilar genotypes.

- The seeds as well as the progeny resulting from the hybridization are known as hybrid or F1. The progeny of F1 obtained by self or inter mating of F1 plants and the subsequent generations are called segregating generations. Today hybridization is the most common method of crop improvement and the majority of the crop varieties have originated from hybridization.
- One of the objectives of hybridization is to create genetic variation. Two genotypically different plants are crossed together to obtain F1 generation. F1 is advanced to generate F2 generation. The degree of genetic variation in F2 and subsequent generation depend on number of heterozygous genes in F1.

Aims of hybridization

1. To transfer of one or few qualitative characters.
2. Improvement in one or more quantitative character.
3. F1 Hybrid as variety.

I. Combination breeding:

This method is used for the transfer of one or more character into or single variety from another variety. Eg: improving the yield by correcting the defect. i.e. disease resistance. The other parent selected for hybridization must have a sufficient intensity of a character under transfer.

II. Transgressive breeding:

It aims at improving yield or its contributing character through transgressive segregation. It refers to the appearance of such plants in F2 generation that are superior to both the parents for one or more character. It is due to accumulation of plus or favourable genes from the parents as a consequence of recombination. The parents used for crossing must combine very well and are genetically diverse. So, pedigree breeding followed by population approach are designed for production of transgressive segregants.

III. Hybrid varieties:

In self-pollinated crops F1 is more vigorous and high yielding than the parents. Two parents should combine well to produce outstanding F1 hybrid.

Types of hybridization

Inter-varietal Hybridization / Intra specific: Parents involved in hybridization belong to the same species. They may be two strains, varieties or races.

Varietal crosses may be simple crosses or complex crosses

a.) Simple crosses: Two parents are crossed to produce F1 (A x B)

b.) Complex crosses: More than two parents are crossed to produce the hybrid (A x B) x Cx F1

Procedure of hybridization:

The breeder has clear cut objective in developing the variety. He has to select the variety accordingly.

1. Choice of parents: One of the parents involved in crosses should be a well-adapted and proven variety in the area. The other variety should be having the character that are absent in this variety. Combining ability of the parents serves as useful guides in the selection of parents, which produce superior F1 and F2.

2. Evaluation of parents: Parents are evaluated for their combining ability.

3. Emasculation: The removal of stamens/anther without affecting the female reproductive organs, hand emasculation is mostly followed.

4. Bagging: Immediately after emasculation the flowers are enclosed in suitable bags to prevent cross pollination.

5. Tagging: The emasculated flowers are tied with a thread. The information on date of emasculation, date of pollination, names of female and male parents are recorded in the tag with pencil. The name of the female parent is written first then male parent.

6. Pollination: Mature fertile and viable pollen from the male parent should be placed on receptive stigma of emasculated flowers to bring about fertilization. Pollen grain is collected, allowed for dehiscence and pollination is carried out with camel hair brush.

7. Harvesting and storing of F1 seeds: The crossed heads/pods should be harvested and threshed. The seeds should be dried and properly stored to protect them from storage pests.

8. Rising the F1 generation: Identify the selfed seeds in the F1 generation by using dominant marker gene. Larger F1 population is desirable, because both the genes are present in heterozygous condition.

9. Selfing: To avoid cross pollination and to ensure self-pollination. In often cross-pollinated crops, they are bagged to prevent cross pollination.

Distant Hybridization

When crosses are made between two different species or between two different genera, they are generally termed as distant hybridization (or) wide hybridization

History

Thomas Fairchild 1717 was the first man to do distant hybridization. He produced a hybrid between two species of Dianthus Dianthus caryophyllus (Carnation) x D. barbatus (Sweet william) Inter generic hybrid produced by Karpechenko, a Russian Scientist in 1928. Raphano brassica is the amphidiploid from a cross between Radish (Raphanus sativus) and cabbage (Brassica oleracea). Triticale was produced by Rimpau in 1890 itself. Triticale is an amphidiploids obtained from cross between wheat and rye. Another example is *Saccharum noblisation* involving three species.

Hybrids in self-pollinated crops - problems and prospects

Exploitation of heterosis through F1 hybrids has hitherto been the prerogative of crosspollinated crops, chiefly due to their breeding systems favouring allogamy. However, possibilities of working for such a proposition have recently been realized in self-pollinated corps also. Indeed, exploitation of hybrid vigour in autogamous/ self-pollinated crops is easy and less time consuming as homozygous inbreds are already available. There is practically no difference with regard to hybrid breeding between self and cross-pollinated crops. But the prospects of hybrids in self-pollinated crops are dependent on three major considerations:

1. How high a heterotic effect can be gained under optimal production conditions?

2. In fact, a breeder's main concern is the magnitude rather than the frequency of occurrence of heterosis in crops. Thus, the consideration is whether or not it is possible to obtain economically viable heterosis.

3. How much of the yield surplus due to high heterosis can offset the extra seed cost? In major self-pollinated crops like wheat, barley, rice, etc., the seed rate per unit area is exorbitant and hence the hybrid seed requirement is also more.

4. How efficient and effective is the mechanism of cross-pollination in self-pollinated crops? By nature, self-pollinated crops are shy pollinators with very poor pollen manoeuvrability (or movability to affect allogamy). Therefore, the efficiency (degree of allogamy) with which cross pollination can take place on a commercial scale is the true determinant of the success of a hybrid programme in self-pollinated crops.

5. Among self-pollinated crops, F1 hybrids have been graduated into the farmer's field in rice, barely, tomato, Sorghum (often-cross-pollinated) and wheat.

Pedigree method

In pedigree method, individual plants are selected from F2 and subsequent generation, their progenies are grown and a record of parent-progeny relationship (pedigree) is maintained. Pedigree method was first outlined by Love in 1927.

Procedure:

1. Hybridization - the selected parents are crossed to produce single or complex cross.

2. F1 generation – F1 seeds are space planted to produce maximum number of seeds.

3. F2 generation – F2 seeds are space-planted to facilitate selection. Usually, 1-10 % selection intensity is practised. When closely related varieties are crossed, the number of F2 individuals selected would be considerably smaller then when the parents are unrelated by descent. When the objective is to breed for quantitative traits, a relatively larger number of F2 plants would be selected. Selection in F2 is based on the traits that are simply inherited.

4. F3 – F5 generation – Individual plant progenies are space planted in F3 and F4 generation. Selection is practiced from within and between the progeny row. If two or

more progenies are coming from the same progeny row are similar, only one of them may be retained. In the F5 generation, variation within the progeny row vanishes and the focus for section should be between progeny row.

5. F6 generation – Individual selected progenies in F5 are planted in multi-row for visual comparison among progeny rows. Superior progenies are bulk harvested as they have become homozygous. Progenies showing segregation are discarded unless the segregants are outstanding. Under such situation, individual plant is selected.

6. F7 generation – PYT with 3 replications are conducted along with standard check. Progenies are evaluated for plant height, lodging and disease resistance flowering and maturity date, yield and quality traits with respect to the check. 2-5 outstanding lines superior to the check would be advanced to the coordinated yield trials.

7. F8 – F10 generation – The superior lines are tested in replicated yield trials at several locations. These are evaluated for plant height, lodging and disease resistance flowering and maturity date, yield and quality traits. A line that is superior to the best commercial variety included in the trial as check in yield and other traits is identified for release as new variety.

8. F11 generation – when the strain is likely to be released as variety, the breeder usually multiplies its seed during the last year in trial. Breeder has the responsibility to supply the breeder seed for production of foundation seed. Thus, in F11 to F12 the seed of the new variety will be multiplied for the distribution

Merits of pedigree method

- ✓ Provides maximum opportunity for the reeder to use his skills for selection of desirable plants from segregating generation.
- ✓ Transgressive segregants for yield and other quantitative traits may be recovered in addition to the improvement in specific trait
- ✓ The breeder may often be able to obtain information about inheritance of qualitative trait from pedigree record.
- ✓ Plants progenies with visible defects and weaknesses are eliminated at an early stage.

Demerits of pedigree method:

- Maintenance of pedigree record is tedious and time consuming job.
- Selection among and within a large number of progenies in every generation is laborious that limit the number of crosses a breeder can handle.
- Success of this method depends on skill of the reeder.
- No opportunity for the natural selection to influence the population.
- Selection for yield in F2/F3 is ineffective. If care is not taken care to retain a sufficient number of progenies, valuable genotypes may be lost in the early segregating generation.

Bulk Method

In bulk method, F2 and subsequent generation are harvested in bulk to raise the next generation until the genotypes attain Homozygosity or the favourable environment for selection is encountered, following which individual plants are selected and evaluated.

Procedure –

1. Hybridization - the selected parents are crossed to produce single or complex cross.

2. F1 generation - F1 generation is space planted and harvested in bulk.

3. F2 – F6 generation - F2 – F6 generation are planted at commercial seed rate and spacings and harvested in bulk. During bulking period, natural selection alters the genotypic frequencies in the population. Artificial selection is generally not done.

4. F7 generation – plants are space planted, seeds from phenotypically superior plants are harvested separately.

5. F8 generation – individual plant progenies are grown in single/multiple row. Superior plant progenies are harvested in bulk.

6. F9 generation – PYT is conducted with standard commercial check. Progenies are evaluated for plant height, lodging and disease resistance flowering and maturity date, yield and quality traits with respect to the check. 2-5 outstanding lines superior to the check would be advanced to the co-ordinated yield trials.

7. F10 – F12 generation - The superior lines are tested in replicated yield trials at several locations. These are evaluated for plant height, lodging and disease resistance flowering and maturity date, yield and quality traits. A line that is superior to the best commercial variety included in the trial as check in yield and other traits is identified for release as new variety.

8. F13 generation - when the strain is likely to be released as variety, the breeder usually multiplies its seed during the last year in trial. Breeder has the responsibility to supply the breeder seed for production of foundation seed.

Merits of bulk method:

- ✓ Natural selection increases the frequencies of superior genotypes.
- ✓ Since large population is grown, there is greater chance of isolation of transgressive segregants than in the pedigree method.
- ✓ Individual plant selection is done after population attains homozygosity, therefore, selection for quantitative traits are more effective.
- ✓ It is particularly suited for small grain crops, since, they are planted at high crop densities.
- ✓ It is suitable for studies on the survival of genes and genotypes in the population.
- ✓ Simple, convenient and inexpensive method allows breeder to focus on other breeding projects.

Demerits of bulk method:

- Natural selection becomes more important only after F10 and bulking may have to be done up to F20 or more which is considerably longer than the time taken in pedigree method.
- Short term bulks are useful for the isolation of homozygous lines but natural selection has little effect on such bulk population
- Provides little opportunity for breeder to exercise his skill.
- Information on inheritance of character cannot be obtained.
- In some cases natural selection may act against agronomically desirable types.

Clonal selection:

A clone may be defined as a group of plants derived from a single plant by vegetative propagation.

The clone can be characterised by the following characters:

(a) Clone is homogeneous,

(b) Individuals of a clone are either homozygous or heterozygous,

(c) Clones are stable in nature,

(d) Variability can be induced through mutation,

(e) Clone is propagated vegetatively.

Selection Procedure:

In view of these considerations, in the earlier stages of clonal selection, when selection is based on single plants or single plots, the emphasis is on the elimination of weak and undesirable plants or clones. The breeder cannot reasonably hope to identify superior' genotypes at this stage.

First Year

From a mixed variable population, few hundreds to few thousand desirable plants are selected. A rigid selection can be done for simply inherited characters with high heritability.

Plants with obvious weaknesses are eliminated.

Second Year

Clones from the selected plants are grown separately, generally, without replication this is done in view of the limited supply of the propagating material for each clone, and because of the large number of clones involved. The characteristics of clones will be clearer now than in the previous generation when the observations were based on individual plants. The number of clones is drastically reduced and inferior clones eliminated.

Third Year

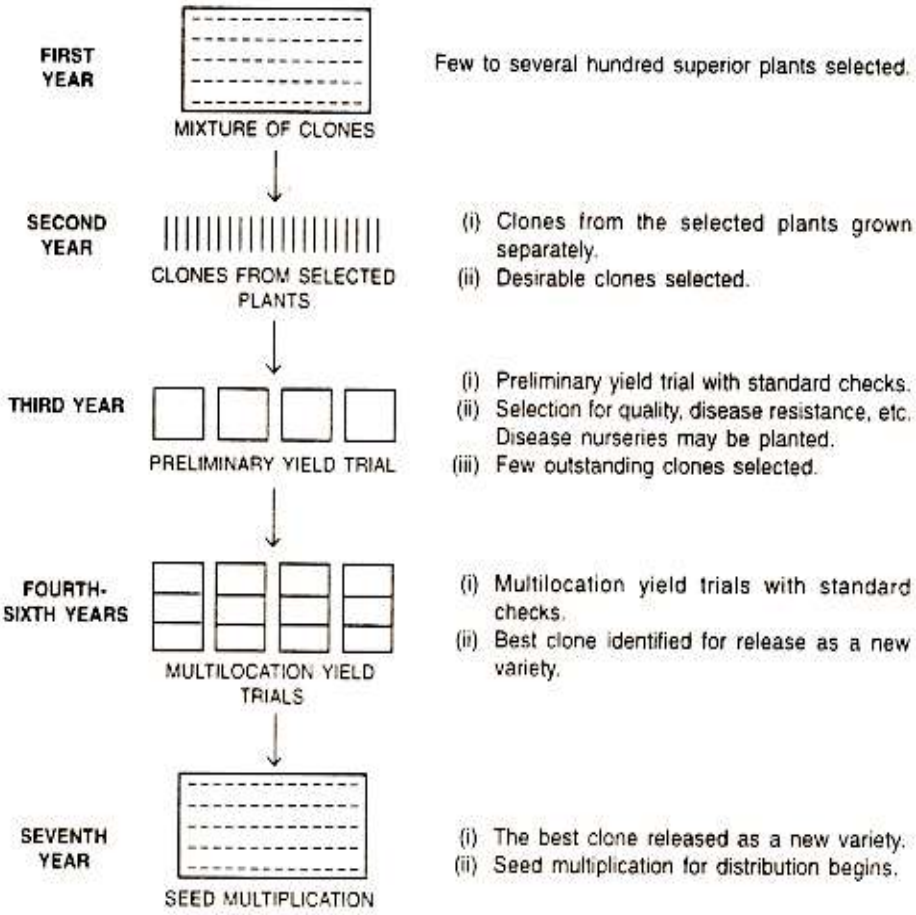
Replicated preliminary yield trial is conducted. Suitable, checks included for comparison. Few superior performing clones with desirable characteristics selected for multilocation trials. At this stage, selection for quality is also done.

Fourth to Sixth Years

Replicated yield trials are conducted at several locations along with a suitable check. The yielding ability, quality and disease resistance, etc. of the clones are rigidly evaluated. The best clone that is superior to the check in one or more characteristics is identified for release as a new variety.

Seventh Year

The superior clone is multiplied released as a new variety.



Merits of Clonal Selection

- ✓ It is the only method of selection applicable to clonal crops. It avoids inbreeding depression, and preserves the gene combinations present in the clones.
- ✓ Clonal selection, without any substantial modification, can be combined with hybridization to generate the variability necessary for selection.
- ✓ The selection scheme is useful in maintaining the purity of clones.

Demerits of Clonal Selection

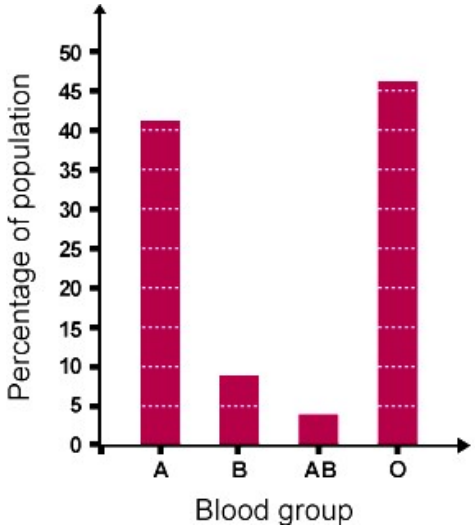
- This selection method utilizes the natural variability already present in the population; it has not been devised to generate variability.
- Sexual reproduction is a prerequisite for the creation of variability through hybridization.

4. Continuous Variation and its Significance: Qualitative traits and discrete variation, Quantitative trait and continuous variation, Polygenes and polygenic inheritance.

Qualitative trait: A qualitative trait is expressed qualitatively, which means that the phenotype falls into different categories. These categories do not necessarily have a certain order. The pattern of inheritance for a qualitative trait is typically monogenetic, which means that the trait is only influenced by a single gene. These types of traits are also referred to as discontinuous traits. Inherited diseases caused by single mutations are good examples of qualitative traits. Another is blood type. The environment has very little influence on the phenotype of these traits.

Variation encompasses the differences observed within a population. Classifying variation into **discrete** and **continuous** categories is essential for understanding genetic diversity and evolution.

Discrete variation: It refers to traits with a limited number of phenotypes, controlled by one or few genes. Discrete traits can be easily classified. Characteristics controlled by a single gene (one copy inherited from each parent) tend to have phenotypes that fall into separate categories. They show discrete variation. Discrete variation in a group of individuals can be shown using a bar chart.



Examples of discrete variation:

- ✓ **Blood Type:** Determined by antigens, human blood types are A, B, AB, and O.
- ✓ **Eye Colour:** Though complex, eye colour has discrete categories like blue, brown, and green.
- ✓ **Tongue Rolling:** Presence or absence of tongue-rolling ability is a discrete trait.

Genetics of Discrete Variation:

- **Single Gene Traits:** Discrete variations often involve one gene with different alleles.
- **Dominant and Recessive Alleles:** Expression may follow the pattern of dominance.

- **Sex-linked Traits:** Some discrete traits like haemophilia are linked to sex chromosomes.
- **Codominance and Incomplete Dominance:** Discrete traits may also follow patterns of codominance or incomplete dominance.

Discrete Variation in Populations: Discrete variation can be easily observed in populations and may lead to rapid evolution if certain traits are favored. This can be seen in cases like antibiotic resistance in bacteria.

Quantitative trait: It deals with the inheritance of traits of degree, viz., heights of length, weight, number, etc. The quantitative traits, however, are economically important measurable phenotypic traits of degree such as height, weight, skin pigmentation, susceptibility to pathological diseases or intelligence in man; amount of flowers, fruits, seeds, milk, meat or egg produced by plants or animals, etc. The quantitative traits are also called metric traits. They do not show clear cut differences between individuals and forms a spectrum of phenotypes which blend imperceptively from one type to another to cause continuous variations. In contrast to qualitative traits, the quantitative traits may be modified variously by the environmental conditions and are usually governed by many factors or genes (perhaps 10 or I00 or more), each contributing such a small amount of phenotype that their individual effects cannot be detected by Mendelian methods but by only statistical methods. Such genes which are non-allelic and effect the phenotype of a single quantitative trait, are called polygenes or cumulative genes. The inheritance of poly genes or quantitative traits is called quantitative inheritance, multiple factor inheritance, multiple gene inheritance or polygenic inheritance. Examples: Kernel Colour in Wheat, Skin Colour in Man, Height in Man, etc. The quantitative inheritance has following characteristics –

i. The segregation phenomenon occurs at an indefinitely large number of gene loci.
ii. If a substitution of a allele occurs in a gene locus then such allelic substitutions have trivial effects.

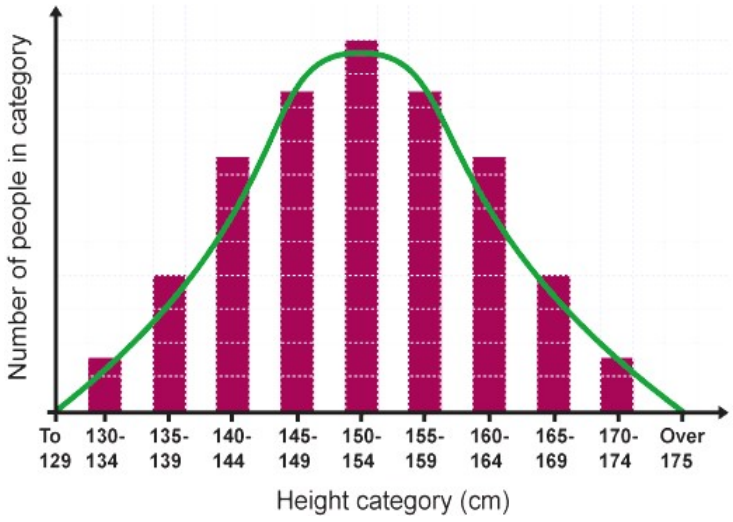
iii. The genes for a multiple trait have different biochemical functions but similar phenotypic effects, therefore, the phenotypic effects of gene substitutions are interchangeable.

iv. Blocks of genes are bound together by inversions and transmitted as units from inversion heterozygotes to their progeny, but such blocks are broken up by crossing over in insersion homozygotes.

v. The polygenes have pleiotropic effects; that is, one gene may modify or suppress more than one phenotypic trait. A single allele may do only one thing chemically but may ultimately affect many characters.

vi. The environmental conditions nave considerable effect the phenotypic expression of polygenes for the quantitative traits. For example, height in many plants (e.g., corn, tomato, pea, and marigold) is genetically controlled quantitative trait, but some environmental factors as soil, fertility, texture, and water, the temperature, the duration and wavelength of incident light, the occurrence of parasites, etc., also affect the height. Similarly, identical twins with identical genotypes, if grow up in different kinds of environments, show different intelligence quotients.

Continuous Variation: It involves traits showing a range without distinct categories. Multiple genes and environmental factors influence them. Most characteristics are controlled by more than one gene and are described as being polygenic. Polygenic



characteristics have phenotypes that can show a wide range of values, with each value following on from the value before. Continuous variation in a group of individuals can be shown using a histogram.

Examples of Continuous Variation:

- ✓ **Height:** Human height exhibits continuous variation.
- ✓ **Weight:** Weight varies based on genetics and lifestyle.
- ✓ **Skin Colour:** Several genes and sunlight influence skin colour, causing it to vary continuously. The structure of proteins plays a significant role in the expression of these genes.

Genetics of Continuous Variation:

- **Polygenic Traits:** Controlled by many genes, these traits show a continuous spectrum. The continuous variation in polygenic characteristics page offers more details on this topic.
- **Environmental Influence:** Factors like nutrition, climate, and upbringing affect the phenotype.
- **Normal Distribution:** Continuous traits often follow a bell-shaped curve in populations.
- **Additive Effects:** The contribution of several genes leads to a cumulative effect on phenotype. This is often seen in the context of DNA replication and its impact on genetic diversity.

Continuous Variation in Populations: In a population, continuous variation allows for a wide array of phenotypes, often leading to a more gradual evolution. The blending of traits allows for more nuanced adaptation to environmental pressures. The concept of evidence of evolution further explores how continuous variation contributes to the evolutionary process.

Comparing Discrete and Continuous Variation:

Discrete Variation

- Finite phenotypes.
- Usually monogenic.
- Categorical.
- Minimally affected by the environment.
- Rapid evolution if a trait is favored.

Continuous Variation

- Infinite phenotypes.
- Polygenic.
- Influenced by environmental factors.
- Gradual evolution due to nuanced adaptation.

The major differences between qualitative and qualitative genetics are asc follows –

Qualitative genetics	**Qualitative genetics**
1. It deals with the inheritance of traits of kind, viz., form, structure, colour, etc.	1. It deals with the inheritance of traits of degree, viz., heights of length, weight, number, etc.
2. Discrete phenotypic classes occur which display discontinuous variations.	2. A spectrum of phenotypic classes occur which contain continuous variations
3. Each qualitative trait is governed by two or many alleles of a single gene.	3. Each quantitative trait is governed by many non-allelic genes or polygenes.
4. The phenotypic expression of a gene is not influenced by environment.	4. Environmental conditions effect the phenotypic expression of polygenes variously.
5. It concerns with individual matings and their progeny.	5. It concerns with a population of organisms consisting of all possible kinds of matings.
6. In it analysis is made by counts and ratios.	6. In it analysis is made by statistical method.

Polygenes and polygenic inheritance:

A **polygene** is a member of a group of non-epistatic genes that interact additively to influence a phenotypic trait, thus contributing to multiple-gene inheritance (polygenic

inheritance, multigenic inheritance, quantitative inheritance), a type of non-Mendelian inheritance, as opposed to single-gene inheritance, which is the core notion of Mendelian inheritance. **Polygenic locus** is any individual locus which is included in the system of genes responsible for the genetic component of variation in a quantitative (polygenic) character.

In modern sense, the inheritance mode of polygenic patterns is called **polygenic inheritance**. Polygenic inheritance occurs when one characteristic is controlled by two or more genes. Often the genes are large in quantity but small in effect. Examples of human polygenic inheritance are height, skin color, eye color and weight. Polygenes exist in other organisms, like *Drosophila*.

Characteristics of Polygenic Inheritance are as follows –

- A gene that employs a minor effect on a phenotype along with other genes is referred to as polygenes.
- The effect of an individual gene is too minor and often remains undetected.
- There is a continuous variation of the phenotype of a trait in a polygenic inheritance.
- Numerous genes exert an equal effect.
- Individually, each allele contributes to the result in a cumulative or additive manner.
- The expression of one gene is not masked by the presence of the other genes, i.e., epistasis is not involved.
- The gene involved in polygenic inheritance is either contributing (active allele) or non-contributing (null allele); there are no genes as dominant or masked genes.
- The polygenic inheritance pattern is difficult to predict and it is highly complex.
- The statistical analysis of polygenic inheritance patterns can help to provide an estimate of population parameters. Most of the polygenic inheritances follow the *normal distribution curve*, wherein the majority of the people fall in the middle range of the curve.

- ❖ The phenotypic expression of the polygenic characters is undergoing considerable modification by environmental influence.
- ❖ The effects of allelic substitution at each of the segregating genes are usually relatively small and interchangeable which results that identical phenotype may be displayed by a great variety of genotypes.
- ❖ Balanced systems of polygenic inheritance in a population contain a great deal of potential genetic variability in the heterozygous condition and released by small increments through genetic recombination between linked polygenes.
- ❖ Polygenic inheritance is different from multiple alleles. In multiple alleles, on the same locus, three or more alleles are present an organism, e.g. human blood group system, i.e., the ABO system, is controlled by three alleles.

5. Self-Incompatibility: Basic concept, Genetic and molecular basis of self-incompatibility, Methods to overcome self-incompatibility in plants.

Self-incompatibility:

Self-incompatibility or intraspecific incompatibility is a well-designed genetic mechanism by which certain plants recognize and reject their own pollen thus forcing outbreeding. It is defined as "inability of the plant producing functional gametes to set seed upon self-pollination". Lundqvist (1964) defined it as "the inability of fertile hermaphrodite seed plant to produce zygotes after self-pollination".

Its genetic system is based on a single locus, the sterility (S) locus, with multiple alleles. Pollen germination or pollen tube growth is blocked when the pollen grain and the stigma upon which it lands have the same allele at the same locus. Besides the genetic

factors, intraspecific incompatibility is also associated with different lengths of stamens and style in flowers on same plant.

This self- incompatibility is acquired nearly one or two days before anthesis as well as in open flowers. Nearly two-thirds of the families of angiosperms exhibit self-incompatibility. The significance of SI in the evolutionary context cannot be overstated, since its possession leads to obligate outbreeding and the maintenance of heterozygosity within a species.

In the crop and ornamental plants, most of the perennial grasses, forage, legumes, and members of Brassicaceae, Asteraceae, Rosaceae, and Solanaceae have SI mechanism of varying kinds and degrees of effectiveness.

General features of Self-incompatibility:

- ✓ Prevent selfing and promotes out-breeding so increases the probability of new gene combinations.
- ✓ Causes may be morphological, physiological, genetical or biochemical.
- ✓ Normal seed set on cross pollination.
- ✓ May operate at any stage between pollination and fertilization.
- ✓ Reduces homozygosity.
- ✓ In plants, self-incompatibility is noften inherited by a single gene (S) with different alleles (e.g. S1, S2, S3 etc.) in the species population.

Causes of Self-incompatibility:

- ❖ Self-imcompatibility pollen grain may fail to germinate on the stigmatic surface.
- ❖ Some may germinate but fails to penetrate the stigmatic surface.
- ❖ Some pollen grains may produce pollen tube, which enters through stigmatic surface, but its growth will be too slow. By the time the pollen tube enters the ovule the flower will drop.
- ❖ Sometime fertilization is effected but embryo degenerates early.

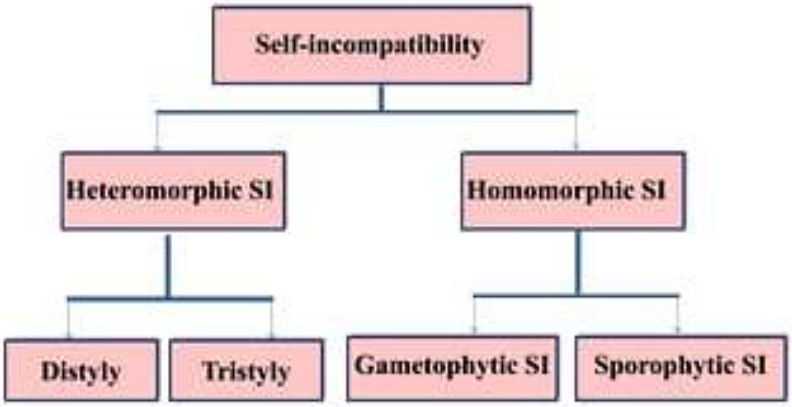
Types of Self-incompatibility:

1) Single-locus self-incompatibility

- Gametophytic self-incompatibility (GSI)
- Sporophytic self-incompatibility (SSI)

2) 2-locus gametophytic self-incompatibility
3) Heteromorphic self-incompatibility
4) Cryptic self-incompatibility (CSI)
5) Late-acting self-incompatibility (LSI)

According to Lewis (1954) the self incompatibility is classified as follows:



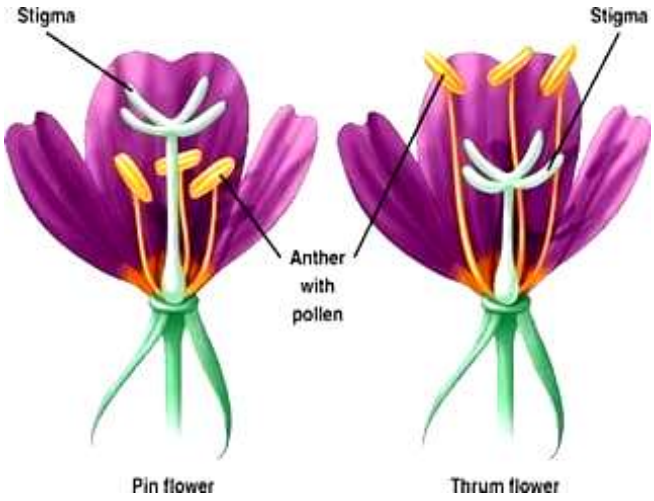
(a) Heteromorphic System:

When the species has two or three-different kinds of arrangement of floral part each type is self-incompatible but compatible with others. Here, the genes or alleles associated with incompatibility are also linked with length of style and filament.

(i) Distyly: In *Primula*, there are two types of flowers –

'Pin type' – long style, short filament, large stigmatic cell, small pollen.

'Thrum type' – short style, long filament, small stigmatic cell, large pollen.



Pin flowers belong to one compatible group, while thrum flowers form other group. Pin and thrum flowers are produced on different plants. Flowers belonging to a single incompatibility group do not set seed when pin flowers are cross-pollinated with each other. Therefore, seeds are produced only when pin flowers are pollinated by thrum flowers and vice-versa. This system is of limited occurrence in crop plants, e.g., in sweet pea, buck wheat, *Primula*.

Both flower morphology and self-incompatibility are governed by a singlelocus S having two alleles (S and s); the controlof SI reaction is sporophytic. The 'thrum' is governed by Ss and 'pin' by ss. As the reaction of pollen is controlled by the gene of the sporophyte, the pollen of the 'thrum' type will behave like S. The s gametes are incompatible with s type but compatible with s type and vice-versa. Mating between pin and thrum flowers produce thrum (Ss) and pin (ss) progeny in 1:1 ratio. It is now known that the locus S is acomplex locus and it has at least six functional genes (*g, s, l_1, l_2, p* and *a*); pin flowers are homozygous recessive for all of them, while thrum flowers are heterozygous for all of them.

Mating		Progeny	
Phenotype	Genotype	Genotype	Phenotype
Pin x Pin	ss x ss	Incompatible mating	
Pin x Thrum	ss x Ss	1 Ss : 1 ss	1 Thrum : 1 Pin
Thrum x Pin	Ss x ss	1 Ss : 1 ss	1 Thrum : 1 Pin
Thrum x Thrum	Ss x Ss	Incompatible mating	

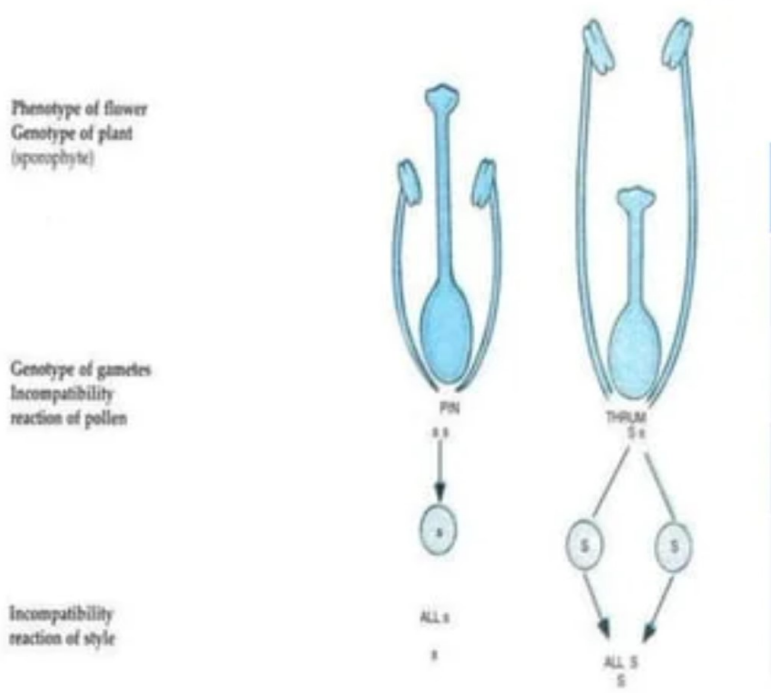


Fig: Heteromorphic system of self-incompatibility in *Primula.*

(ii) Tristyly:

In *Lythrum*, three types of flowers wih different stylar length exist. Here the stylar length is governed by two independent loci M and S. Plants with S have short style irrespective of the nature of other allele. The three different morphological types are self-incompatible but cross compatible.

	Long style	**Medium style**	**Short style**
Genotype	mmss	Mmss/MMss	MmSs/mmSs/ mmSS/MMSS/ MMSs/MmSSS

(b) Homomorphic System:

In this case, there is no morphological distinction between the self-incompatible flowers and the incompatibility is governed by multiple alleles.

This system can be of two types –

1. Pollen tube growth is controlled by the genotype of sporophyte; and
2. Pollen tube growth is governed by genotype of the pollen.

(i) Sporophytic self-incompatibility:

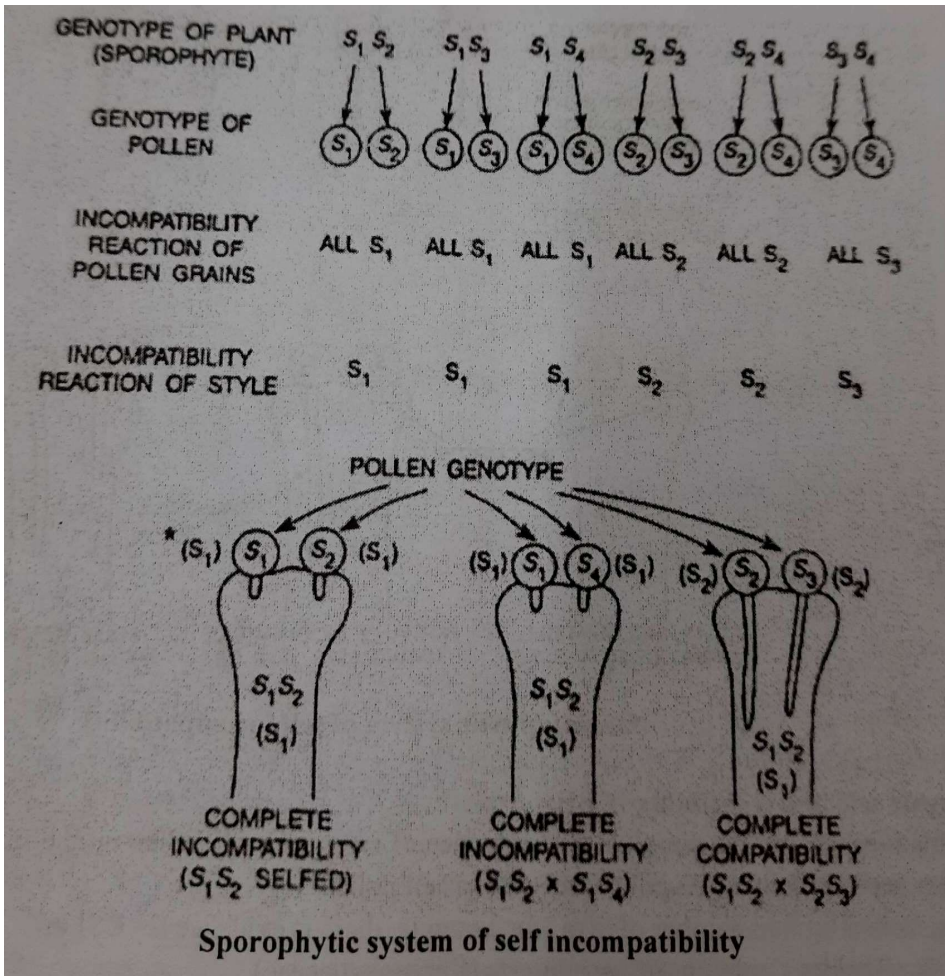
This system was reported by Hughes and Babcock in 1950 in *Crepis foetida* and by Gerstel in *Parthenium argentatum*. It occurs in radish, diplod *Brassica* sp., sunflower, Sinapis etc. Here the incompatibility is governed by multiple alleles but has the dominant-recessive reaction. Here, the pollen tube growth is not controlled by the genotype of pollen but by the genotype of the plant on which it is being produced. Actually the behaviour of gametophyte is controlled by dominant allele of the genotype of sporophyte.

In this system, SI reaction of a pollen grain is determined by the genotype at the S locus of the plant, which produced it. The SI reaction is governed by a single *S* locus with multiple S_1, S_2, S_3, S_4; S_1 is dominant over others, S_2 is dominant over S_3 and S_4, S_3 is dominant over S_4, etc. Irrecpective of the gametophytic genotype, the reaction will be of dominant type of the sporophyte.

S_1S_2 will produce all S_1 type gametophyte;

S_3S_4 will produce all S_3 type gametophyte;

S_2S_3 will produce all S_2 type gametophyte.



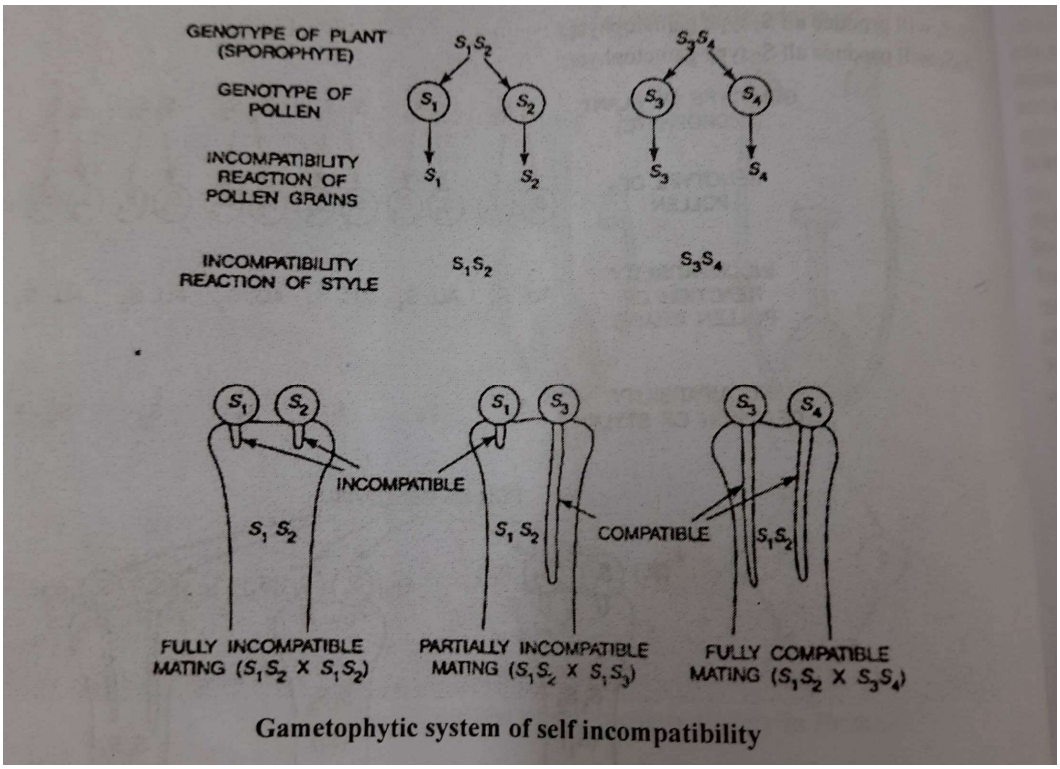
Sporophytic system of self incompatibility

(ii) Gametophytic self-incompatibility:

This SI system was first described in 1925 by East and Mangelsdrof in *Nicotiana sanerae*. It is found in rye, sugar beet, pineapple, diploid clover, alfalfa and several wild species. In this system (1) the SI reaction of pollen grains is determined by their own genotypes. This is because the substance involved in SI reaction of pollen is nproduced after mneiosis, (2) In style, the two S alleles show co-dominance, (3) In many species like *Nicotiana* sp., *Lycopersicon* sp., *Solanum* sp., *Petunia* sp., etc., the SI reaction is specified by a single gene S with multiples S1, S2, S3 etc. In such cases, polyploidy may eliminate SI, (4) In some species, e.g., *Phalaris*, *Physalis*, etc., two loci (S and Z) govern SI, while in some others like sugar beet, *Papaver*, etc., three loci are involved. In these

cases, polyploidy has no effect on SI, (5) A mutant allele Sf of the S locus allows the pollen grains carrying it to affwect fertilization of flowers with any S genotype.

If a pollen grain lands on a stigma, which has the same S allele as the pollen (e.g., S_1 pollen on S_1S_2, S_1S_3, S_1S_4, etc. stigma), it geminates normally. But the pollen tube growth is so slow that fertilization does not take place. However, if the stigma does not have the same S allele that is present in the pollen, pollen tube grows normally and fertilization is effected. Therefore, depending on the S locus genotype, matings between different plants are of the following three types: (1) fully compatible (e.g., $S_1S_2 \times S_3S_4$), (2) partially compatible (e.g., $S_1S_2 \times S_1S_3$) and (3) fully incompatible (e.g., $S_1S_2 \times S_1S_2$).



Gametophytic system of self incompatibility

Cryptic self-incompatibility (CSI):

In this mechanism, the simultaneous presence of cross and self pollen on the same stigma, results in higher seed set from cross pollen, relative to self pollen. It exists in a limited number of taxa (for example, there is evidence for CSI in (i) Bladder Campion – *Silene vulgaris* (Caryophyllaceae), (ii) Viper's Bugloss or Blueweed – *Echium vulgare*

(Boraginaceae), (iii) Waterwillow or swamp loosestrife – *Decodon verticillatus* (Lythraceae).

However, as opposed to 'complete' or 'absolute' SI, in CSI, self-pollinationwithout the presence of competing cross pollen, results in successive fertilization and seed set; in this way, reproduction is assured, even in the absence of cross-pollination. CSI acts, at least in some species, at the stage of pollen tube elongation and leads to faster elongation of cross pollen tubes, relative to self polletubes. The cellular andmolecular mechanisms of CSI have not been described.

The strength of a CSI response can be defined, as the ratio of crossed to selfed ovules, formed when equal amounts of cross and self pollen, are placed upon the stigma; in the taxa described up to this day, this ratio ranges between 3.2 and 11.5.

Late-acting self-incompatibility (LSI):

In this mechanism, self pollen germinates and reaches the ovules, but no fruit is set. LSI can be pre-zygotic (e.g. deterioration of the embryo sac prior to pollen tube entry, as in *Narcissus triandrus*) orpost-zygotic (malformation of the zygote or embryo, as in certain species of *Asclepias* and in *Spathodea campanulata*).

Other types of Self Incompatibility:

Criteria	Types
Genes involved (number)	Monoallelic (governed by single gene)
	Diallelic (governed by two genes)
	Polyallelic (governed by many genes)
Cytology of pollen	Binucleate (pollen with two nuclei)
	Trinucleate (pollen with three nuclei)
Expression site	Ovarian (expression site is ovary)
	Stylar (expression site is style)
	Stigmatic (expression site is stigma)

Mechanism of Self Incompatibility:

This is quite complex and is poorly understood. The various phenomena observed in self incompatibility are grouped into three categories:

1. Pollen – Stigma interaction
2. Pollen tube – Style interaction
3. Pollen tube – Ovule interaction

1. Pollen – Stigma interaction

This occurs just after the pollen grains reach the stigma and generally prevents pollen from germination. Previously it was thought that binucleate condition of pollen in gametophytic system and trinucleate condition in sporophytic system was the reason for self-incompatibility. But later on it was observed that they are not the reason for self-incompatibility. Under homomorphic system of incompatibility, there rae differences in the stigmatic surface which prevents pollen germination. In gametophytic system the stigma surface is plumose having elongated receptice cells which are commonly known as wet stigma. The pollen grain germinates on reaching the stigma and incompatibility reaction occrs at a later stage.

In the sporophytic system, the stigma is papiliate and dry and covered with hydrated layer of protein known as pellicle. This pellicle is involved in incompatibility reaction. With in few minutes of reaching the stigmatic surface the pollen releases exine exudates which are either protein or glycerol protein. This reacts with pellicle and induces callose formation, which further prevents the growth of polen tube.

2. Pollen tube – Style interaction

Pollen grains germinate and pollen tube penetrates the stigmatic surface. But in incompatible combinations the growth of pollen tube is retarded with the style as in *Petunia*, *Lycopersicon* and *Lilium*. The protein and polysaccharine synthesis in the pollen tube stops resulting in bursting up o pollen tube and leading to death of nuclei.

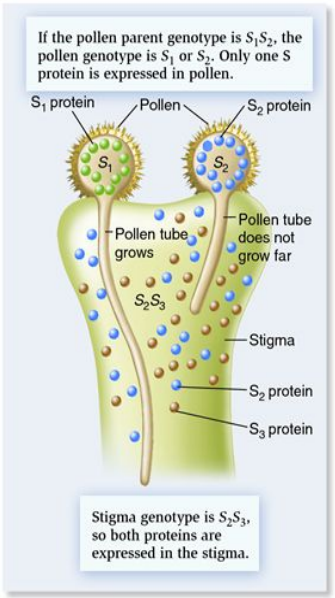
3. Pollen tube – Ovule interaction

In *Theobroma cacao*, pollen tube reaches the ovule and fertilization occurs but the embryo degenerates later due to some biochemical reaction.

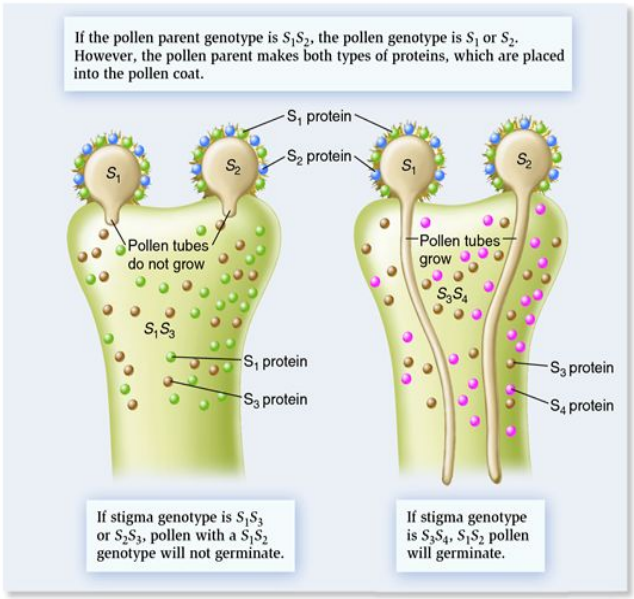
Molecular Mechanisms of single-locus self-incompatibility:

The best studied mechanisms of SI act by inhibiting the germination of pollen on stigmas, or the elongation of the pollen tube in the styles. These mechanisms are based on protein-protein interactions, and the best-understood mechanisms are controlled by a single locus termed S, which has many different alleles in the species population. Despite their similar morphological and genetic manifestations, these mechanisms have evolved independently, and are based on different cellular components; therefore, each mechanism has its own, unique S-genes.

The S-locus contains two basic protein coding regions - one expressed in the pistil, and the other in the anther and/or pollen (referred to as the female and male determinants, respectively). Because of their physical proximity, these are genetically linked, and are inherited as a unit. The units are called S-haplotypes. The translation products of the two regions of the S-locus are two proteins which, by interacting with one another, lead to the arrest of pollen germination and/or pollen tube elongation, and thereby generate an SI response, preventing fertilization. However, when a female determinant interacts with a male determinant of a different haplotype, no SI is created, and fertilization ensues. This is a simplistic description of the general mechanism of SI, which is more complicated, and in some species the S-haplotype contains more than two protein coding regions.



(a) Gametophytic SI: If pollen *S* allele does not match either stigma allele, pollen will germinate.



(b) Sporophytic SI: If pollen coat S proteins do not match either stigma S protein, pollen tubes will grow.

Gametophytic self-incompatibility (GSI):

In gametophytic self-incompatibility (GSI), the SI phenotype of the pollen is determined by its own gametophytic haploid genotype. This is the more common type of SI. Two different mechanisms of GSI have been described in detail at the molecular level, and their description follows –

- ✓ ***The RNase mechanism:*** The female component of GSI in the Solanaceae was found in 1989. Proteins in the same family were subsequently discovered in the Rosaceae and Plantaginaceae. Despite some early doubts about the common ancestry of GSI in these distantly related families, phylogenetic studies and the finding of shared male determinants (F-box proteins) clearly established homology. Consequently, this mechanism arose approximately 90 million years ago, and is the inferred ancestral state for approximately 50% of all plants. In this mechanism, pollen tube elongation is halted when it has proceeded approximately one third of the way through the style. The female component ribonuclease, termed S-RNase probably causes degradation of the ribosomal RNA (rRNA) inside the pollen tube, in the case of identical male and female S alleles, and consequently pollen tube elongation is arrested, and the pollen grain dies.

  The male component was only recently putatively identified as a member of the "F-box" protein family. Despite some fairly convincing evidence that it may be the male component, several features also make it an unlikely candidate.

  The block within incompatible polen tubves is created by an S-locus-encoded ribonuclease (SRNase) which is

  - ❖ Synthesized within the style
  - ❖ Enters the pollen tube
  - ❖ Destroys its RNA molecules
  - ❖ Haalting pollen tube growth

  The RNase molecules contain a **hypervariable region**, eacg coded by adifferent allele, which establishes each S specificity (S1, S2, S3, etc.) The pollen tube express a protein designated **SLF (S-locus F-box protein)** that binds SRNase.

SLF also exists in different S specificities (S1, S2, S3, etc.) In compatible ('nonself') tubes, the SLF or SCF (Skp1-Cu11-F box protein ubiquitin ligase) triggeres and degradation (in proteosome) of the SRNase thus permitting RNAs in the pollen tube to survive and growth to continue, In incompatible ('self') tubes, the interaction of, fr example, the S1 SCF with the SRNase blocks its degradation so the RNAs of the pollen tube are destroyed and growth is halted.

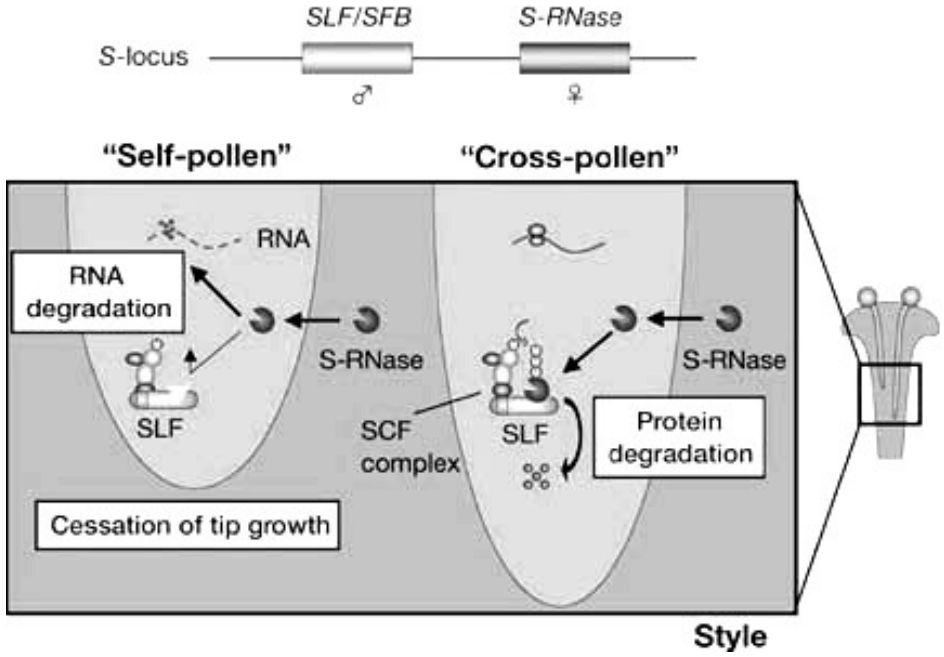


Fig: Molecular model of the SI in Solanaceae.

- ✓ ***The S-glycoprotein mechanism:*** The following mechanism was described in detail in *Papaver rhoeas*. In this mechanism, pollen growth is inhibited within minutes of its placement on the stigma.

  The female determinant is a small, extracellular molecule, expressed in the stigma; the identity of the male determinant remains elusive, but it is probably some cell membrane receptor. The interaction between male and female determinants transmits a cellular signal into the pollen tube, resulting in strong

influx of calcium cations; this interferes with the intracellular concentration gradient of calcium ions which exists inside the pollen tube, essential for its elongation. The influx of calcium ions arrests tube elongation within 1–2 minutes. At this stage, pollen inhibition is still reversible, and elongation can be resumed by applying certain manipulations, resulting in ovule fertilization.

Subsequently, the cytosolic protein p26, a pyrophosphatase, is inhibited by phosphorylation, possibly resulting in arrest of synthesis of molecular building blocks, required for tube elongation. There is depolymerization and reorganization of actin filaments, within the pollen cytoskeleton. Within 10 minutes from the placement on the stigma, the pollen is committed to a process which ends in its death. At 3–4 hours past pollination, fragmentation of pollen DNA begins, and finally (at 10–14 hours), the cell dies apoptotically.

- Stigmatic S-proteins isolated through in vitro assays of pollen tube inhibition the male determinant is believed to be a receptor located at the pollen plasma membrane.
- SBP (S-proteins binding protein) specifically binds S-proteins, but without haplotype specificity
- Interaction of self-pollen with pollen with the stigma induces a calcium-dependent signalling cascade leading to programmed cell death in the pollen (Thomas & Frnklin-Tong 2004)

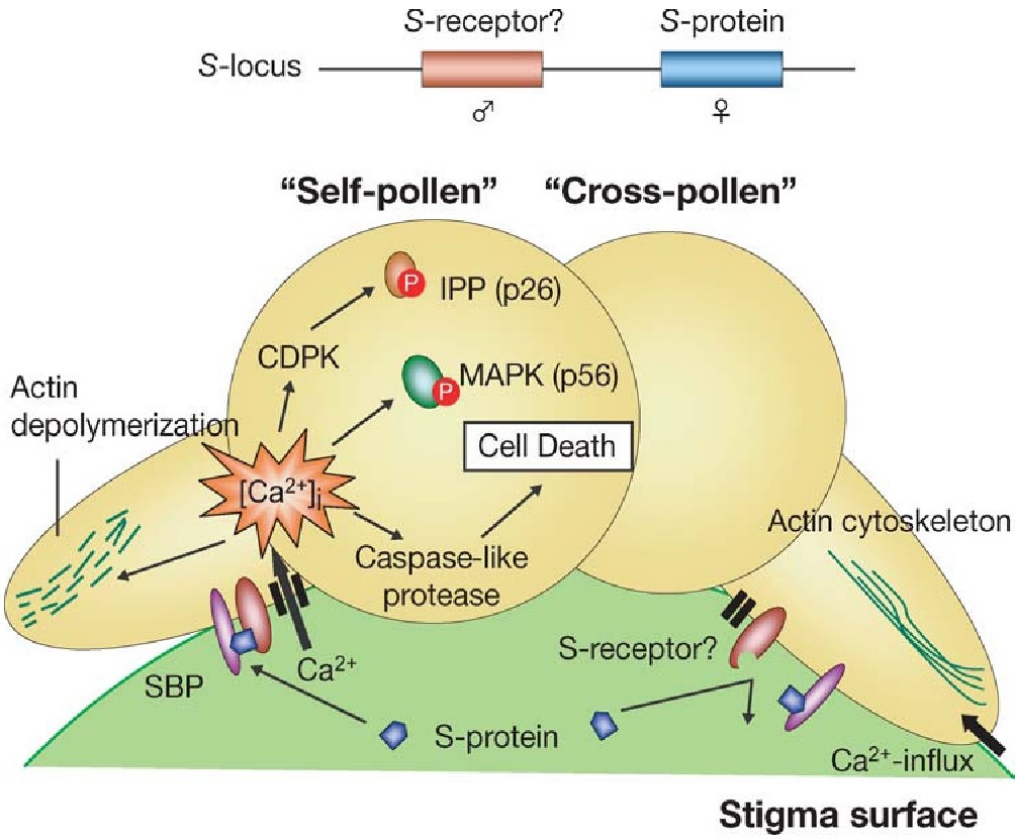


Fig: Molecular model of the SI in Papaveraceae.

Incompatible pollen undergoes an S-haplotype-specific interaction. Secreted stigmatic S-proteins interact with the pollen S receptor. An haplotype-specific interaction such as binding S1 protein to S1 pollen results in triggering an intracellular Ca^{2+} signalling cascade(s), involving large-scale Ca^{2+} influx and increases in Ca^{2+}. A series of events then occur in the incompatible pollen. Within 1 min there is a dissipation of the tip-focused calcium gradient that is required for continued pollen growth and the activation of calcium dependent protein kinase (CDPK). The CDPK phosphorylates Pr-p26.1, a soluble inorganic pyrophosphatase (sPPase). Both calcium and phosphorylation inhibit sPPase activity, resulting in a reduction in the biosynthetic capability of the pollen, therby inhibiting growth. Dramatic changes to pollen cytoskeleton organization are apparent within 1 min, with extensive depolymerization of the Factin accompanying this, also predicted to cause rapid arrest of tip growth. 56-MAPK is activated and may

signal to PCD, PCD is triggered, involving key features of PCD including caspase-like activity, cytochrome c leakage and DNA fragmentation. This ensures that incompatible pollen does not start to grow again.

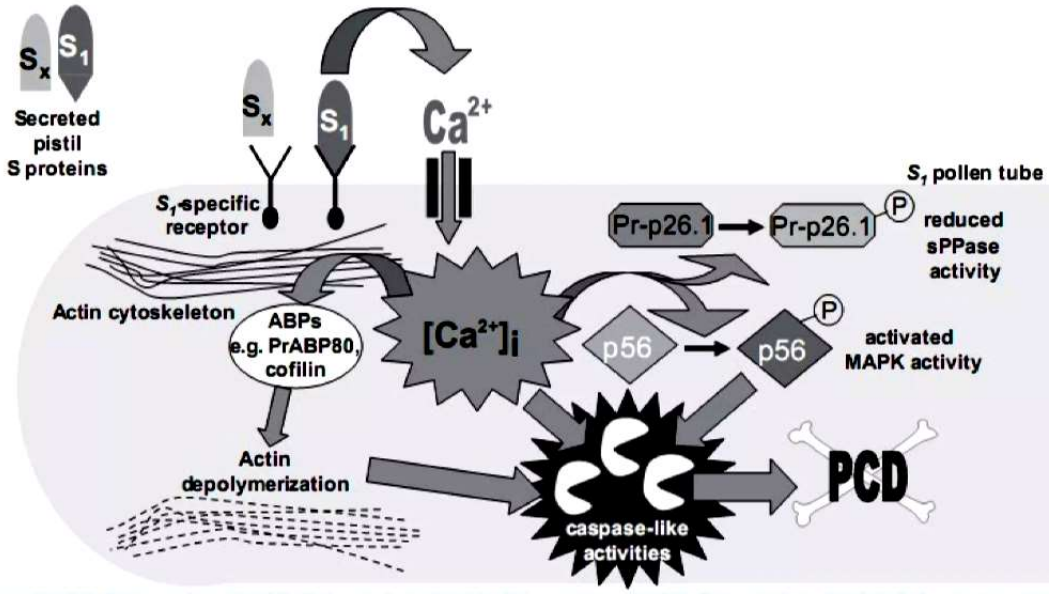


Fig: Molecular model of the SI in Papaveraceae.

Sporophytic self-incompatibility (GSI):

In sporophytic self-incompatibility (SSI), the SI phenotype of the pollen is determined by the diploid genotype of the anther (the sporophyte) in which it was created. This form of SI was identified in the families: Brassicaceae, Asteraceae, Convolvulaceae, Betulaceae, Caryophyllaceae, Sterculiaceae and Polemoniaceae. Up to this day, only one mechanism of SSI has been described in detail at the molecular level, in *Brassica* (Brassicaceae).

Since SSI is determined by a diploid genotype, the pollen and pistil each express the translation products of two different alleles, i.e. two male and two female determinants. Dominance relationships often exist between pairs of alleles, resulting in complicated patterns of compatibility/self-incompatibility. These dominance relationships also allow the generation of individuals homozygous for a recessive S allele.

Compared to a population in which all S alleles are co-dominant, the presence of dominance relationships in the population, raises the chances of compatible mating between individuals. The frequency ratio between recessive and dominant S alleles, reflects a dynamic balance between reproduction assurance (favoured by recessive alleles) and avoidance of selfing (favoured by dominant alleles).

The SI mechanism in *Brassica*:

As previously mentioned, the SI phenotype of the pollen is determined by the diploid genotype of the anther. In Brassica, the pollen coat, derived from the anther's tapetum tissue, carries the translation products of the two S alleles. These are small, cysteine-rich proteins. The male determinant is termed SCR or SP11, and is expressed in the anther tapetum as well as in the microspore and pollen (i.e. sporophytically). There are possibly up to 100 polymorphs of the S-haplotype in Brassica, and within these there is a dominance hierarchy.

The female determinant of the SI response in *Brassica*, is a transmembrane protein termed SRK, which has an intracellular kinase domain, and a variable extracellular domain. SRK is expressed in the stigma, and probably functions as a receptor for the SCR/SP11 protein in the pollen coat. Another stigmatic protein, termed SLG, is highly similar in sequence to the SRK protein, and seems to function as a co-receptor for the male determinant, amplifying the SI response.

SP11 is predominantly expressed in anther tapetum and accumulates in the pollen coat during poillen maturation. On pollination, SP11 penetrates the papilla cell wall and binds to the receptor complex consisting of SRK and SLG (or its relatives). This binding induces the autophosphorylation of SRK, which triggers the sihnalling cascade that results in the rejection of self-pollen. Another protein essential for the SI response is MLPK, a serine-threonine kinase, which is anchored to the plasma membrane from its intracellular side.

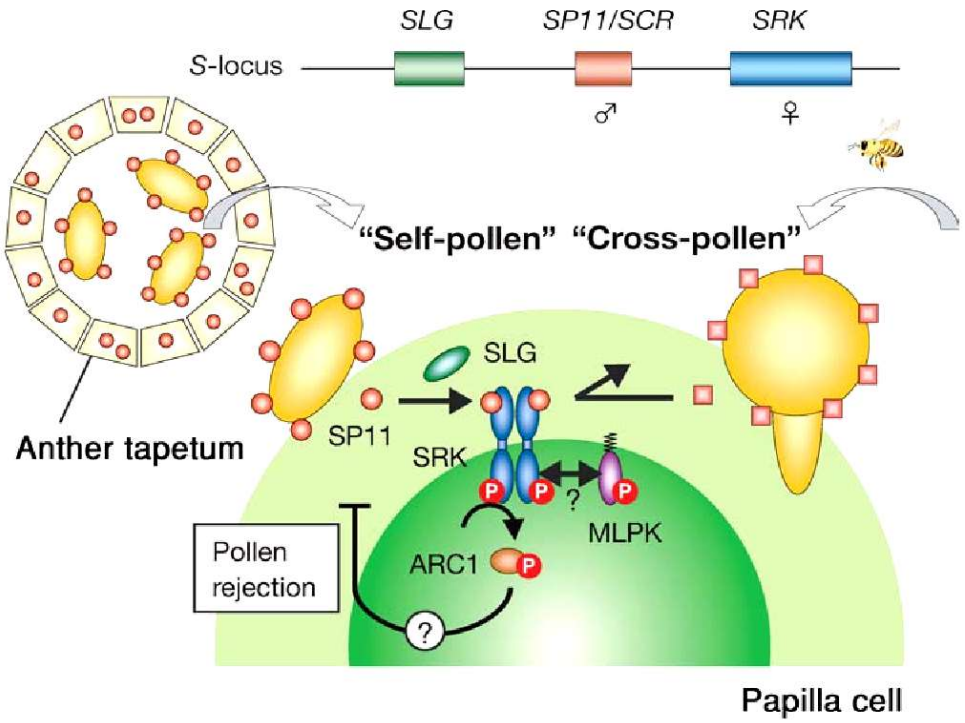


Fig: Molecular model of the SI in Brassicaceae.

Methods to Overcome Self-Incompatibility in Plants

The following methods have been used to overcome self-incompatibility in plants.

- **Method 1. Bud Pollination:** It is the most successful method in both the gametophytic and sporophytic systems. The most effective stage for the buds to overcome self-incompatibility is 2-7 days before anthesis. In *Petunia* inhibition is nullified if the buds are self- pollinated two days before anthesis. At the bud stage, the stigma lacks exudates, which appear only during anthesis. Thus, if the stigma is self-pollinated at bud stage, when the factors (present in the exudates) responsible for the self-incompatibility are not appeared, the pollen tubes will grow normally and effect fertilization.

- **Method 2. Mixed Pollination:** In this method the stigma is camouflaged from recognizing the incompatible pollen. This is achieved by pollinating the stigma with a mixture of compatible and incompatible pollens.It is presumed that when a stigma is pollinated with compatible pollen along with incompatible pollen, there the proteins released from the compatible pollen cover-up the inhibition reaction for the incompatible pollen at the surface of the stigma. By this method self-incompatibility can be successfully overcome in Cosmos (sporophytic self-incompatibility) and *Petunia hybrida* (gametophytic self- incompatibility).

- **Method 3. Deferred Pollination:** It has been observed that if pollination is deferred for few days, incompatible pollen tubes pass through the style. In *Brassica* and *Lilium* delayed pollination has been successful in overcoming self-incompatibility.

- **Method 4. Test tube Pollination:** Such a method to overcome self-incompatibility was first reported by Kanta (1962) in *Papaver somniferum* and later success also achieved in *Argemone mexicana*, *Nicotiana rustica* and *N. tabacum* of Solanaceae.

  In this method, stigmatic, stylar, and ovary wall tissues are completely removed from the path of pollen tube. The bare ovules are directly dusted with pollen grains. Successfully pollinated ovules are cultured in a nutrient medium that supports germination as well as development of fertilized ovules into seeds.

- **Method 5. Stub Pollination:** Those incompatibilities that are restricted to the stigma or to the length of the style that is larger than the maximum length attained by the pollen tubes have been overcome by removing the stigma and part of the style. The stigmatic surface of Ipomoea trichocarpa is the primary site of incompatibility and if the stigmatic lobe is removed and the cut surface pollinated then the pollen tube grows uninhibited in to the ovule.

- **Method 6. Intra-Ovarian Pollination:** In cases where the zone of incompatibility lies in the stigma or in the style, there pollen suspension can be

applied directly in the ovary to overcome incompatibility. Viable seeds have been obtained by this method in, *Argemone mexicana* by Kanta and Maheshwari (1963).

In this method the ovary is at first surface sterilized, followed by injecting the aqueous pollen suspension (with or without specific substance for germination) by a hypodermic syringe followed by sealing the holes with petroleum jelly. The introduced pollen grains germinate and achieve fertilization. The method has also been successful in other members of Papaveraceae, like *Papaver rhoeas* and *P. somniferum*.

- **Method 7. In Vitro Pollination:** This method was developed by Kanta (1962) in *Papaver somniferum* to overcome pre zygotic barriers to fertility. The exposed ovules, achieved by removing the stigmatic, stylar, and ovary wall tissues were directly dusted with pollen grains and then cultured in a suitable nutrient medium that supported both the germination of pollen as well as the development of fertilized ovules.

- **Method 8. Elevated Temperature Treatment:** Incompatibility reactions are affected by high temperature treatment. Hot water treatment of *Lilium longiflorum* detached or intact styles at 50°C for 6 minutes before pollination help to overcome self-incompatibility.

- **Method 9. Irradiation:** X-ray irradiation of flower buds at pollen mother cell stage helps to overcome self incompatibility. In fact irradiation damages the physiological mechanism of self incompatibility in the style, thus allowing the pollen tube to pass through the style.

- **Method 10. Surgical Method:** The stigma becomes the zone of inhibition, in plants with sporophytic type of incompatibility. Thus in such cases removal of such barrier is effective in overcoming self incompatibility. Defacement or decapitation of the stigma before pollination or deposition of pollen grains

directly into the stylar tissue through a slit has helped in overcoming self incompatibility.

- **Method 11. Application of Chemicals:** Different chemicals including growth hormones have been recorded to be effective in overcoming self-incompatibility. Olivomycin and cycloheximide, the inhibitors of RNA and protein synthesis could overcome self- incompatibility in *Petunia hybrida*, when injected into the flower buds just 2-3 days before anthesis. The treatment of *Brassica oleracea* stigma before pollination with hexane was found to be effective in fruit set.

- **Method 12. Protoplast Fusion:** In cases where sexual incompatibility does not permit to raise hybrids by the conventional methods, there the fusion of isolated protoplasts have achieved great success. Since it involves the fusion of somatic protoplast, the method is described as parasexual hybridization.

  The technique involves three basic steps, viz., isolation of protoplasts, fusion of the isolated protoplasts, and culture of hybrid protoplast to regenerate whole plants.

Significance of Self-incompatibility in plant breeding:

Self-incompatibilty effectively prevents self-pollination; as a result, it has a profound effect on plant breeding approaches and objectives.

1. In self-incompatible fruits trees, it is necessary to plant two cross-compatible varieties to ensure fruitfulnesss.
2. Self-incompatibilty may be used in hybrid seed production. For that, two self-incompatible but cross-compatible lines are to be interpolated; seeds obtained from both the lines would be hybrid seed.
3. Self-incompatibilty provides a way for hybrid seed production without emasculation and without resorting to genetic or cytoplasmic male sterility.
4. Self-incompatibilty system permits combining of desirable genes in as ingle genotype from two or more different sources through natural cross pollination which is not possible in self compatible species.

5. In case of pineapple, commercial clones are self-incompatible. As aresult, the fruits develop parthecarpically and are seedless.

Limitations of Self-incompatibility:

1. It is very difficult to produce homozygous inbred lines in a self-incomtible species.
2. Bud pollination has to be made to maintain the parental lines.
3. Self-incompatibility is affected by environmental factors such as temperature and humidity. Incompatibility is reduced or broken down at high temperature and humidity.
4. There is a limited use of self-incompatibility due to problems associated with the maintenance of inbred lines through hand pollination as it is tedious and costly.

6. Male Sterility: Overview; Types of male sterility; Mechanisms, Maintenance of male sterile line, Transgenic male sterility, Induction of male sterility, utilization in crop improvement.

Introduction:

Male sterility is defined as an absence or non-function of pollen grain in plant or incapability of plants to produce or release functional pollen grains as a result of formation or development of functional stamens, microspores or gametes. Main reason is mutation. The use of male sterility in hybrid seed production has a great importance as it eliminate the process of mechanical emasculation.

Manifestations of Male Sterility:

- Absence or malformation of male organs.

- Failure to develop normal microsporogenous tissueanther
- Abnormal microsporogenesis (deformed or inviable pollen)
- Abnormal pollen maturation
- Non dehiscent anthers but viable pollen, sporophytic control
- Barriers other than incompatibility preventing pollen from reaching ovule

History of Male Sterility:

- J.K. Koelreuter (1763) observed anther abortion within species & species hybrids.
- Genic male sterility has been reported in cabbage (Rundfeldt 1960), cauliflower (Nieuwhof 1961)
- Male sterility systems have been also developed through genetic engineering (Williams et al. 1997) and protoplast fusion (Pelletier et al. 1995)
- Male sterility were artificially induced through mutagenesis (Kaul 1988)

Why Male Sterility?

- Reduced the cost of hybrid seed production.
- Production of large scale of F1 seeds.
- Avoids enormous manual work of emasculation and pollination.
- Speed up the hybridization programme.
- Commercial exploitation of hybrid vigour.

Classification of Male Sterility:

Kaul (1988) Classified Male Sterility in three major groups, –

1. Phenotypic Male Sterility (Morphological)
    - Structural or Staminal Male Sterility
    - Pollen Male Sterility
    - Functional Male Sterility
2. Genotypic Male Sterility
    - Genetic Male Sterility (GMS)
        - Environmental Sensitive (EGMS)

a) Thermo sensitive genetic male sterility (TGMS)
b) Photoperiod sensitive genetic male sterility (PGMS)

- ❖ Environmental non-sensitive
- ➢ Cytoplasmic Male Sterility (CMS)
- ➢ Cytoplasmic Genetic Male Sterility (CGMS)
- ➢ Transgenic Male Sterility (TMS)

3. Chemically Induced Male Sterility (CHA)

Phenotypic male sterility:

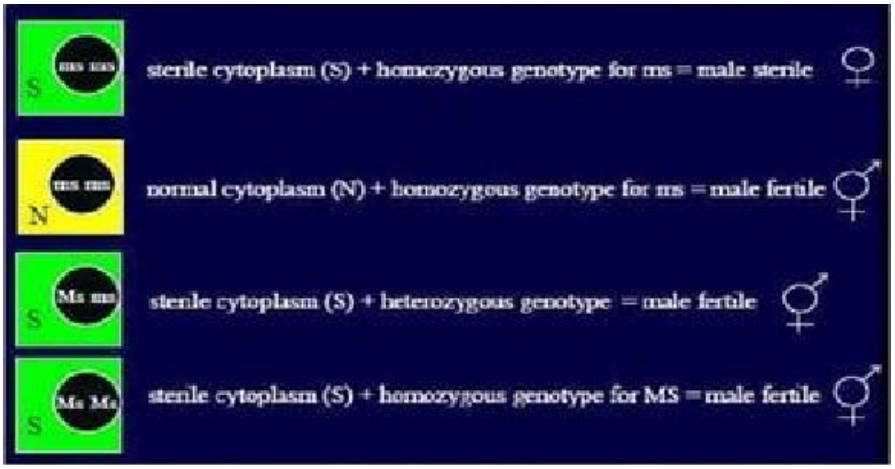
1. ***Pollen sterility:*** in which male sterile individuals differ from normal only in the absence or extreme scarcity of functional pollen grains (the most common and the only one that has played a major role in plant breeding).
2. ***Structural or staminal male sterility:*** in which male flowers or stamen are malformed and non functional or completely absent.
3. ***Functional male sterility:*** in which perfectly good and viable pollen is trapped in indehiscent anther and thus prevented from functioning

Cytoplasmic male sterility:

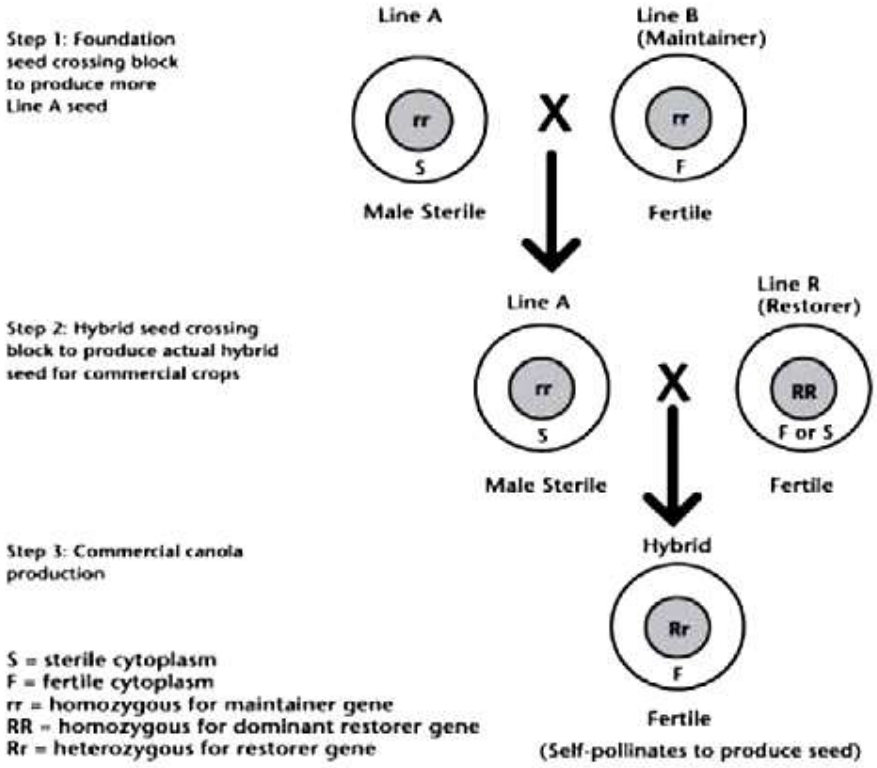
- Determined by the cytoplasm (mitochondrial or chloroplast genes).
- Result of mutation in mitochondrial genome (mtDNA)- Mitochondrial dysfu nction.
- Progenies would always be male sterile since the cytoplasm comes primarily from female gamete only.
- Nuclear genotype of male sterile line is almost identical to that Of the recurrent pollinator strain.
- Male fertile line (maintainer line or B line) is used to maintain the male sterile line (A line).
- CMS is not influenced by environmental factors (temperature) so is stable.

Utilization of CMS in plant breeding:

It may be used in hybrid seed production of certain ornamental species or in species where a vegetative part is of economic value. But not for crop plants where seed is the economic part because the hybrid progeny would be male sterile. This type of male sterility found in onion, fodder jowar, cabbage etc.



Use of CMS lines:



Transfer of CMS to new strain:

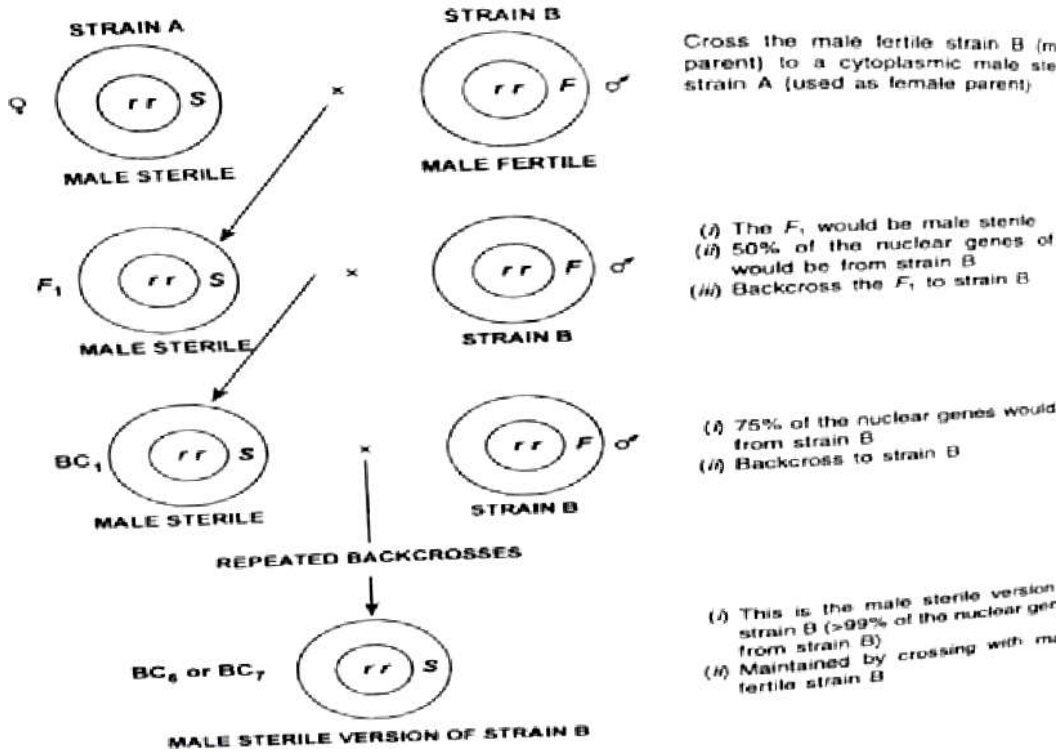


Fig: Transfer of male sterile cytoplasm from strain A to strain B. The strain B is used as maintainer of the new male sterile line.

Genetic male sterility:

It is governed by nuclear genes. It is wide occurrence in plants. Male sterility genes are generally recessive (ms ms) but dominant gene governing male sterility are also occur in safflower, and arise through spontaneous mutation or may be induced mutagen treatments. In rice, 25 ms genes are known. '*ms*' alleles may affect stamina inititation, stamen or anther sac development, PMC formation, meiosis, pollen formation, maturation and dehiscence.

Inheritance and Maintenance of Male Sterile Line:

When a male sterile plant (*ms ms*) is crossed with a male fertile one (*Ms Ms*), the F_1 (*Ms ms*) is male fertile. In F_2, a 3 male fertile: 1 male sterile ratio is obtained.

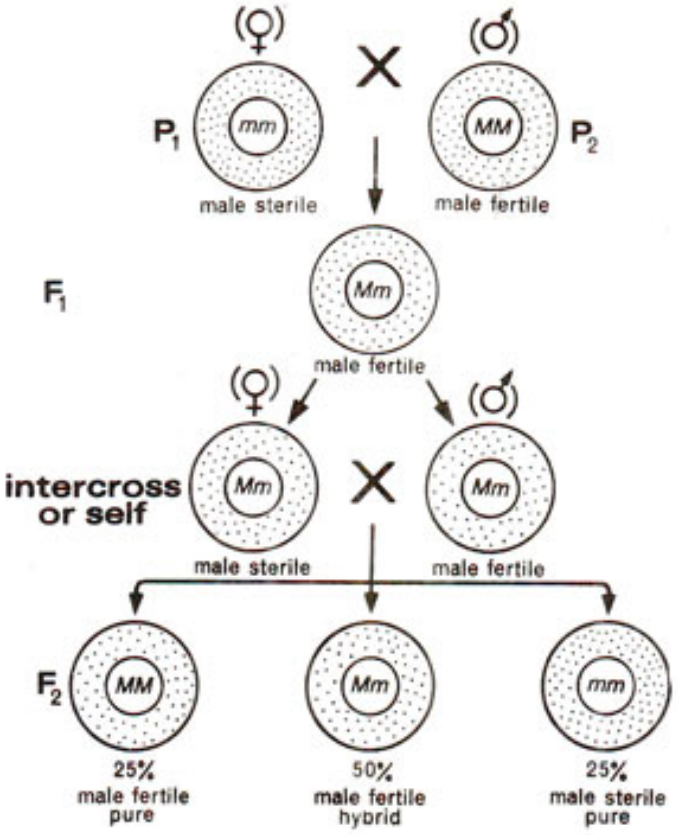
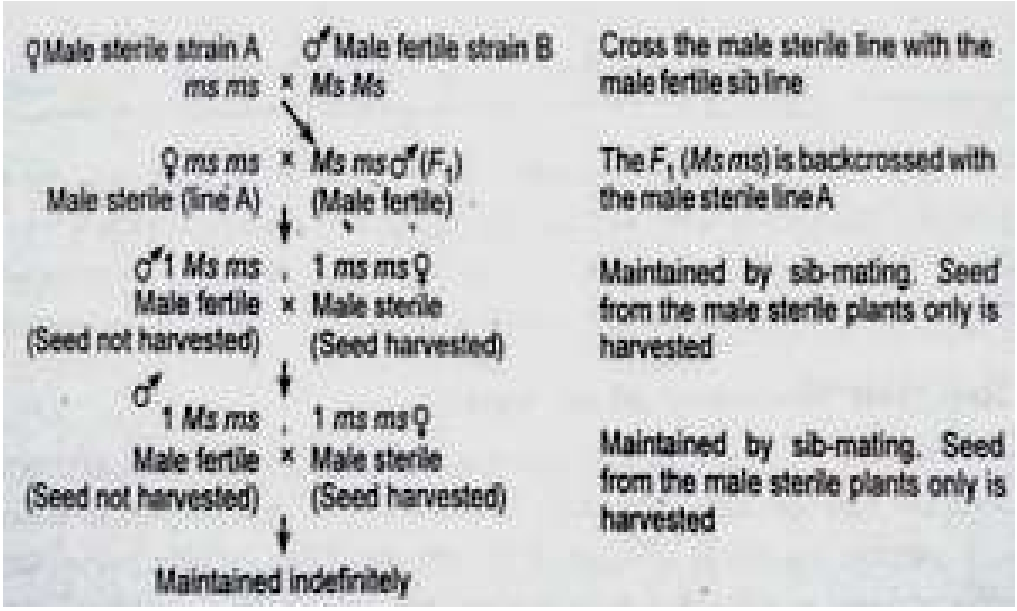


Fig: Inheritance pattern of genetic male sterility.

A male sterile line (*ms ms*) is maintained by crossing it with a heterozygous male fertile (*Ms ms*) line. This is simply acheieved by harvesting the seeds produced only on the male sterile plants present in a segregating generation like F_2. The plants derived from such seeds will be of two types: some will be male sterile (*ms ms*) and the rest will be male fertile (*Ms ms*). In these subsequent generations, seeds are harvested only from the male sterile plants; these seeds will give to 1 male sterile: 1 male fertile plants.



Difficulties in use of GMS:

- Maintenance of GMS requires skilled labour to identify fertile and sterile line. Labelling is time comsuming and costly.
- In hybrid, seed production plot identification of fertile line and removing them is costly.
- Use of double the seed rates of GMS line is costly.
- In crops like castor, high temperature leads to break down of male sterility.

Types of GMS:

- **Environment insensitive GMS:** ms gene expression is much less affected by the environment.
- **Environment sensitive GMS:** ms gene expression occurs within a specified range of temperature and /or photoperiod regimes (Rice, Tomato, Wheat etc.).

1. **TGMS:** Sterility is at particular temperature e.g. In rice TGMS line (Pei- Ai645) at 23.30C (China).

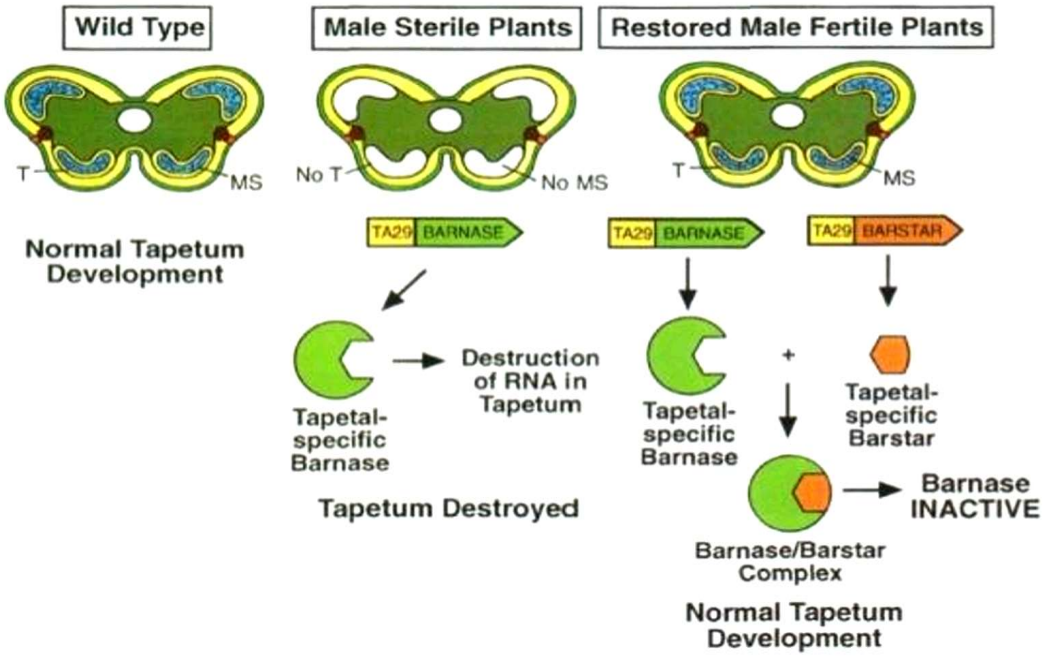
- ✓ TGMS at high temperature is due to failure of pairing of two chromosomes at metaphase was evident.
- ✓ This abnormality led to abnormal meiosis, abnormal or sterile pollens.
- ✓ Anthers were shriveled and non-dehiscence-Male sterile.
- ✓ However, these lines produced normal fertile pollen at low temp.
- ✓ Sensitive period: PMC formation to Meiosis

2. PGMS:

- ✓ Governed by 2 recessive genes.
- ✓ Sterility is obtained in long day conditions while in short days, normal fertile plant.
- ✓ Rice:- Sterile under Long day conditions (13 hr. 45 min + Temp.
- ✓ 23-290 C) but fertile under short day conditions.
- ✓ Sensitive period: Differentiation of secondary rachis branches to
- ✓ PMC formation

3. Transgenic male sterility:

A gene ontroduced into the genome of an organism by recombinant DNA technology or genetic engineering is called transgene. Many transgenes have been shown to produce genetic male sterility, which is dominant to fertility. Consequently, it is essential to develop effective fertility restoration system if these are to be utilized for hybrid seed production. An effective restoration system is available in at least one case called Barnase or Barstar System.



The Barnase gene of *Bacillus amyloliquefaciens* encodes RNAs. When Barnase gene is driven by TA 29 promoter, it is expressed only in tapetum cells causing their degeneration. Transgenic tobacco and *Brassica napus* plants expressing Barnase were completely male sterile. Another gene, Barstar, from the same bacterium encodes a protein, which is a highly specific inhibitor of Barnase RNase. Therefore, transgenic plants expressing both Barstar and Barnase are fully male fertile.

The Barnase gene has been tagged with bar gene, which specifies resistance to the herbicide phosphinothricin. This male sterile line is maintained by crossing with a male fertile line. The progeny so obtained contain 1 male sterile: 1 male fertile plant; the latter are easily eliminated at seedling stage by aphosphinothricin spray. The male sterile plants are crossed with the Barstarline to obtain male fertile hybrid progeny. This system of male sterile is yet to be commercially used.

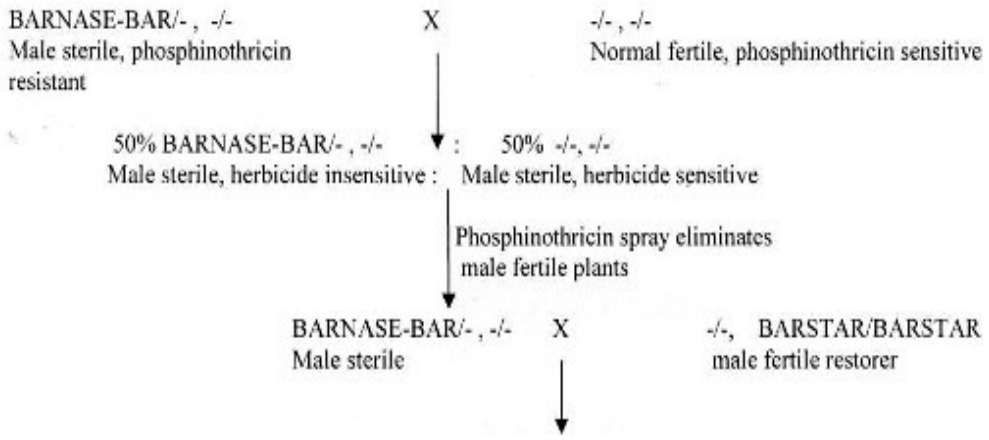


Fig: Hybrid seed production using Barnase/Barstar system.

Utilization of GMS in plant breeding:

This system (GMS) of male sterility has been used for hybrid seed production in castor, tomato and pigeon pea. Proposal for use in other crops like barley, wheat, cotton have been made but they are yet to be realized. Some of serious limitations of this system are briefly describe below:

1. The line used as female parent in hybrid seed production is the progeny from *ms ms × Ms ms* cross. Therefore, the female parent hasd 50% male fertile (Ms ms) plants, which have to be removed before flowering. This is usually quite difficult and costly to achieve. This problem is not faced when TGMS or PGMS are used. These male sterilwe lines are maintained by growing them at temperatures/ under photoperiods, which produce male fertility in male sterile lines. The selfed progeny of such lines therefore are 100% male sterile when grown under appropriate conditions.
2. Pollen dispersal from the male parent is generally poor.

Cytoplasmic Male Sterility:

In this system, male sterility is determined by the cytoplasm, and the plasmagenes producing male sterility are ordinarily located in mtDNA just as is the case of CMS. But a nuclear gene, called restorer gene, restores male fertility and thereby eliminates the effects of male sterile cytoplasm. The restorer gene is generally dominant, and is found in some varieties of the some varieties of the same species or in a related species. CGMS is known in several crops, and in many cases it is being commercially used. Repeated back cross are used for the transfer of male sterile cytoplasm, and the male sterile line is maintained in the same way as in the case of CMS. But the transfer of restorer genes using a backcross programme is a little more involved.

Plants having the male sterile cytoplasm will be male sterile only if their nuclear genotype is *rr* (*r* is the recessive allele of the fertility restorer gene *R* and is incapable of fertility restoration). But if the nuclear genotype is either *Rr* or *RR*, they will be male fertile.

- **A line or ms line:** This term represents a male sterile line belonging to anyone of the above categories. The A line is always used as a female parent in hybrid seed production.
- **B line or maintainer line:** This line is used to maintain the sterility of A line. The B line is isogenic line which is identical for all traits except for fertility status.
- **R line and restoration of fertility:** It is other wise known as Restorer line which restores fertility in the A line. The crossing between A × R lines results in F_1 fertile hybrid seeds which is of commercial value.

Utilization of CGMS in plant breeding: CGMS is being commercially used to produce hybrid seed in bajra, jowar, maize, rice, wheat and many other crops. The male sterile line is maintained by crossing with a maintainer line; this line has rr nuclear genotype. For hybrid seed production, the male sterile line is crossed with a restorer line so that the F_1 are fertile. In addition to the restorer line, it must be combining well with the

male sterile line to producer a high yielding F_1 hybrid. CGMS is at present commercially the most extensively used MS system.

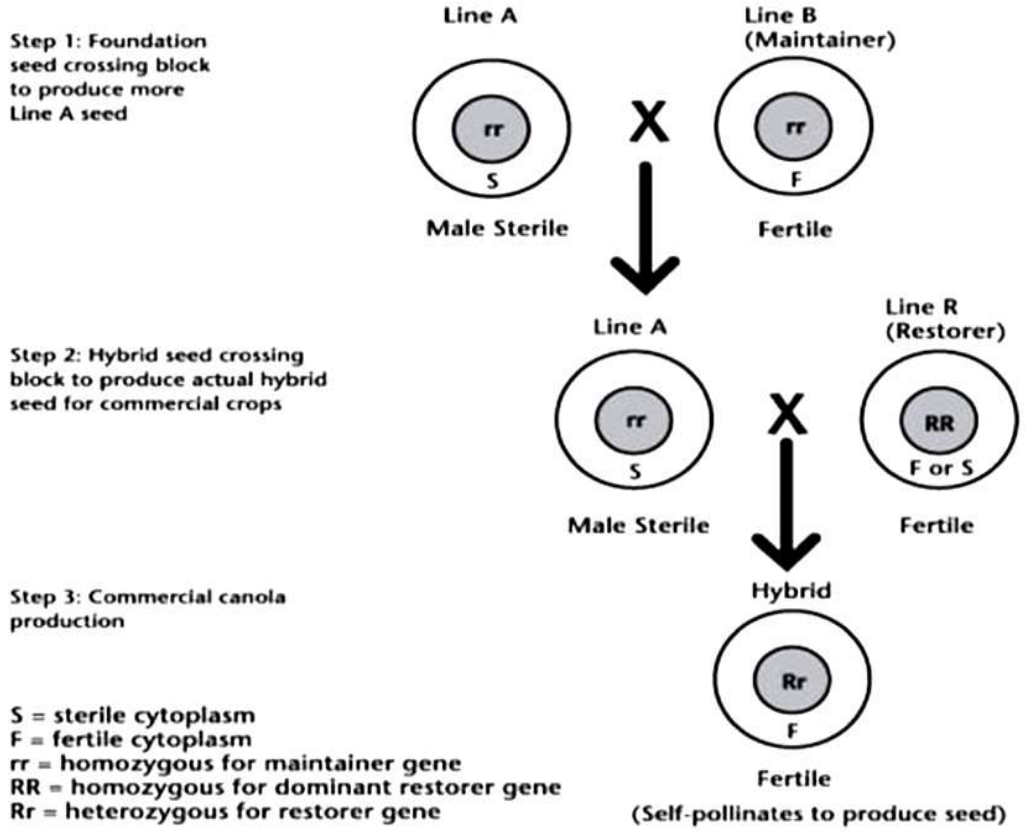
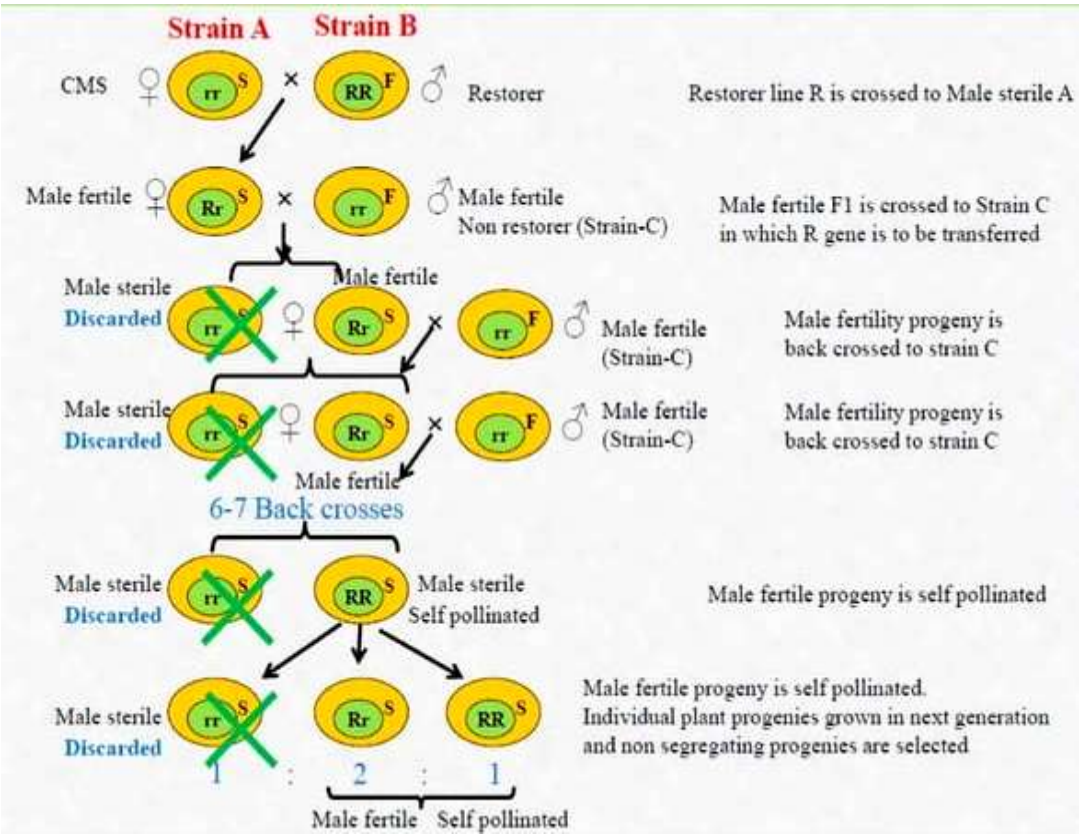
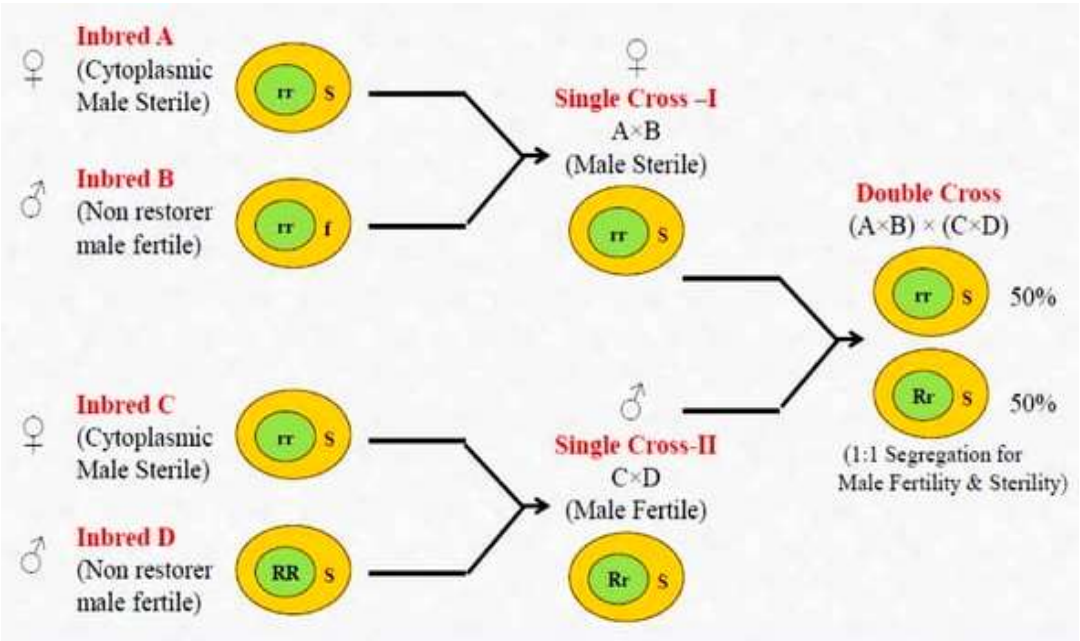


Fig: Hybrid seed production using CGMS system.

Maintenance:


Strain A
Strain B
CMS
rr S
RR F
Restorer
Restorer line R is crossed to Male sterile A
Male fertile
Rr S
rr F
Male fertile
Non restorer (Strain-C)
Male fertile F1 is crossed to Strain C
in which R gene is to be transferred
Male sterile
Discarded
Male fertile
Male fertile
(Strain-C)
Male fertility progeny is
back crossed to strain C
Male sterile
Discarded
Male fertile
(Strain-C)
Male fertility progeny is
back crossed to strain C
Male fertile
6-7 Back crosses
Male sterile
Discarded
RR S
Male sterile
Self pollinated
Male fertile progeny is self pollinated
Male sterile
Discarded
Rr S
RR S
Male fertile progeny is self pollinated.
Individual plant progenies grown in next generation
and non segregating progenies are selected
1 : 2 : 1
Male fertile
Self pollinated


Inbred A
(Cytoplasmic
Male Sterile)
rr S
Inbred B
(Non restorer
male fertile)
rr f
Single Cross –I
A×B
(Male Sterile)
rr S
Double Cross
(A×B) × (C×D)
rr S
50%
Rr S
50%
(1:1 Segregation for
Male Fertility & Sterility)
Inbred C
(Cytoplasmic
Male Sterile)
rr S
Inbred D
(Non restorer
male fertile)
RR S
Single Cross-II
C×D
(Male Fertile)
Rr S

Sources of CMS & Restorer genes in some Crops:

Crop species	Cytoplasm	Restorer Genes
Rice	CMS-CW	*O. spontanea*
	CMS-bo	*O. sativa* boroll (single dominant)
	CMS-WA	*O. spontanea* (WA, four genes)
	CMS-W18	*O. rufipogon*
Wheat (*T. aestivum*)	*T. timopheevi*	*Rf1* and *rf2*
	A. caudata	-
T. darum	*Aegilops ovata*	-
Maize	CMS-C	*Rf4*
	CMS-S	*Rf3*
	CMS-T	*Rf1* and *Rf1*
Tobacco	*N. debneyi*	-
	N. megalosiphon	-
	N. bigelovii	-
Cotton		

Limitations of Cytoplasmic – Genetic male sterility:

- Undesirable effects of the cytoplasm
- Unsatisfactory fertility restoration
- Unsatisfactory pollination
- Spontaneous reversion
- Modifying genes
- Contribution of cytoplasm by male gamete
- Environmental effects
- Non availability of a suitable restorer line

Chemical Induced Male Sterility:

This type of male sterility is induced by treatment with certain chemicals and is therefore, confined to the generation of chemical treatment. These chemicals are called as Male gametocides, also called male sterilants, selective male sterilants, pollen suppressants, pollenocide, androcide etc.. The first report was given by Moore and Naylor (1950), they induced male sterility in Maize using maleic hydrazide (MH). They are applied during certain development stages sensitive to the treatment, and cause pollen abortion. Generally, repeated applications of chemical are required. It is being used to develop rice hybrids in China; three hybrids have so far been released for cultivation. Chemicals inducing male sterility are ethrel, GA_3, maleic hydrazide, naphthalene acetic acid and sodium/zinc methyl arsenate. Unfortunately, none of them is an ideal male gametocide. For example, they often produce incomplete male sterility, repeated applications are needed, female fertility is also reduced and often undesirable side effects are produced. But they have the unique advantage of application with any line/variety of a crop whenever desired.

Properties of an Ideal CHA:

- ✓ Must be highly male or female selective.
- ✓ Should be easily applicable and economic in use.
- ✓ Time of application should be flexible.
- ✓ Must not be mutagenic.
- ✓ Must not be carried over in Fl seeds.
- ✓ Must consistently produce >95% male sterility.
- ✓ Must cause minimum reduction in seed set.
- ✓ Should not affect out crossing.
- ✓ Should not be hazardous to the environment.

Some important CHAs

Serial No.	CHAs	Critical stage	Crop species
1.	Zinc-Methyl-Arsenate Sodium-Methyl-Arsenate	5 days before heading	Rice
2.	Ethephon/Ethrel	Depends on crop	Barley, Oat, Bajra,

			rice
3.	Mendok	Depends on crop	Cotton, sugarbeet
4.	Gibberellic acid	1-3 days before meiosis	Maize, Barley, Wheat, Rice, Sunflower
5.	Maleic Hydrazide	Early microsporogenesis	Maize, Wheat, Cotton, Onion

Hybrid Seed Production based on CHAs

Conditions required:-

- Proper environmental conditions (Rain, Sunshine, temp, RH etc.)
- Synchronisation of flowering of Male & Female parents.
- Effective chemical emasculation and cross pollination
- CHA at precise stage and with recommended dose
- GA_3 spray to promote stigma exertion.
- Supplementary pollination to maximise seed set
- Avoid CHA spray on pollinator row.

Advantages CHAs

- Any line can be used as female parent.
- Choice of parents is flexible.
- Rapid method of developing male sterile line.
- No need of maintaining A, B & R lines.
- Hybrid seed production is based on only 2 line system.
- Maintenance of parental line is possible by self pollination.
- CHA based F2 hybrids are fully fertile as compared to few sterile hybrids in case of CMS or GMS.

Limitations CHAs

* Expression and duration of CHA is stage specific.

- Sensitive to environmental conditions.
- Incomplete male sterility produce selfed seeds.
- Many CHAs are toxic to plants and animals.
- Possess carryover residual effects in Fl seeds.
- Interfere with cell division.
- Affect human health.
- Genotype, dose application stage specific.

Significance male Sterility in Plant Breeding

- Male sterility a primary tool to avoid emasculation in hybridization.
- Hybrid production requires a female plant in which no viable pollens are borne. Inefficient emasculation may produce some self fertile progenies.
- GMS is being exploited (Eg.USA-Castor, India-Arhar).
- CMS/ CGMS are routinely used in Hybrid seed production in corn, sorghum, sunflower and sugarbeet, ornamental plants.
- Saves lot of time, money and labour.

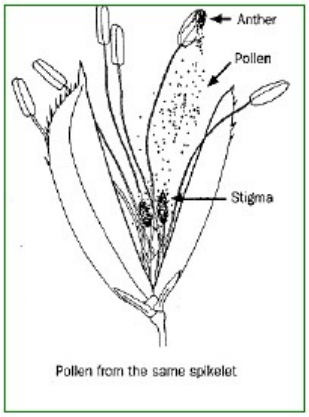
Limitations in using Male Sterile line

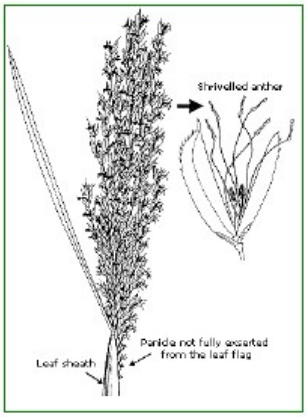
- Existence and maintenance of A, B & R Lines is laborious and difficult.
- If exotic lines are not suitable to our conditions, the native/adaptive lines have to be converted into MS lines.
- Adequate cross pollination should be there between A and R lines for good seed set.
- Synchronization of flowering should be there between A & R lines,
- Fertility restoration should be complete otherwise the FI seed will be sterile Isolation is needed for maintenance of parental lines and for producing hybrid seed.

Application of male sterility in hybrid seed production: Male sterility system in Rice hybrid seed production –

In rice following types of male sterility systems are used, like

- Cytoplasmic Male Sterility (Three line breeding)
- Genetic Male Sterility (Two line breeding)
- Male sterility induced by chemicals
- Hybridizing agents

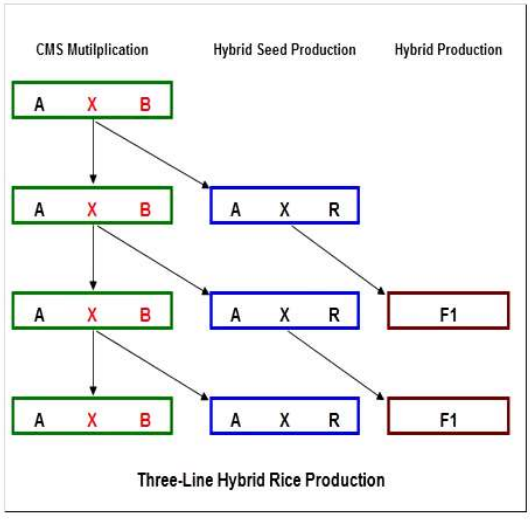


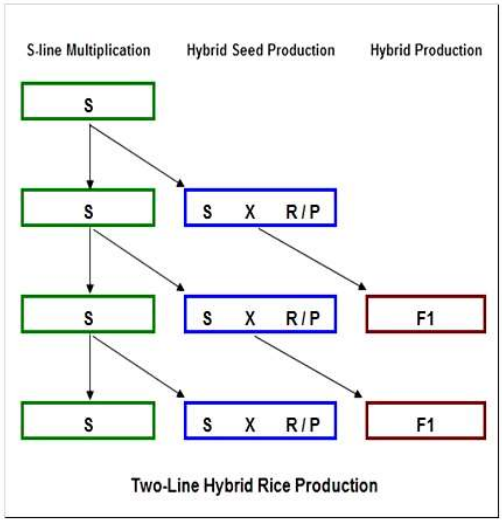


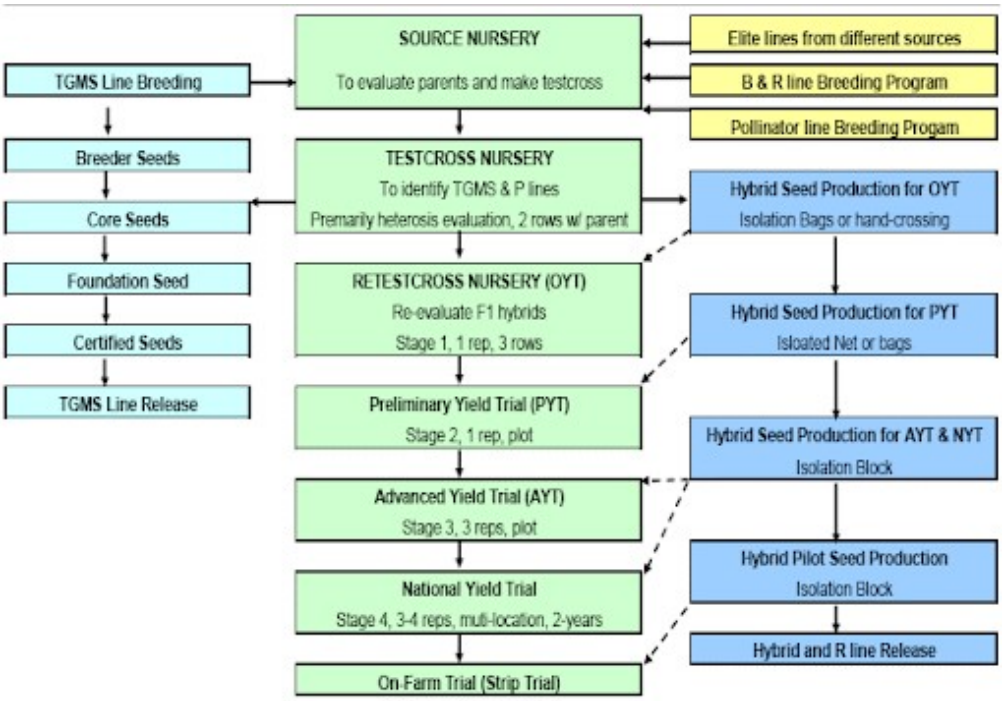
Normal Rice Spikelet
(self pollinated crop)

Sterile Rice Spikelet
(Male Sterility)

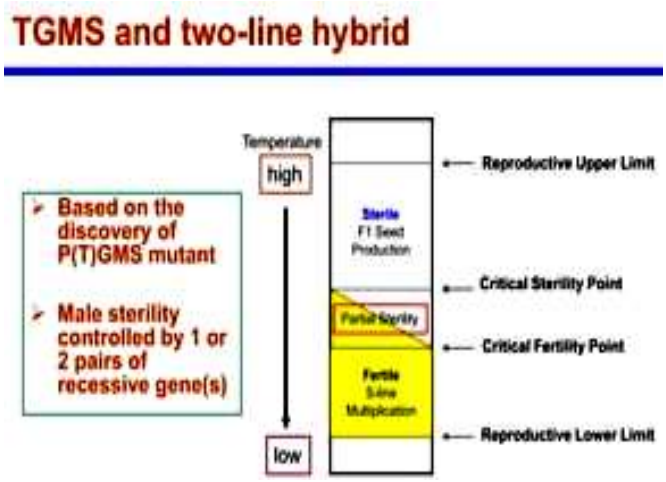
Two Commercial Systems for Hybrid Rice







Flowchart of 2-line Hybrid Rice Evaluation and Seed Production.



Model of Sreility Fertility Expression for TGMS rice.

Advantages of 2-line hybrid rice system

- ✓ Simplified procedure of hybrid seed production

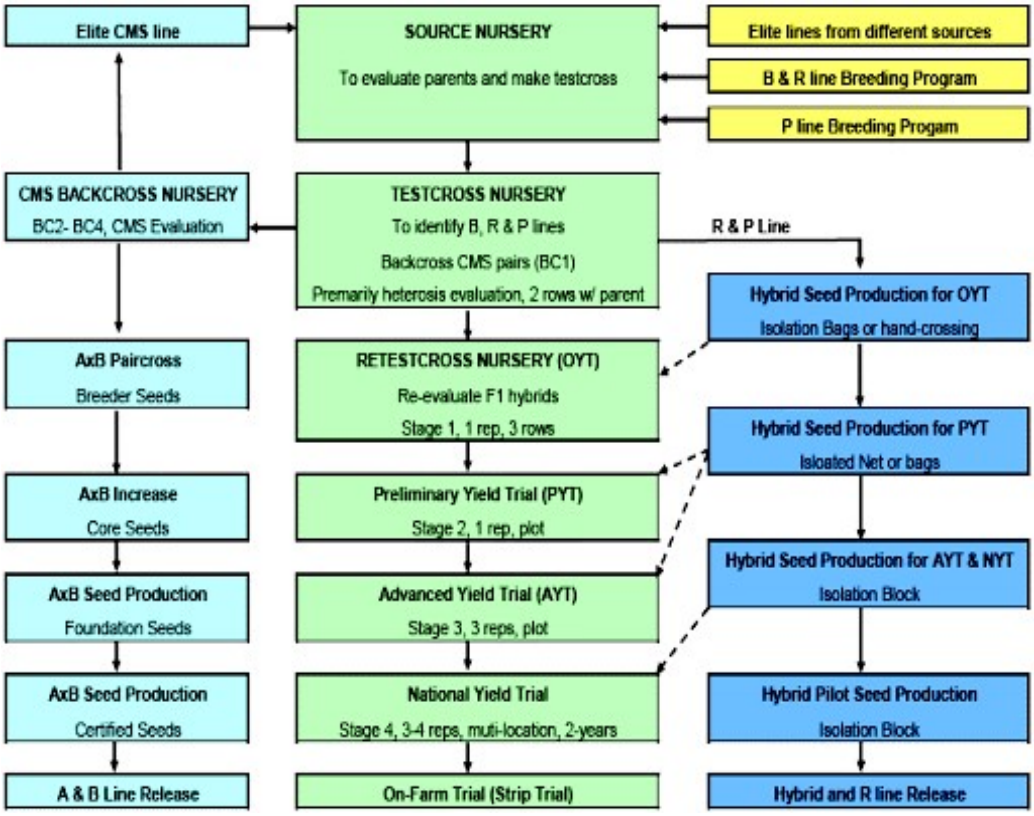
- ✓ Multiple and diverse germplasm available as parents 1) Any line could be bred as female, 2) 97% (2-line) vs 5% (3-line) of germplasm as male.
- ✓ Increased chance of developing desirable & heterotic hybrids
- ✓ Multiple cytoplasm courses as female parents

Disadvantages of 2-line hybrid rice system

- Environmental effect on sterility could cause seed purity problem

Requirements for 3-line in CMS System:

- **A-line**
  - Stable Sterility
  - Well developed floral traits for outcrossing Easily, wide-spectum, & strongly to be **restored B-line**
- **Restorer B-line**
  - Well developed floral traits with large pollen load
  - Good combining ability
- **R-line**
  - Strong restore ability
  - Good combining ability
  - Taller than A-line
  - Large pollen load, normal flowering traits and timing



Flowchart of 3-line Hybrid Rice Evaluation and Seed Production.

Advantages of 3-line hybrid rice system

- ✓ Stable male sterility

Disadvantages of 3-line hybrid rice system

- Limit germplasm source (CMS, Restorer)
- Dominant CMS cytoplasm in large area (WA)
- One more step for parental seed production
- Time consuming of CMS breeding

Male sterility system in Maize hybrid seed production

Different ways of inducing male sterility in maize

i. Manual/mechanical emasculation (detasselling)
ii. Genic male sterility
iii. Cytoplasmic genetic male sterility
iv. Gametocides

1. Genetic Male sterility

Male sterility determined by single recessive gene 40 loci involved have been identified (msl to ms52) ms5 —cloned

Problem: impossible to maintain male sterile inbred detasselling required

2. Cytoplasmic Male sterility

A. CMS-T (Texas) (Rogers and Edwardson, 1952)

- Highly stable under all environmental conditions
- Characterized by failure of anther exertion and pollen abortion
- Susceptible to race T of the southern corn leaf blight (Coch/iobolus heterostrophus = Bipolaris maydis)
- Widespread use of T-cytoplasm for hybrid corn production led to epidemic in 1970 with the widespread rise of Race T.
- Toxin produced by C heterostrophus = T-toxin.
- Fertility restoration is sporophytic
- Rfl (chr. 3) & Rf2 (chr.9) are responsible for fertility restoration

T-urf13 gene in T cytoplasm maize

Mitochondrial gene **T-urf13** is a unique chimeric sequence

Effect of URF13 protein-

- Degeneration of the tapetum during microsporogenesis
- Disruption of pollen development leading to male cell abortion

B. CMS-C (Charrua) (Beckett, 1971)

- Mutations in three genes viz atp6, atp 9 and cosll- confer CMS phenotype
- Fertility restoration is Sporophytic

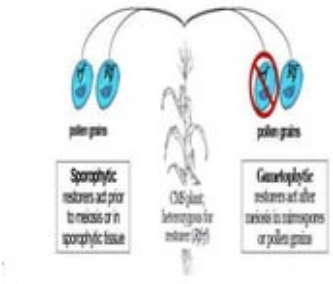
- Rf4, Rf5, Rf6 are responsible for fertility restoration

C. CMS-s (USDA) (Jones,1957)

- Sterility associated with orf355-orf77 chimeric mt gene
- Fertility restoration is Gametophytic
- Rf3 (chr. 2) are responsible forfertility restoration
- Plasmid like element Sl & S2

Reversion to fertility:

- The reversion of CMS strain to male fertility is based on genetic change
- Reversion can be spontaneous or mutagen induced.
- S-cytoplasm revert_rather frequently to male fertility (than T & C).



Maize-CMS Restoration of fertility system: different classes of pollen grains are produced, but not all of them are viable

Male sterility system in Brassica hybrid seed production

Cytoplasmic male-sterile:

Stamen (anther and filament) and pollen grains are affected It is divided into:

a) ***Autoplasmic***: Arisen within a species as a result of spontaneous mutational changes in the cytoplasm, most likely in the mitochondrial genome.

b) ***Alloplasmic***: Arisen from intergeneric, interpecific or occasionally intraspecific crosses and where the male sterility can be interpreted as being due to incompatibility or poor co-operation between nuclear genome of one species and the organellar genome.

Another CMS can be a result of interspecific protoplast fusion.

Various CMS systems:

- *Raphanus* or *ogu* system

- Polima or *pol* system
- Shiga-Thompson or nap system.
- *Diplotaxis muralis* or mur system
- Tournefortii (tour) system
- *Moricandia arvensis* or mori system
- *Chinese juncea* or jun system

17 systems are available, only difference is the use of male sterile cytoplasmic sources differs for each system

- **Nap system—** *B. napuus* cross between winter & spring varieties
- **pol system —** *B. napus* var polima
- **mur system —** *Diplotaxis muralis* x *B. campestris* cv y*ukina*
- **tour system—** *B. juncea* collections

Ogu system:-

- First discovered in Japanese radish (*Raphanus sativus*) by Ogura, 1968
- *B. napus* genome was transferred into the back round of *R. sativus* (*mst*) through intergeneric crosses followed by back crossing with *B. napus*.
- CMS seedling under low temperature showed chlorosis, because chloroplast of *R. sativus* is sensitive to cold, it is governed by cpDNA, but *mst* is governed by mt DNA.
- Protoplast fusion of R.sativus with B.napus carried out to have normal green plants with ogu CMS characterisitics.
- This system now has been used for developing alloplasmic male sterile line in *B. juncea* and *B. campestris*.

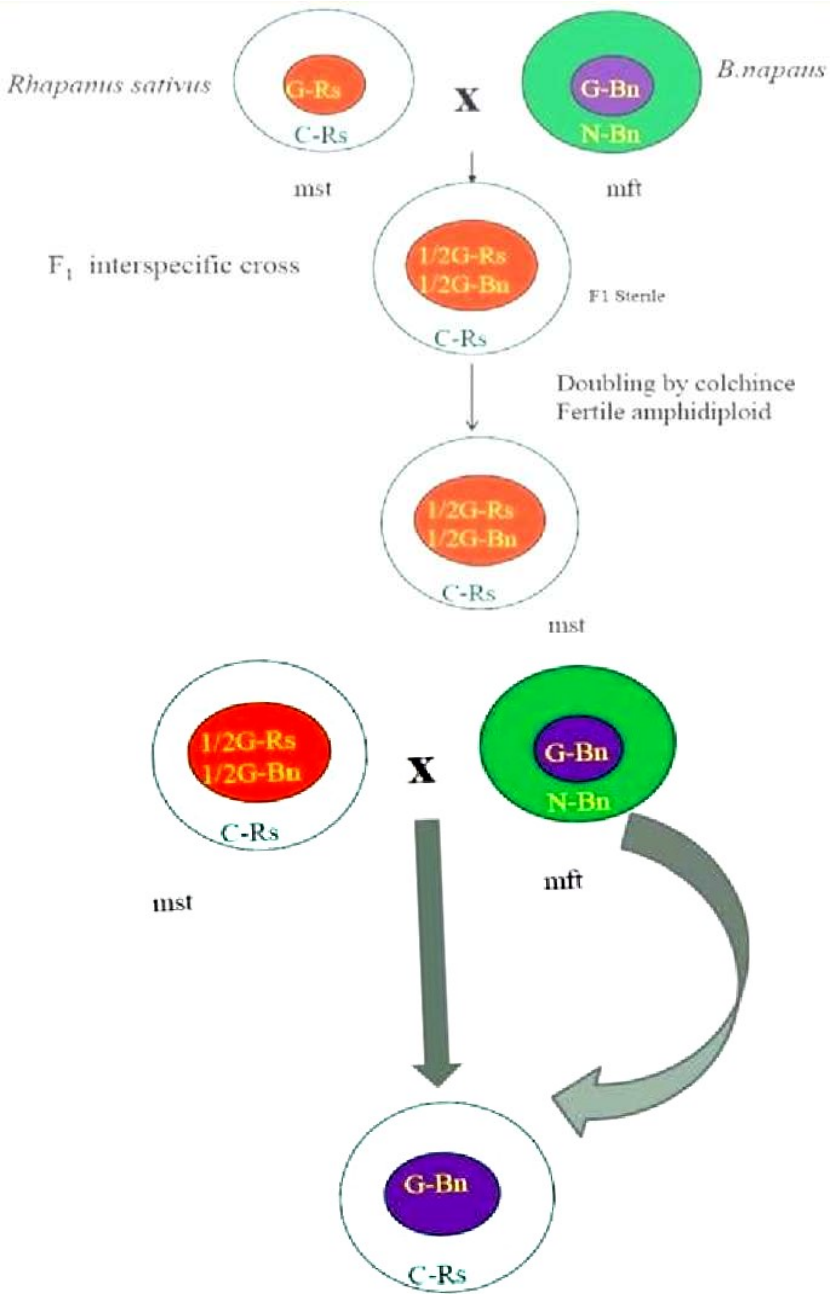
Genetic Male Sterility

GMS is governed by two genes either recessive or dominant genes (Kaul,1988). One more dominant gene is associated with development of male sterility in B.napus type by means of transgenic male sterility.

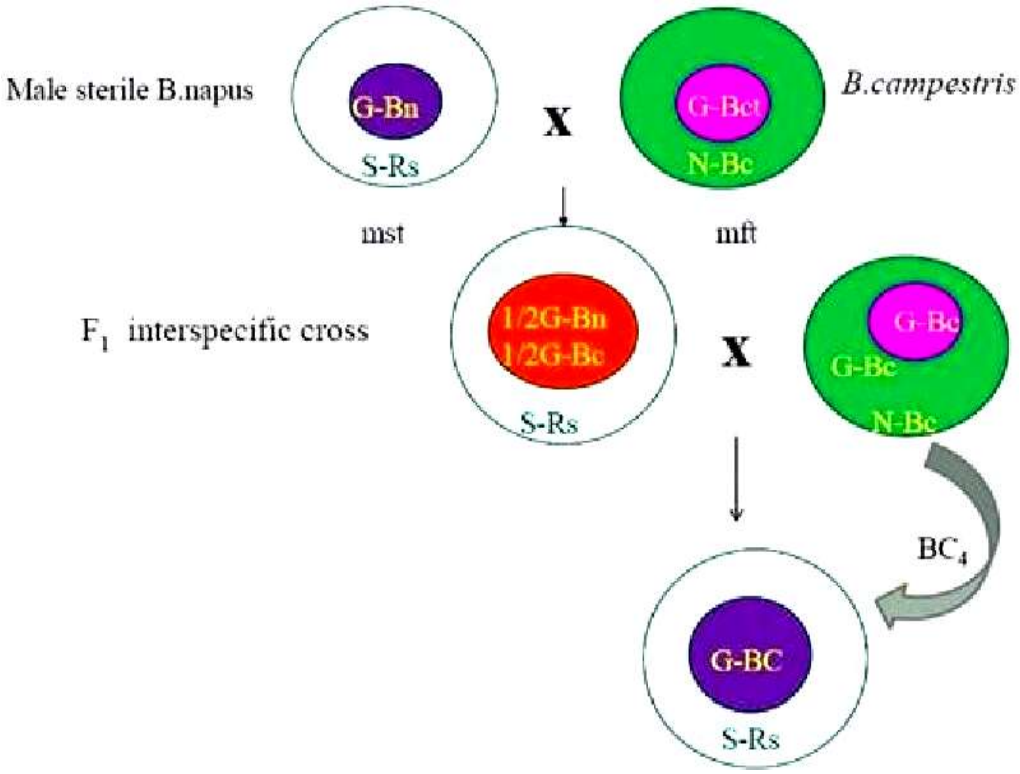
Chemical Male Sterility

- **Enthrel** – *Brassica juncea*
- **Zinc methy arsenate** – *B. napus*
- **GA** – *B. oleracea* var *capitata*

Development of Male sterile *B. napus* from *R. sativus*



Development of Alloplasmic Male sterile *Brassica campestris*



Male sterility system in Sunflower hybrid seed production

Genetic Male sterility (GMS)

- **Complete male sterility**
  - ✓ *ms1-ms5* = male sterility in sunflower recessive gene
- **Two types of g-mst**
  - ✓ Type 1-gmst-Bloomington type
  - ✓ Type 2-gmst-Modern type
- Cultivated Sunflower variety Karlik-68(Dwarf 68)- two recessive genes *msi1*, *msi2* (Stable and complete male sterile)
- **Partial male sterility —p mst**

CGMS	
H. petiolaris x *H. anmms*	Repeated backcross of H.annuus results in crnsl which is extensively used mst in hybrid seed production of sunflower all over the world
H. giganteus x *H. annuus*	Cmss (S cytoplasm source)
H. annuus subspp *lenticuiaris* x *H. anmms* CV *commander*	Indiana I

Male sterility system in Cotton hybrid seed production

All three types of male sterility occurs (g mst, c mst,gc mst) in cotton Genetic Male Sterility (GMS):

- **Genetic Male Sterility (GMS)**
  - Reported in upland, Egyptian and arboreum cottons.
  - In tetraploid cotton, male sterility is governed by both recessive and dominant genes.
  - However, male sterility governed by recessive genes is used in practical plant breeding
  - Sixteen different genes in tetraploid cottons (13 in *G. hirsutum* and 3 in *G. barbadense*) and two in *G. arboreum* have been identified for genetic male sterility.
  - Sterility is conditioned by dominant alleles at five loci viz, MS4, MS7, MSIO, MSII and MS12 by recessive allele at other loci viz. msl, ms2, ms3, ms13, ms14 (Dong A), ms15 (Lang A) and ms16 (81 A).
  - *G. hirsutum* line Gregg (MS 399) from USA is the basic source of GMS possessing ms5 ms6 gene for male sterility.

Gene	Species
ms_1	*G hirsutum*
ms_2	*C. hirsutum*
ms_3	*G hirsutum*
ms_4	*G hirsutum*
ms_5ms_6	*C. hirsutum*
ms_7	*G hirsutum*
ms_8ms_9,	*G hirsuturn*
ms_{10}	*G hirsutvm*
ms_{11}	*G barbadense*
ms_{12}	*G barbadense*
ms_{13}	*G barbadense*

Male Sterility System	Genotypes
GMS (4%)	LRA 5166, SRT 1, DGMS l , HGMS 2, GAK 32A, SHGMS-9, DGMS2, SHC,W5
CMS	Germplam – G 67, DMS A-8, RCMS A-2. GSCMS-IS, 34, CAK32A, C 1412, C 1998, CAK 1234, LCMS 6, JK 119, DMSA IC 1547
	Varieties – Rajat, LH 900, Supriya, G, Cot 10, Laxmi, Abadhita, BN, K2, LRA 5166, H 777, G. Cot 14, Ganganagar Ageti, F 4 14, Bhagya, kh3, Narmada, Devira
GMS (2X)	GMS 4, GMS 2, GAK20A, GAK 09, SGMS 2, SGMS 4, RGMS A-2, ROMS 3,SGMS 13, GMS 4-1, GAK ISA, GAK26A, sujay, GAK 423, GAK8615
R line	NH 258, AKH 545, GSR 22, AKH 39R, LR29, AKH 26k, AKH 1167, GSR 6, DR6 DR I AKH-0l-143, LR 104

CMS System

In case of CMS, the originally discovered CMS sources involving *G. arboreum* and *G anomalum* cytoplasmic systems having interaction with ms3 locus were not found effective or stable under different environments. The only stable and dependable CMS source under varied environment was developed through the utilization of *G. harknessii*. The complete genome of *G. hirsutum* was transferred into the *G. harknessii* cytoplasm.

A single dominant gene 'Rf' from *G. harknessii* is essential for fertility restoration.

Fertility enhancer factor 'E' for this CMS restorer system was obtained from a *G. barbadense* stock. The harknessii system is reported to contribute to good agronomic properties and attraction to honey bees.

Sources of Male Sterility in Cotton

Source of ms cytoplasm	Nuclear genome
G. anomalum, *G. arboreum*, *G. harknessii*	*G. hirsutum*
G. anomalum, *G. arboreum*	Heat sensitive, less stable
G. harknessii x *G. hirsutum*	Stable CMS all over the environment
New sources of CMS	
G. aridum Skovt. x *G. hirsutum*	(D4)
G. trilobum x *G. hinsutum*	CMS 8 (D-8)
G. sturtianum x *G. hirsunnn*	CMS-C1
New sources of CGMS	
G. anomalum x *G. thurberi*	Cg-mst

Mutation

G. arboreum, the first spontaneous male sterility mutant was identified in variety DS-5

Chemical based male sterility

- ✓ FW 450(Sodium B-Dichloro-iso-butyrate)
- ✓ MH-30 (Maleic hydrazide)
- ✓ Ethidium bromide

Male sterility based hybrid Production

- ✓ GMS system. CPH2 (Suguna), First hybrid based on GMS released at CICR, RS, Coimbatore
- ✓ *G. harknessii* based cms with fertility restoration gene sources were used in developing the hybrid CAHH 468 (PKV Hy-3).

7. Heterosis: Concept, Types of heterosis, genetic and molecular basis of heterosis and inbreeding, utilization in crop improvement.

The term heterosis was first introduced by Shull in 1914.

It may be defined as the superiority of an F_1 hybrid over both of its parents in terms of yield or some other character. Generally, heterosis is manifested as an increase in vigour, size, growth rate, yield or some other characteristic. Usually, Hybrid vigour is used as synonym of heterosis. Some other terms used to describe one or the other feature of heterosis and the manner of their estimation are briefly discussed below –

Estimation of heterosis (Types of Heterosis):

1. Average heterosis:

It is the heterosis where F_1 is superior to **mid parent value** i.e. average of the two parental means. It is also term as relative heterosisand is estimated by following formula

$$\text{Average heterosis} = [(\bar{F}_1 - \overline{MP}) / \overline{MP}] \times 100$$

Where,

F_1 = Mean performance of F_1 hybrid

MP = Average of mean performances of the two parents $(\overline{MP} = (\bar{P}_1 + \bar{P}_2)/2]$

This type of heterosis is only of theoretical significance; it has no practical value. Further, it should be considered as a case of partial or complete dominance unless F_1 hybrid surpasses the parental range.

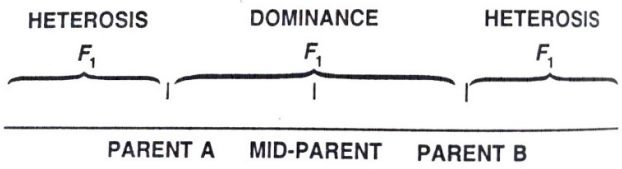


Fig: Heterosis and dominance depending on the performance of F_1 in relation to its parents.

2.

Heterobeltiosis: It refers to the superiority of an F_1 over the better parent. This term is uncommon in use and in fact it is called as heterosis. It is estimated as follows.

$$\text{Heterobeltiosis} = [(\bar{F}_1 - \overline{BP}) / \overline{BP}] \times 100$$
$$= \textit{Heterosis}$$

Where, BP = Mean performance of the better parent.

3. Economic heterosis:

It describes the superiority of an F_1 compared to the high yielding commercial variety in a particularcrop. This gives an idea of the usefulness of an F_1 hybrid as a commercial variety. It is also called useful heterosis or standard heterosis and is estimated by the following formula:

Economic heterosis= (F– cv)/cv*100
cv= commercial cultivar/variety mean value

Heterosis is of two types:

True heterosis (euheterosis) and pseudo-heterosis.

1. True heterosis:

It is inherited.

It can be further divided into two types:

(a) Mutational true heterosis:

It is the sheltering or shadowing of the deleterious, un-favourable, often lethal, recessive mutant genes by their adaptively superior dominant alleles.

(b) Balanced true heterosis:

It arises out of balanced gene combinations with better adaptive value and agricultural usefulness.

2. Pseudo-heterosis:

Crossing of the two parental forms brings in an accidental, excessive and un-adaptable expression of temporary vigour and vegetative overgrowth. It is also called **luxuriance**.

Luxuriance: Luxuriance is the increased vigour and size of interspecific hybrids. The principal difference between heterosis and luxuriance lies in the reproductive ability of the hybrids. Heterosis is accompanied with an increased fertility, while luxuriance is expressed by interspecific hybrids that are generally sterile or poorly fertile. In addition, luxuriance may not result from either masking of deleterious genes or from balanced gene combinations brought together into the hybrid. Therefore, luxuriance does not have any adaptive significance.

History:

- **Koelreuter** (1763) was the first to report hybrid vigour in the hybrids of tobacco, Datura etc.
- **Mendel** (1865) observed this in pea crosses.
- **Darwin** (1876) also reported that inbreeding in plants results in deterioration of vigour and the crossing in hybrid vigour.
- **Beal** (1877-1882) concluded that F_1 hybrids yield as much as 40 percent more of the parental varieties.
- The dominance hypothesis was proposed **Davenport** in 1908 and it was later elaborated by **Keeble 88 and Pellew in 1910**
- The overdominance hypothesis was put forth by **East and Shull** in the same year, i.e., 1908. In 1912, East and Hays advocated heterosis breeding as an alternative plant breeding strategy.
- The concept of double cross hybrids was proposed by **Jones in 1917**, while that of top cross hybrids was advanced by **Davis** in 1927.

Heterosis in Cross- and Self-PollinatedSpecies:

In general, cross-pollinated species show heterosis, particularly when inbred lines are used as parents. In many cross-pollinated species, heterosis has been commercially exploited, for example, in maize, bajra, jowar, cotton, sunflower, onion (*A. cepa*), alalfa, etc. Many crosses in self-pollinated species also show heterosis, but the magnitude of heterosis is generally smaller than that in the case of cross-pollinated species. But in some self-pollinated crops, heterosis is large enough to be used for the production of hybrid varieties. Hybrid varieties are commercially used in some vegetables, such as tomato, where a single fruit produces a large number of seeds, and in crops like rice. The chief drawback in the use of hybrid varieties in self-pollinated crops is the great difficulty encountered in the production of large quantities of hybrid seed.

Manifestation of Heterosis:

Performance or expression of any character or trait is influenced by many genetic factors — some are positive (stimulating) and others are negative (decreasing). Expressivity of the genes or the degree of manifestation of a character is the result of genetic balance in the action of differently directed factors.

The various manifestations of heterosis may be summarised as follows:

1. Increased Yield:

Increase in yield which may be measured in terms of grain, fruit, seed, leaf, tuber or the whole plant is one of the most important manifes-tations of heterosis.

2. Increase in Size and General Vigour:

Heterosis results in more vigorous growth which ultimately leads to healthier and faster growing plants with increase in size than the parents.

3. Better Quality:

In many cases heterosis yields better quality which may be accompanied with higher yield.

4. Greater Adaptability:

Hybrids are generally more adapted to environmental changes than the inbred lines due to heterozygosity.

5. More Disease Resistant:

Heterosis sometimes results into development of more disease resistant character in the hybrids.

6. Increased Reproductive Ability:

Hybrids exhibit heterosis by expressing high fertility rate or reproductive ability, which is ultimately expressed in yield character.

7. Increase in Growth Rate:

In many cases the hybrids show faster growth rate than the parents, but that does not always produce larger plant size than the parents.

8. Early Flowering and Maturity:

In many cases the hybrids may show early-ness in flowering and maturity than the parents, for some crops these are the desir-able characters for crop improvement. All these manifestations of heterosis can be traced at all levels of hybrid plant organisation.

Genetic bases of heterosis and inbreeding depression:

Heterosis and inbreeding depression are closely related phenomena. In fact, they may be regarded as the opposite sides of the same coin. Therefore, genetic theories that explain heterosis also explain inbreeding depression. There are three main theories to explain heterosis and, consequently, inbreeding depression: (1) dominance, (2) over dominance, and (3) epistatis hypotheses.

(A) Dominance Hypothesis:

This hypothesis was proposed by Davenport and further expanded by others. This hypothesis suggests that at each locus dominant allele has the favourable character, whereas the recessive allele has the unfavourable character.

When they are combined together; i.e., in heterozygous condition in the hybrids, the favourable characters get expressed whereas the unfavourable characters are masked. So the heterosis results from the masking of harmful effects of recessive alleles by their dominant alleles.

Dominance Hypothesis has Assumptions:

(a) Dominant genes are beneficial and recessive genes are deleterious.

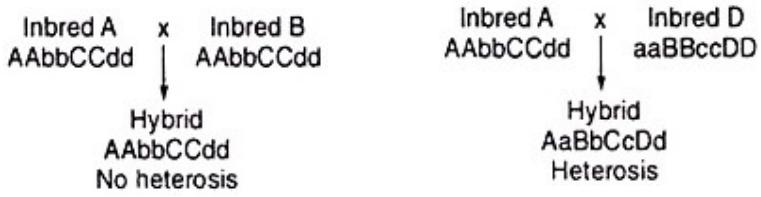
(b) The loci show addition effects, non-allelic interactions are absent.

(c) No recombination barrier between the genes.

With the help of following example heterosis can be explained:

In a cross between Inbred A (AAbbCCdd) with Inbred B (AAbbCCdd), there will be no heterosis in F1 hybrid, there is no masking of recessive gene in hybrid. But in another cross, Inbred A (AAbbCCdd) is crossed with Inbred D (aaBBccDD), where the F1 hybrid is (AaBbCcDd) with all the genes having dominant allele.

As a result, the harmful effects of a, b, c, d are hidden by the dominant alleles A, B, C and D. Thus, some parents produce heterotic progeny while others do not. Generally, parents of diverse or different origin are more likely to produce heterotic progeny than those of similar origin.



Objection:

1. Failure in Isolation of Inbreds as Vigorous as Hybrids:

According to domi-nance hypothesis it should be possible to get the inbred line with all the dominant genes. Such inbreds should be as vigorous as the F_1 hybrids, but such inbreds have not been isolated.

2. Symmetrical Distribution in F_2:

According to dominance hypothesis, the quan-titative characters should not show symmetrical distribution as because dominant and recessive alleles should segregate in the proportion of 3:1, but generally the F_2 shows symmetrical distribution.

Explanations for the Objections:

In 1917, **Jones** suggested that since quantitative characters are governed by many genes, these genes are likely to show linkage. It may be expected that dominant and recessive genes governing a character would be linked together. In such a case, inbreds containing all the dominant genes cannot be isolated because this would require several

precisely placed crossovers. It would also explain the symmetrical curves obtained in F2. This explanation is often known as the **dominance oflinked genes hypothesis**.

Later in 1921, **Collins** showed that if the number of genes governing a quantitative character was large, symmetrical distribution would be obtained even without linkage. Further, it is unlikely that a plant containing all the dominant genes would be recovered if the number ofgenes were large even if they were not linked. The distribution curve would further become symmetrical due to the effects of environment, that is, due to less than 100 per cent heritability.

(B) Over-dominance Hypothesis:

This hypothesis was independently proposed by **East and Shull**. This is sometimes known as single gene heterosis, super-dominance, cumulative action of divergent alleles and stimulation of divergent alleles. According to this hypothesis, heterozygotes are superior to both the homozygotes.

So the heterozygote Aa would be superior to both the homozygotes AA and aa. Consequently, heterozygosity is essential for the cause of heterosis. In case of maize, the gene ma affects maturity. The heterozygote Ma/ma is more vigorous with late maturity than the homozygotes Ma/Ma or ma/ma.

Another proposal by East was that there are several alleles, e.g., a_1, a_2, a_3, a_4…….. etc. with increasingly different functions. Heterozygotes between more divergent alleles would be more heterotic than those involving less divergent genes, e.g., a_1a_4 is more heterotic than a_1a_2, a_2a_3, a_3a_4, etc. In these cases, due to presence of divergent alleles the hybrids have the capacity to perform different functions which is not possible by any of the heterozygotes.

Objection:

1. There are many examples where the superiority is due to the epistatic affect of several non-allelic genes, not due to over-dominance (which is the interaction between allelic genes).

2. There is another objection against over-dominance hypothesis that there are many examples where the homozygotes are superior to the heterozygotes.

Comparison between Dominance and Overdominance Hypotheses

The two hypotheses lead to similar expectations, but they do differ from each other with respect to some expectations. The similarities and differences between them are listed below -

Similarities:

The two hypotheses have the following similarities.

1. Inbreeding would produce inbreeding depression.
2. Outcrossing would restore vigour and fertility.

3. The degree of heterosis would depend upon the genotypes of the two parents. In general, the greater the genetic diversity between the parents, the higher the magnitude of heterosis.

Differences:

The chief differences between the two hypotheses are

1. Heterozygotes are superior to the two homozygotes according to the overdominance hypothesis, while according to the dominance hypothesis they are as good as the dominant homozygote.

2. Inbreds as vigorous as the F, hybrid can be isolated according to the dominance hypothesis, but it will be impossible according to the overdominance hypothesis.

3. According to dominance hypothesis, inbreeding depression is due to homozygosity of harmful recessive alleles, while as per overdominance hypothesis, it is due to homozygosity itself.

4. According to the overdominance hypothesis, heterosis is the consequence of heterozygosity per se. But as per dominance hypothesis it is the result of dominant alleles masking the deleterious effects of their recessive alleles, and heterozygosity itself is not the cause of heterosis.

Table: Comparison between Dominance and Overdominance Hypotheses

Feature	*Hypothesis*	
	Dominance	*Overdominance*
Effects of inbreeding	Deleterious	Deleterious
Cause of inbreeding depression	Homozgosity of deleterious recessive alleles	Homozygosity *per se*
Effects of homozygosity	Deleterious *only in case of recessive alleles*	Deleterious
Crossing between divergent lines/strains	Results in heterosis	Results in heterosis
Cause of heterosis	Deleterious effects of recessive alleles masked by their dominant alleles	Heterozygosity *per se*
Heterozygote performance	Comparable to that of the homozygote for all dominant alleles	Superior to homozygotes
Effects of heterozygosity	Heterosis: *only if the two parents have different dominant alleles.*	Heterosis
The degree of heterosis	Increases with the diversity of	Increases with the diversity parents of parents
Isolation of inbreds as good as F_1 hybrid	Possible	Impossible
Most likely contribution to practical cases of heterosis	Major	Minor

Epistasis Hypothesis:

In 1952, **Gowen** had suggested that influence of one locus on the expression of another may be involved in heterosis. Subsequently, considerable data has accumulated to implicate epistasis as a cause of heterosis. For example, a majority of heterotic crosses show significant epistasis. But all heterotic crosses do not show epistasis, and all crosses that show epistasis are not heterotic. In many cases, the effects of a single homozygous successive allele are epistatic to almost the whole genetic make-up of an inbred. When the effects of such an allele are masked by its dominant allele, the effects on heterosis are usually dramatic (Stuber 1994).

However, epistatic variance usually forms only a much smaller component of the total genetic variance than do additive and dominance variances.

Theoretically, epistatic interactions will lead to the maximum heterosis when the following two conditions are met with. (1) First, the epistasis should be predominantly of complementary type, i.e., the estimates of h (dominance effects) and / (dominance x

dominance interaction effects) have the same sign so that they do not cancel each other out. Second, the interacting pairs of genes should be dispersed in both the parents. It has been suggested that in the absence of overdominance, dispersion (between the two parents of hybrids) of genes showing complementary epistasis seems to be the major cause of heterosis. In many experiments, multiplicative interaction has been reported as a cause of heterosis; it was concluded that in such cases, epistatic effects are nonlinear functions of the one-locus involve several mutually interacting genes.

Molecular Basis:

Molecular analysis was performed to assess protein, epigenetic, transcription, and other gene regulatory components that contribute to heterosis to investigate the underlying structure that impacts the degree of hybrid vigour divergence between hybrids and parental inbreds.

- **Transcriptome studies:**

The transcriptome analysis of successful parental inbred lines and hybrids has been carried out in order to categorize diverse gene expression designs into types of gene activity in a hybrid combination as opposed to its parental inbred lines, as well as to link those alterations to improvements in biological yield and yield production.

Gene interaction between the nucleus and the cytoplasm happens during the hybridization of two inbred parental lines, resulting in cellular and molecular changes as well as a shift in gene expression pattern. These alterations in gene expression and genome function in the F_1 hybrid via its inbred parental lines have been seen in a number of cereal hybrid crops, including maize, wheat, and cotton. Transcriptome analysis, and its capacity to quantify the degree of contribution of each allele in hybrid progeny, might be seen as a transitional phase between phenotypic expression and genetic information in plants. Early transcriptome investigations on a range of crops revealed that hybrids outperformed parental inbred lines in terms of gene expression patterns. Although transcriptomic investigations in reciprocal hybrids were enhanced in order to discover allele-specific expression, the value of maternal or paternal influences on gene expression patterns could not be identified. Maize and *Arabidopsis* have

recently shown increased biomass as a result of epigenetic modifications in circadian clock genes and variances in gene expression patterns caused by differentially generated short. Surprisingly, it has been shown that a single blossoming gene's over-dominant manner of gene activity generates yield heterosis. In any case, it is crucial to recognize that distinct geneexpression patterns in inbred lines and hybrids do not necessarily result in varied protein production.

- **Proteomics studies:**

Although changes in primary transcriptional activity may not always result in proteins with altered gene expression, and detecting heterosis is reliant on post-transcriptional regulation and translation processes, proteins play a vital role in heterosis detection. As a consequence of the lack of stable protein levels, parental inbred lines have enhanced protein metabolism, which requires a significant amount of energy to suppress, resulting in a lack of liveliness for biological synthesis, vegetative growth, and production. Inbred parental lines' genetic makeup is primarily due to a lack of intra-allelic interaction in their own homozygous state, whereas F_1 hybrids will have multiple alleles and produce many more allelic combinations, allowing for higher development caused by rapid cell division and resulting in hybrid vigour. The majority of the DEPs responsible for heterosis have been found in tissue samples from major cereal crop species such as rice, maize, and wheat leaves, embryos, and. The majority of DEPs found in parental inbred lines and their hybrids are due to non-additive gene effects, and these DEPs are linked to a variety of plant metabolic pathways, including photosynthesis, transcriptional regulation, disease resistance, glycolysis, carbon metabolism, protein, amino acid metabolism, and others.

- **Epigenomic studies**:

When two distinct parental inbred lines are crossed, epigenetic changes such as histone acetylation, chromatin remodeling, modest RNAi regulation, and DNA methylation occur. In most crop species, DNA methylation is the most essential regulator of genome-related activity and cellular development. Most crops have their DNA methylated as a consequence of the deposition of DNA methyl transferase at the 5th position of cytosine. The overall frequency of DNA methylation in hybrids varies according to the genetic

variety of the parental inbred lines. The repressininitiated transcription pathway, which either blocks the regulatory genetic causes of inbreeding depression or promotes gene expression for heterosis, is primarily responsible for the appearance of heterosis through DNA methylation. The methylation sites of inbred parental lines are often connected to methylation sites in hybrid progenies. Certain methylated regions in parental inbred lines are covered by siRNA levels, suggesting that DNA methylation is connected to RNA (RNA-directed DNA Methylation), which may promote remodelling in DNA methylated regions of hybrid progenies to exploit. Histones undergo post-translational changes such acetylation, methylation, and phosphorylation for amino acids at N-Terminal Tails. The majority of these changes take place in histone proteins like H3K9ac and H3K4me3, which are present in actively expressed euchromatic sites. Histones are in charge of the transcriptome of a maize hybrid.Endosperm transcriptome and histone HTA112 endosperm transcriptome revealed much higher gene expression variety than inbred parental lines.

Quantitive Trait Loci (QTL) and Heterosis:

The essential concept that aided molecular understanding of heterosis by making molecular markers accessible, allowing for a more precise method to mapping genes and detecting them in complicated phenotypes. These molecular markers aid in the identification of genomic sequences involved in heterosis. The quantitative trait loci (QTL) for specific variables associated in the formation of heterosis in parental inbred lines were found using Marker Assisted Selection. However, it is a complicated concept that is difficult to apply well. Several marker-assisted QTL investigations have failed to detect epistasis or the degree of epistasis. The difficulties in identifying specific heterotic traits and the loci that regulate them when employing RIL (Recombinant Inbred Line), backcross, and F_2 populations are mostly due to epistasis effects across several segregating loci of the whole genome. Although QTL does not generally rule a single agronomic trait, in nature it controls all of them and is mediated by a number of genes at several loci. Advances in QTL and genetics enabled the identification of the

expression. Brem et al. (2005) made great strides in finding genetic connections between heterosis-related genes. QTL Analysis is the future, and it will have a significant influence on current approaches in QTL analysis for genetic dissection and trait manipulation. Several genetic techniques will be employed to aid in the research, appraisal, and interpretation of heterosis in order to get a better understanding of it.

Inbreeding:

Mating between individuals related by descent or ancestry is called **inbreeding**. When the individuals are closely related, e.g., in brother-sister mating or sib mating, the degree of inbreeding is high. The highest degree of inbreeding is achieved by selfing. The chief effect of inbreeding is an increase in homozygosity in the progeny, which is proportionate to the degree of inbreeding. The degree of inbreeding of an individual is expressed as **inbreeding coefficient (F)**. The degree inbreeding is proportional to degree of homozygosity.

Inbreeding depression may be defined as the reduction or loss in vigour and fertility as a result of inbreeding.

$$\textbf{Inbreeding depression} = F_1\text{-}F_2 \,/\, F_1 \times 100$$

Effects of inbreeding:

Inbreeding is accompanied with a reduction in vigour and reproductive capacity i.e. fertility. There is a general reduction in the size of various plant parts and in yield. In many species, harmful recessive alleles appear after selfing; plants or lines carrying them usually do not survive. The different effects of inbreeding are:

- Inbreeding is accompanied with a reduction in vigour and reproductive capacity i.e. fertility.
- **Appearance of Lethal and Sublethal Alleles**: Inbreeding results in appearance of lethal; sublethal and subvital characters. Eg: Chlorophyll deficiencies, rootless seedlings, flower deformities.
- **Reduction in vigour:** General reduction in vigour size of various plant parts.

- **Reduction in Reproductive ability:** Reproductive ability of population decreases rapidly. Many lines reproduce purely that they cannot be maintained.
- **Separation of the population into distinct lines:** population rapidly separates into distinct lines i.e. due to increase in homozygosity. This leads to random fixation of alleles in different lines. Therefore, lines differ in genotype and phenotype. It leads to increase in the variance of the population.
- **Increase in homozygosity**: Each lines becomes homozygous. Therefore, variation within a line decreases rapidly. After 7-8 generations of selfing the line becomes more than 99% homozygous. These are the inbreds. These have to be maintained by selfing.
- **Reduction in yield**: Inbreeding leads to loss in yield. The inbreds that survive and maintained have much less yield than the open pollinated variety from which they have been developed.

Degrees of inbreeding depression:

The degree of inbreeding depression depends on the plant species concerned. But within species, the extent of depression is related to the value of F and the relative fitness of the trait in question. Inbreeding depression is common in the case of such traits that form an important component of fitness, while those that contribute little to fitness usually show littleor no inbreeding depression. The extent of inbreeding depression observed in various plantspecies may be grouped into the following four categories:

1. High inbreeding depression:

In such species, as alfalfa, carrot, etc., a largeproportion of plants produced by inbreeding/selfing does not survive. Loss in vigour andfertility is so high that only few lines can be maintained after 3-4 generations of inbreeding.The yields of surviving inbred lines are usually <25% of that of the parent open-pollinatedvarieties.

2. Moderate inbreeding depression:

In species like maize, jowar (sorghum), bajra(pearlmillet), etc., a large proportion of plants can be maintained under self-pollination. Inbred lines may yield as much as 50% ofthe parent open-pollinated varieties.

3. Low inbreeding depression:

Eg: Onion, many Cucurbits, Rye and Sunflower etc. show a small degree of inbreeding depression. A small proportion of the plants show lethal or subvital characteristics. The loss in vigour and fertility is small; rarely a line cannot be maintained due to poor fertility. There is little or no reduction in yield and someinbred lines may yield as much as the parent open-pollinated varieties.

4. Lack of inbreeding depression: The self-pollinated species do not show inbreeding depression, although they do show heterosis. It is because these species reproduce by self-fertilization and as a result, have developed homozygous balance.

Utilization of heterosis in crop improvement

Commercially heterosis is observed in almost every crop species studied. Often the degree of heterosis is considerably high to permit its commercial exploitation. Heterosis is commercially used in the form of hybrid or synthetic varieties. Such varieties have been most commonly used in cross-pollinated and often cross-pollinated crop species. In several selfpollinated species also hybrid varieties have been commercially used. Attempts have been made to utilize heterosis higher price than in the case of those that fetch a lower price. Further, the quantum of additional production will increase with the level of useful heterosis, and also with the average yield/ha of the standard varieties of the crop. Therefore, the level of heterosis required to generate a given quantum of additional yield will depend on the average yield of crop; it will be lower in crops having higher yields than in crops having lower yields. The commercial significance of hybrid technology may be illustrated with the singular success of hybrid maize in U.S.A. The yield of open-pollinated maize varieties ranged between -20 and -32 bushels per acre between 1870 and 1930. Around this time, double cross maize hybrids were introduced; their yields increased steadily from -25 bushels per acre during 1935 to -55 bushels/acre during 1960s. The introduction of single cross hybrids around this time

marked a quantum jump in maize yields; it started from -62 bushels/acre in 1960 and rose to -120 bushels/acre by 1990. These data, and those from many other countries, demonstrate the unquestionable superiority of single cross maize hybrids over other varietal forms. Similarly, hybrid rice has become quite popular in China. The first hybrid variety of rice was released in 1976, and by 1997 hybrid rice occupied -54% of the total paddy area and contributed nearly 64% of the total paddy production in China.

A list of some examples of plant and animal species where heterosis is being commercially exploited

Category	*Examples*
Crop species	1. *Asexually propagated species* 2. *Cross-pollinated species*: maize, jowar, bajra, sugarbeets, sunflower, forage grasses, castor, forage legumes, and cotton 3. *Self-pollinated crops*: rice, pigeonpea (India)
Vegetable crops	Tomato, brinjal (*Solanum melongena*), onion, Brussel's sprouts, Watermelon, pepper, winter sqash, muskmelon, cabbage, broccoli, spinach, red beets, carrot cauliflower, celery, asparagus
Fruit trees	In almost all the fruit trees

8. Molecular Markers: Development of molecular markers; trends and progress, RFLP, PCR based, single locus and multi-locus markers, NGS based markers; Applications in crop improvement.

Molecular Markers: Development of molecular markers; trends and progress:

The last 30 years have witnessed a continuous evolution of new molecular marker systems from restriction fragment length polymorphisms, random amplified

polymorphic DNAs, and amplified fragment length polymorphisms to present-day popular marker systems such as simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), and diversity array technologies. Advent of low-cost and high throughput sequencing technologies, commonly called next-generation sequencing (NGS) technologies have increased the speed of SSR and SNP discovery. NGS technologies in combination with restriction enzymes are now ready for detecting genome-wide polymorphism and new marker systems like RAD-tag sequencing, genotyping by sequencing are becoming popular. It seems that NGS-based marker systems will be dominating marker systems in future. These new emerging marker systems are expected to facilitate enhanced adoption of modern genetics and breeding approaches like genome-wide association studies and genome-wide selection that generally require markers at high-density in crop plants.

Marker development:

Molecular markers have changed the entire scenario of life sciences with their broad application to understand the cellular and molecular responses, providing breeders a simple but powerful weapon for precision selection of a desired genotype. Generally, transformation techniques depend on the availability of the candidate gene and efficient transformation regeneration protocols. The availability of a variety of molecular markers and high-density genetic linkage maps has broadened the field of conventional breeding for the identification of the desired lines with complex traits via, marker-assisted selection (MAS).

A molecular marker is a DNA sequence with a known position on a linkage map/chromosome that may or may not be linked with phenotypic expression of a gene that can easily distinguish between two closely related individuals (These are effectively used for a variety of applications, viz., forensics, paternity assessment, disease diagnosis, systematics, phylogenetic studies, conservation biology, etc., and provide an efficient way linking genotypic and phenotypic variation.

The ideal properties of markers are:

- The marker should be polymorphic between the two individuals of the choice.

- The inheritance pattern of co-dominant with even distribution throughout the genome.
- The marker should be simple, inexpensive and quick to assess.
- The marker should require less quantity tissue or DNA samples for its analysis.
- Sequence-based genome information is not a prerequisite.
- The marker should be highly reproducible facilitating data exchange between different laboratories.
- They should have an easy way to assess showing strong heritability from generation to generation.
- The marker should have a close association with desired phenotype selectively neutral to environmental conditions.

For any kind of a single marker system, it is very difficult to have all of the above properties. Depending on the experimental goals, every marker system has its advantages and disadvantages that differ in its total genome coverage, locus specificity, reproducibility, and stable inheritance, the ease with which it can be detected and total cost-effectiveness per genotype analysis.

Marker types:

A **bio-marker, or biological marker** is a measurable indicator of some biological state or condition.

Markers are of four types, viz: (i) Morphological, (ii) Biochemical, (iii) Cytological, and (iv) Molecular or DNA markers.

These are briefly discussed as follows:

i. Morphological:

In plant breeding, markers that are related to variation in shape, size, colour and surface of various plant parts are called morphological markers. Such markers refer to available gene loci that have obvious impact on morphology of plant. Genes that affect form, coloration, male sterility or resistance among others have been analyzed in many plant species.

In rice, examples of this type of marker may include the presence or absence of awn, leaf sheath coloration, height, grain color, aroma etc. In well-characterized crops like maize, tomato, pea, barley or wheat, tens or even hundreds of such genes have been assigned to different chromosomes.

There are several demerits of morphological markers as given below:

a. They generally express late into the development of an organism. Hence their detection is dependent on the development stage of the organism.

b. They usually exhibit dominance.

c. Sometimes they exhibit deleterious effects.

d. They exhibit pleiotropy.

e. They exhibit epistasis.

f. They exhibit less polymorphism.

g. They are highly influenced by the environmental factors.

ii. Biochemical:

Markers that are related to variation in proteins and amino acid banding pattern are known as biochemical markers. A gene encodes a protein that can be extracted and observed; for example, isozymes and storage proteins.

iii. Cytological:

Markers that are related to variation in chromosome number, shape, size and banding pattern are referred to as cytological markers. In other words, it refers to the chromosomal banding produced by different stains; for example, G banding.

iv. Molecular Markers:

A molecular marker is a DNA sequence in the genome which can be located and identified. As a result of genetic alterations (mutations, insertions, deletions), the base composition at a particular location of the genome may be different in different plants.

These differences, collectively called as polymorphisms can be mapped and identified. Plant breeders always prefer to detect the gene as the molecular marker, although this

is not always possible. The alternative is to have markers which are closely associated with genes and inherited together.

The molecular markers are highly reliable and advantageous in plant breeding programmes:

- **Polymorphism:**
  Markers should exhibit high level of polymorphism. In other words, there should be variability in the markers. It should demonstrate measurable differences in expression between trait types and/or gene of interest.
- **Co-Dominant:**
  Marker should be co-dominant. It means, there should be absence of intra-locus interaction. It helps in identification of heterozygotes from homozygotes.
- Even distribution across the whole genome (not clustered in certain regions)
- Clear distinct allelic features (so that the different alleles can be easily identified)
- Single copy and no pleiotropic effect
- Low cost to use (or cost-efficient marker development and genotyping)
- Easy assay/detection and automation
- High availability (un-restricted use) and suitability to be duplicated/multiplexed (so that the data can be accumulated and shared between laboratories)
- Genome-specific in nature (especially with polyploids)
- No detrimental effect on phenotype

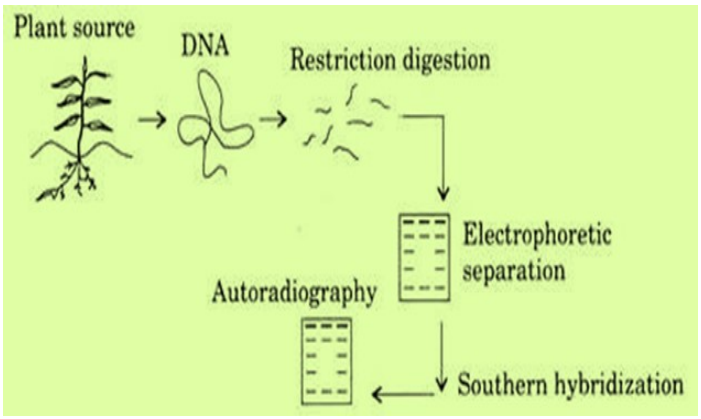
DNA based markers can be classified as hybridization-bascd markers and polymerase chain reaction (PCR)-based markers. In hybridization-based markers a labeled probe is used to visualize the DNA profile of restricted DNA vz. RFLP. Whereas, PCR based markers involve in vitro amplification of particular DNA sequences or loC1, with the heip ot specifically or arbitrary chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments (amplicons) are separated clectrophoretically and banding pattern are detected by different methods such as staining and autoradiagraphy, as in the case of RAPD, microsatellites, STMS and EST.

Types and description of DNA markers:

Markers Based on DNA Hybridization:

The DNA piece can be cloned, and allowed to hybridize with the genomic DNA which can be detected. Marker-based DNA hybridization is widely used. The major limitation of this approach is that it requires large quantities of DNA and the use of radioactivity (labeled probes).

Restriction fragment length polymorphism (RFLP):



RFLP markers were used for the first time in the construction of genetic maps by Botstein et al. in 1980. The RFLPs markers are relatively highly polymorphic, codominantly inherited and highly reproducible. The polymorphism in restricted fragments due to DNA rearrangements that occur due to evolutionary processes, point mutations with in the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over. In RFLP, DNA polymorphism is detected by hybridizing a chemically labelled DNA probe to a Southern blot of DNA digested by restriction endonucleases, resulting in differential DNA fragment profile. Labelling of the probe may be performed with a radioactive isotope or with alternative nonradioactive stains, such as digoxigenin or fluorescein The hybridization results can be visualized by autoradiography, or using chemiluminescence. RFLPs correspond to DNA fragments, usually within the range of 2-10 kb, that have resulted from the digestion of genomic DNA with restriction enzymes. The differential profile is generated due to nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms. RFLPs are applied in diversity and phylogenetic

studies ranging from individuals within populations or species, to closely related species.

Steps:

Following are the steps involved in a typical RFLP assay

(1) Restriction digestion of genomic DNA using restriction endonuclease

(2) Resolving restriction genomic fragments through gel electrophoresis

(3) Transfer of resolved fragments from gel to nitrocellulose membrane using southern blotting

(4) Membrane containing DNA fragments hybridized with labeled probe using southerm hybridization

(5) Detection of polymorphism through autoradiography or chemiluminescent technique.

Based on the presence of restriction sites, DNA fragments of different lengths can be generated by using different restriction enzymes. In the Fig., two DNA molecules from two plants (A and B) are shown. In plant A, a mutation has occurred leading to the loss of restriction site that can be digested by EcoRI.

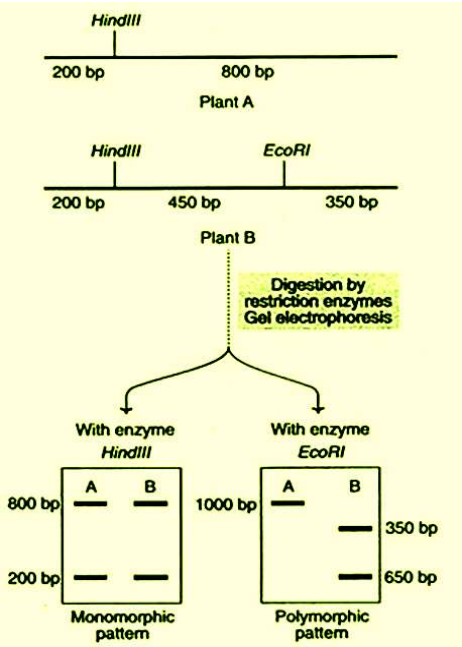


Fig. 53.3 : A schematic representation of restriction fragment length polymorphism (RFLP) analysis as molecular marker.

The result is that when the DNA molecules are digested by the enzyme Hindlll, there is no difference in the DNA fragments separated. However, with the enzyme EcoRI, plant A DNA molecule is not digested while plant B DNA molecule is digested. This results in a polymorphic pattern of separation.

Advantages of RFLP:

- ✓ Present everywhere,
- ✓ Mendelian inheritance,
- ✓ Co-dominant expression,
- ✓ No pleiotropic effects,
- ✓ Independent of the environment,
- ✓ Present at each developmental stage,
- ✓ Long stability of cDNA probes,
- ✓ Different loci may be identified by one probe,
- ✓ Heterologous genes may be used as probes,
- ✓ Any number of probes can be produced,
- ✓ Probes are producible for coding and silent sequences,
- ✓ Probes show the variability of flanking sequences,
- ✓ Several characters can be screened in the same sample.

Disadvantages:

Developing sets of RFLP probes and markers is labour intensive. This technique requires large amount of high-quality DNA. The multiplex ratio is low, typically one per gel. The genotyping throughput is low. It involves use of radioactive chemicals. RFLP finger prints for multi-gene families are often complex and difficult to score. RFLP probes cannot be shared between laboratories.

Uses:

They can be used in determining paternity cases. In criminal cases, they can be used in determining source of DNA sample. They can be used to determine the disease status of an individual. They are useful in gene mapping, germplasm characterization and marker

assisted selection. They are useful in detection of pathogen in plants even if it is in latent stage.

PCR based Marker:

Randomly Amplified Polymorphic DNA (RAPD):

In 1991, Welsh and McClelland developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD) This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer an arbitrary nucleotide (8-12 bp) sequence. The primer anneals to complimentary sequence in template DNA in forward or reverse direction at multitude location of genonme. The amplification occurs between forward and reverse annealing generally 150-4000 bp apart, by resolving the resulting amplicons, profile with multiple bands can be secen. No knowledge of the DNA sequence for the targetes gene is required, as the primers will bind somewherc in the sequence, but it is not certain exactly where. This marker show lack of reproducibility and the assay is sensitive variation in DNA concentration. They are dominant markers and hence have limtaions in their use as markers for mappng, which can be overcome to some extent by selecting those markers that are linked in coupling.

RAPD assay has been used by several groups as efficient tools for identication of markers linked to agronomicaly important traits, whch are introgressed during the development of near isogenic lines (NILs).

Steps:

Important steps of RAPD can be summarized as follows.

Extraction of DNA

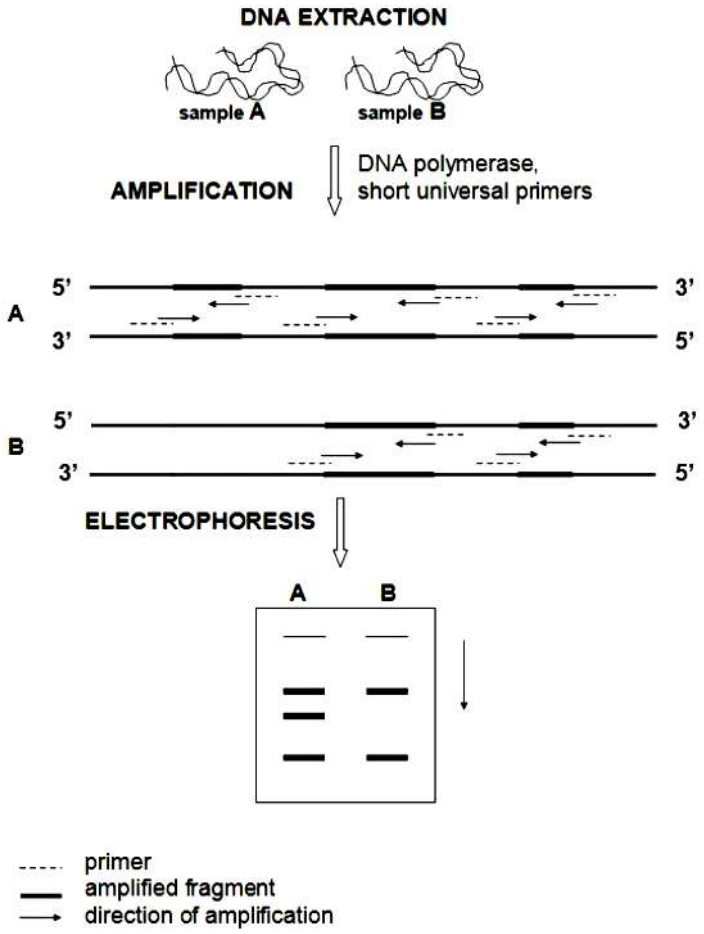
DNA is extracted by various methods. It also depends upon the species of plant under study but the basic procedure remains the same. Genomic DNA is basically extracted and purified from plant cell using proteinase K digestion and standard phenol: Chloroform extraction as per the standard protocol.

Selection of Primers

Random Amplified Polymorphic DNA (RAPD) is a multiplex marker system that conventionally uses single primer PCR to amplify random DNA fragments. Because of its

multiplex nature, it is frequently used in Bulked Segregant Analysis (BSA). In view of the very large numbers of markers BSA often requires the use of mixtures of primers as a method of increasing the number of markers available. Theoretically, if a singleprimer reaction produces x bands on average, an unrestrained PCR process using a primers should produce xa2 bands. A total of 40 random oligonucleotide primers were used for amplification. All the random primers were 10 bp long and with high GC content and were custom synthesized from M/s Bangalore Genei, Bangalore, India a Yadav and Yadav (2007). The standard RAPD technology utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR.

PCR Amplification



The polymerase Chain Reaction (PCR) is a relatively simple but powerful technique that amplifies a DNA template to produce multiple copies of specific DNA fragment in vitro. The bases (complementary to the template) are coupled to the primer on the 3' end (the polymerase adds dNTP's from 5'to 3', reading the template from 3'to 5' side; bases are added complementary to the template).

Using fresh clean tips, all the reagents are added to an autoclaved microfuge tube placed on ice. The PCR machine is programmed for the specific reaction conditions desired. After completion of the PCR reaction, the tubes are removed from the temperature block. The reaction products are separated according to size by agarose gel electrophoresis and visualized after staining the gel with ethidium bromide.

Agarose Gel Electrophoresis of PCR

After completion of the PCR programme, the products are checked in 2% agarose for the amplification. Before loading into the wells, gel loading dye (bromophenol blue in glycerol) is added to the sample and the samples are run under constant voltage condition (80 V) till the two dyes get separated. Amplified products appear as sharp orange color bands under UV Transilluminator due to the intercalation of ethidium bromide. To ensure that the amplified DNA bands originated from genomic DNA and not primer artifacts, negative control are carried out for each primer/breed combination (Galli and Satti, 2009) No amplification is detected in control reactions. All amplification products are found to be reproducible when reactions are repeated using the same reaction conditions.

Advantages:

RAPD primers are readily available being universal. They provide moderately high genotyping throughput. This technique is simple PCR assay (no blotting and no radioactivity). It does not require special equipment. Only PCR is needed. The start-up cost is low.

RAPD marker assays can be performed using very small DNA samples (5 to 25 ng per sample). RAPD primers are universal and can be commercially purchased. RAPD markers can be easily shared between laboratories. Locus-specific, co-dominant PCR-

based markers can be developed from RAPD markers. It provides more polymorphism than RFLPs.

Disadvantages:

The detection of polymorphism is limited. The maximum polymorphic information content for any bi-allelic marker is 0.5. This technique only detects dominant markers. The reproducibility of RAPD assays across laboratories is often low. The homology of fragments across genotypes cannot be ascertained without mapping. It is not applicable in marker assisted breeding programme.

Uses:

This technique can be used in various ways such as for varietal identification, DNA fingerprinting, gene tagging and construction of linkage maps. It can also be used to study phylogenetic relationship among species and sub-species and assessment of variability in breeding populations.

Amplified Fragment Length Polymorphisms (AFLP):

To overcome the limitation of reproducibility associated with RAPD, AFLP technology was developed. It combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers. The DNA is cut with two restriction enzymes, one being a frequent cutter and the other an infrequent cutter. This is followed by ligation of adapters, including restriction motifs followed by a two-step PCR amplification of selected fragments. The selective amplification uses primers composed of the adapters and 1 to 3 selected nucleotides at the 3' end. It limits the number of fragments to a resolvable range. The PCR-amplified fragments can then be separated by gel electrophoresis and banding patterns visualized. A range of enzymes and primers are available to manipulate the complexity of AFLP fingerprints to suit application. The AFLP banding profiles are the result of variations in the restriction sites or in the intervening region.

Steps

Step 1: DNA Extraction:

In the first step of AFLP clean and high molecular weight DNA is extracted using CTAB procedure.

Step 2: Restriction Digestion:

Restriction fragments of the genomic DNA are produced by using two different restriction enzymes: a frequent cutter (the four-base restriction enzyme Msel) and a rare cutter (the six-base restriction enzyme EcoRI).

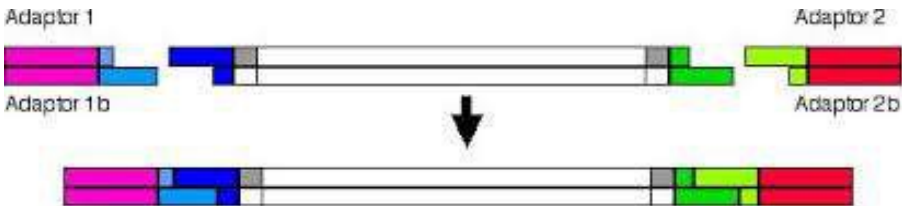
The frequent cutter serves to generate small fragments, which amplify well and which have the optimal size range for separation on a sequence gel, whereas the rare cutter limits the number of fragments to be amplified.

MseI	5'TTAA3'
EcoRI	5'GAATTC3'

Step 3: Ligation of Oligonucleotide Adapters:

Double-stranded adapters consist of a core sequence and an enzyme-specific sequence. Therefore, adapters are specific for either the EcoRI site or the Msel site. Usually restriction and ligation take place in a single reaction.

Ligation of the adapter to the restricted DNA alters the restriction site in order to prevent a second restriction from taking place after ligation has occurred. The core sequence of the adapters consists of a known DNA sequence of 20 nucleotides, which will be used later as primer in the PCR.



Step 4: Pre-Amplification:

This step is a normal PCR where the adapters are used as primers. This first PCR, called pre-amplification, allows a first selection of fragments by only amplifying the DNA restriction fragments that have ligated an adaptor to both extremities.

Step 5: Amplification:

The aim of this step is to restrict the level of polymorphism and to label the DNA. For this second amplification, we added three more nucleotides at the 3′ end of the primer sequence used for the pre-amplification (adaptors sequence + 3 nucleotides). These two additional nucleotides make the amplification more selective and will decrease the number of restriction fragments amplified (polymorphism).

Step 6: Electrophoresis:

The PCR products are denaturized and run on acrylamide gel.

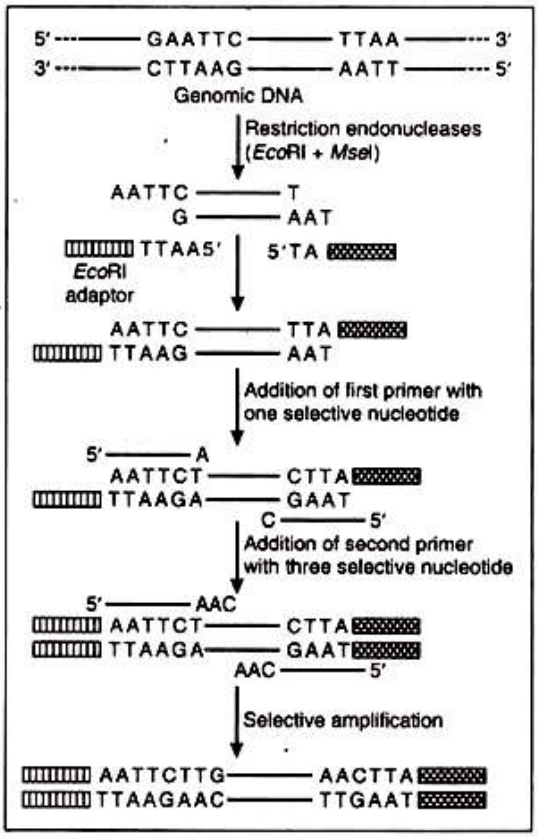


Fig. 8.4: Steps involved in AFLP

Advantages:

- ✓ It provides very high multiplex ratio and genotyping throughput. These are highly reproducible across laboratories.
- ✓ No marker development work is needed; however, AFLP primer screening is often necessary to identify optimal primer specificities and combinations.
- ✓ No special instrumentation is needed for performing AFLP assays; however, special instrumentation is needed for co-dominant scoring.
- ✓ Start-up costs are moderately low. AFLP assays can be performed using very small DNA samples (typically 0.2 to 2.5 pg per individual).
- ✓ The technology can be applied to virtually any organism with minimal initial development.

Disadvantages:

- The maximum polymorphic information content for any bi-allelic marker is 0.5. High quality DNA is needed to ensure complete restriction enzyme digestion. DNA quality may or may not be a weakness depending on the species. Rapid methods for isolating DNA may not produce sufficiently clean template DNA for AFLP analysis.
- Proprietary technology is needed to score heterozygotes and ++ homozygotes. Otherwise, AFLPs must be dominantly scored. Dominance may or may not be a weakness depending on the application.
- The homology of a restriction fragment cannot be unequivocally ascertained across genotypes or mapping populations. Developing locus specific markers from individual fragments can be difficult and does not seem to be widely done. The switch to non-radioactive assays has not been rapid. Chemiluminescent AFLP fingerprinting methods have been developed and seem to work well.
- The fingerprints produced by fluorescent AFLP assay methods are often difficult to interpret and score and thus do not seem to be widely used. AFLP markers often densely cluster in centromeric regions in species with large genomes, e.g., barley (*Hordeum vulgare* L.) and sunflower (*Helianthus annuus* L.)

Uses:

This technique has been widely used in the construction of genetic maps containing high densities of DNA marker. In plant breeding and genetics, AFLP markers are used in varietal identification, germplasm characterization, gene tagging and marker assisted selection.

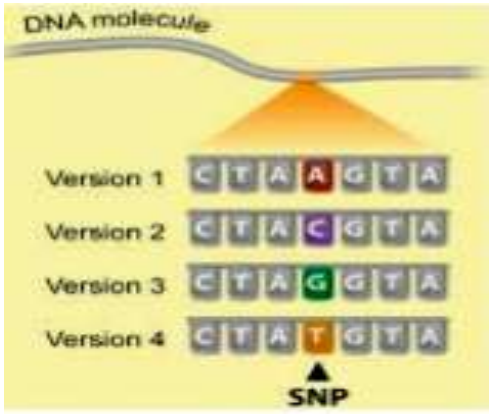
Single Nucleotide Polymorphism (SNP):

The variations which are found at a single nucleotide position are known as single nucleotide polymorphisms or SNP. Such variation results due to substitution, deletion or insertion. This type of polymorphisms has two alleles and also called bialleleic loci. This is the most common class of DNA polymorphism. It is found both in natural lines and after induced mutagenesis. Main features of SNP markers are given below.

1. SNP markers are highly polymorphic and mostly bialleleic.
2. The genotyping throughput is very high.
3. SNP markers are locus specific.
4. Such variation results due to substitution, deletion or insertion.
5. SNP markers are excellent long-term investment.
6. SNP markers can be used to pinpoint functional polymorphism.
7. This technique requires small amount of DNA.

SNPs are found in

- coding and (mostly) noncoding regions.
- Occur with a very high frequency
- about 1 in 1000 bases to 1 in 100 to 300 bases.
- The abundance of SNPs and the ease with which they can be measured make these genetic variations significant.
- SNPs close to particular gene acts as a marker for that gene.
- SNPs in coding regions may alter the protein structure made by that coding region.



SNPs Discovery

1. Sequence databases searches
2. Target specific SNP discovery and development

-Conformation-based mutation scanning

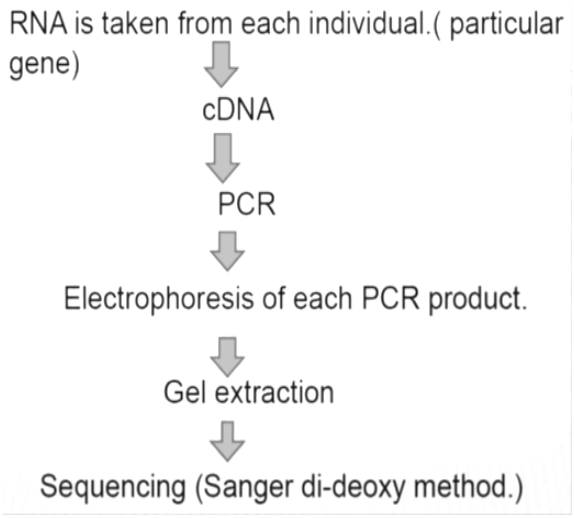
-Direct DNA sequencing

Identification of Target Specific SNPs

Steps:

1. Amplify the genes of interests with PCR
2. Scan for mutation with various methods
    - -Conformation-based mutation scanning
    - - Single -strand conformation polymorphism analysis
    - - Gel electrophoresis
    - - Chemical and enzymatic mismatch cleavage detection
    - - Denaturing gradient gel electrophoresis
    - - Denaturing HPLC
3. Align sequences from different sources to find SNPs
4. Sequence positive PCR products
    - -Sequence multiple individuals
    - -Sequence heterozygotes

Development of SNP (direct sequencing Method)



Technologies for Detecting Known SNPs

Gel-Based Methods

-PCR-restriction fragment length polymorphism analysis

-PCR-based allelic specific amplification

-Oligonucleotide ligation assay genotyping

-Minisequencing(10~20base)

Non-Gel-Based High Through Genotyping Technologies

-Solution hybridization using fluorescence dyes

-Allelic specific ligation

-Allelic specific nucleotide incorporation

1. High resolution separation

2. Chemical color reaction

-DNA microarray genotyping

Allele-Specific Codominant PCR Strategy

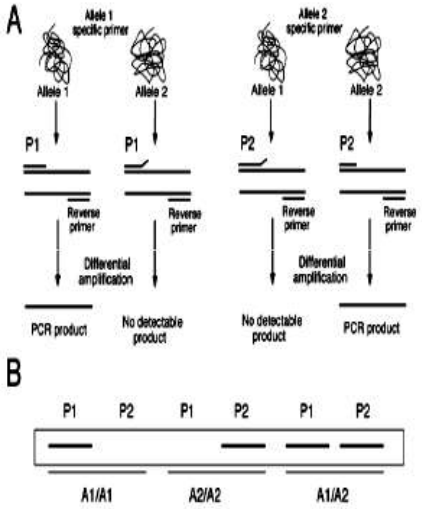


Figure. Schematic representation of the allele-specific codominant PCR strategy. Oligonucleotide primers with 3' nucleotides that correspond to an SNP site are used to preferentially amplify specific alleles.
A, Primer P1 forms a perfect match with allele 1 but forms a mismatch at the 3' terminus with the DNA sequence of allele 2. Primer P2 similarly forms a perfect match with allele 2 and a 3' terminus mismatch with allele 1.
B, Schematic of agarose gel analysis showing the expected outcome for the amplification of organisms homozygous and heterozygous for both alleles using primers P1 and P2. P1, Primer 1; P2, primer 2; A1, allele 1; A2, allele 2.

Eliana Drenkard et al. 2000 Plant Physiol 124: 1483-1492

Advantages:

SNP markers are useful in gene mapping. SNPs help in detection of mutations at molecular level. SNP markers are useful in positional cloning of a mutant locus. SNP markers are useful in detection of disease causing genes.

Disadvantages:

Most of the SNPs are bialleleic and less informative than SSRs. Multiplexing is not possible for all loci. Some SNP assay techniques are costly. Development of SNP markers is labour oriented. More (three times) SNPs are required in preparing genetic maps than SSR markers.

Uses:

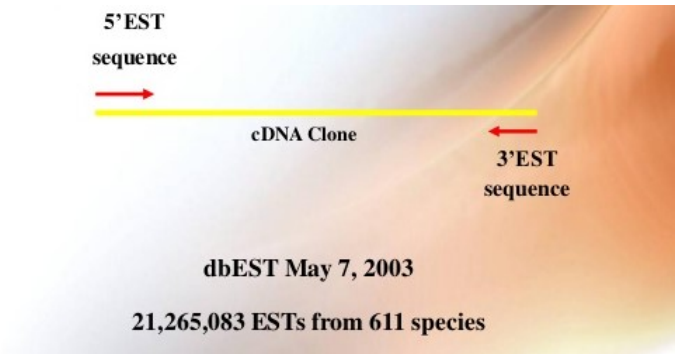
SNPs are useful in preparing genetic maps. They have been used in preparing human genetic maps. In plant breeding, SNPs have been used to lesser extent.

Expressed Sequence Tags (EST):

Expressed Sequence Tags (ESTs) are small pieces of DNA and their location and sequence on the chromosome are known. The variations which are found at a single nucleotide position are known. The term Expressed Sequence Tags (ESTs) was first used by Venter and his colleagues in 1991. Main features of EST markers are given below.

1. ESTs are short DNA sequences (200-500 nucleotide long).
2. They are a type of sequence tagged sites (STS).
3. ESTs consist of exons only.

Single-pass sequencing reads from randomly selected cDNA clone



Steps for ESTs

•cDNA libraries (containing many of the expressed genes of an organism)

•pick cDNA clones randomly

•rapidly determine some of the sequence of nucleotides from the end of each clone.

•These ESTs could then be compared to all known sequences using a program called BLAST.

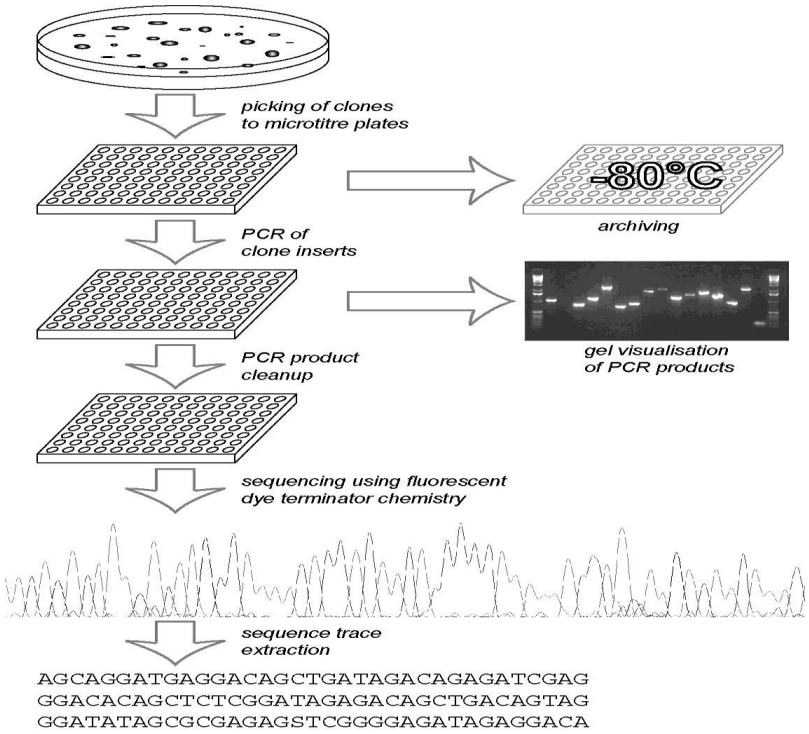
An exact match to a sequenced gene means that the gene encoding that EST is already known.

If the match was close but not exact one could conclude that the EST is derived from a gene with a function similar to that of the known gene.

The EST sequences with their putative identification are then deposited in the GenBank and the clones from which they were derived are kept in a freezer for later use.

Overview of the EST sequencing process

Clones are picked from petriedishes into microtitre plates, and archived for later use. All subsequent manipulations (PCR, clean up and sequencing) are carried out in microtitre plates to yield medium-throughput.



Advantages:

It is a rapid and inexpensive technique of locating a gene. ESTs are useful in discovering new genes related to genetic diseases. They can be used for tissue specific gene expression.

Disadvantages:

ESTs have lack of prime specificity. It is a time consuming and labour oriented technique. The precision is lesser than other techniques. It is difficult to obtain large (> 6kb) transcripts. Multiplexing is not possible for all loci.

Uses:

ESTs are commonly used to map genes of known function. They are also used for phylogenetic studies and generating DNA arrays.

Sequence Tagged Sites (STS):

In genomics, a sequence tagged site (STS) is a short DNA sequence that has a single copy in a genome and whose location and base sequence are known. Main features of STS markers are given below.

1. STSs are short DNA sequences (200-500 nucleotide long).
2. STSs occur only once in the genome.
3. STS are detected by PCR in the presence of all other genomic sequences.
4. STSs are derived from cDNAs.

Advantages:

STSs are useful in physical mapping of genes. This technique permits sharing of data across the laboratories. It is a rapid and most specific technique than DNA hybridization techniques. It has high degree of accuracy. It can be automated.

Disadvantages:

Development of STS is a difficult task. It is time consuming and labour oriented technique. It requires high technical skill.

Uses:

STS is the most powerful physical mapping technique. It can be used to identify any locus on the chromosome. STSs are used as standard markers to find out gene in any region of the genome. It is used for constructing detailed maps of large genomes.

Microsatellites and mini-satellites:

The term microsatellites were coined by Litt and Lutty (1989), while the term mini-satellites were used by Alec Jeffrey (1985). Both are multi-locus probes creating complex banding patterns. They essentially belong to the repetitive DNA family. Fingerprints generated by these probes are also known as oligo-nucleotide fingerprints.

Mini-satellites are tandem repeats of DNA sequence with 10-100 bp repeat motifs whereas, microsatellites are tandem repeat of DNA sequence with 2-6 bp repeat motifs. These are also referred to as Variable Number Tandem Repeats (VNTRS) and this is one

of the basis of polymorphism at a locus. Many alleles exist in a population, the level of heterozygosity is high and they follow Mendelian inheritance.

Simple Sequence Repeats (SSRs):

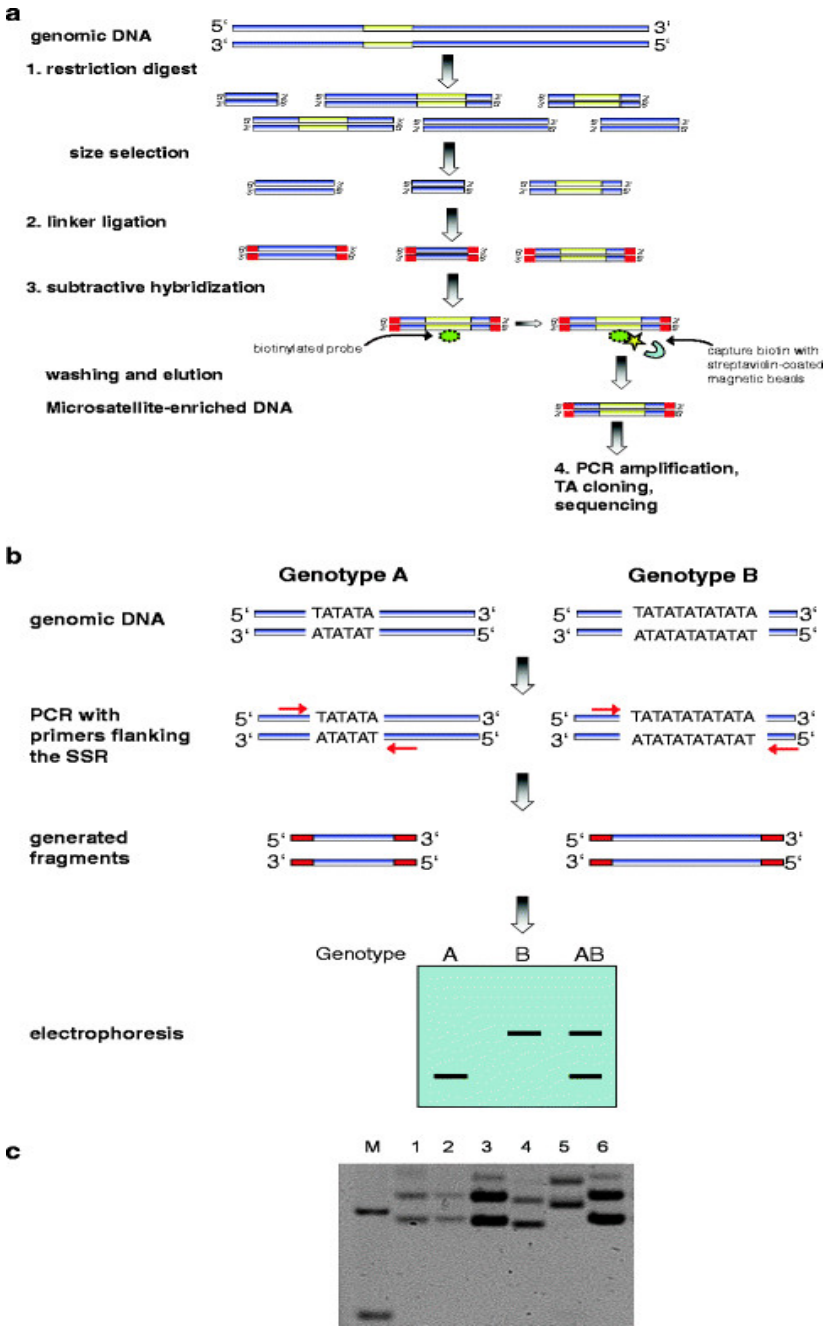
Simple sequence repeats (SSRs) or microsatellites are tandemly repeated mono-, di-, tri-, tetra-, penta-, and hexanucleotide motifs. SSR length polymorphisms are caused by differences in the number of repeats. SSR loci are individually amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence.

Jeffreys (1985) showed that some restriction fragment length polymorphisms are caused by VNTRs. The name **"mini satellite"** was coined because of the similarity of VNTRs to larger satellite DNA repeats.

Advantages:

SSR markers tend to be highly polymorphic. The genotyping throughput is high. This is a simple PCR assay. Many SSR markers are multi-allelic and highly polymorphic. SSR markers can be multiplexed, either functionally by pooling independent PCR products or by true multiplex- PCR. Semi-automated SSR genotyping methods have been developed. Most SSRs are co-dominant and locus specific.

No special equipment is needed for performing SSRs assays; however, special equipment is needed for some assay methods, e.g., semi-automated fluorescent assays performed on a DNA sequences. Start-up costs are low for manual assay methods (once the markers are developed). SSR assays can be performed using very small DNA samples (~100 ng per individual). SSR markers are easily shared between laboratories.



Disadvantages:

The development of SSRs is labor intensive. SSR marker development costs are very high. SSR markers are taxa specific. Start-up costs are high for automated SSR assay

methods. Developing PCR multiplexes is difficult and expensive. Some markers may not multiplex.

Uses:

SSR markers are used for mapping of genes in eukaryotes.

Variable Number Tandem Repeat (VNTR):

A Variable Number Tandem Re-peat (VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and of-ten show variations in length between individuals. Each variant act as an in-herited allele. Due to this reason VNTR can be used for personal or parental identification.

Use of VNTRs in Genetic Analysis:

VNTRs are frequently used in the develop-ment of linkage maps. Now that many ge-nomes have been sequenced, VNTRs have be-come essential to forensic crime investiga-tions, via DNA fingerprinting. When removed from surrounding DNA by the PCR or RFLP methods, and their size determined by gel electrophoresis or Southern blotting, they produce a pattern of bands unique to each individual.

When tested with a group of inde-pendent VNTR markers, the likelihood of two unrelated individuals having the same allelic pattern is extremely improbable. In the ex-ample considered in the diagram below locus A is a tandem repeat of the motif GC: there are four alleles, with two, three, four, or five repeats (A2, A3, A4, and A5, respectively).

Locus B is a tandem repeat of the motif AGCT: there are only two alleles, with two or three repeats (B2 and B3, respectively). Individual 1 is heterozygous at Locus A (A2/A5) and ho-mozygous at Locus 2 (B2/B2) which gives a single-banded phenotype in the fingerprint.

Individual 2 is heterozygous at both loci (A4/ A3 and B3/B2). The two individuals are dis-tinguishable at either locus. Typical finger-prints include a dozen or more VNTR loci.

VNTR analysis is also being used to study ge-netic diversity and breeding patterns in populations of wild or domesticated animals.

Inter-Simple Sequence Repeats (ISSR):

The generation of ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite repeated sequence and in some cases primer also contains 1-4 base anchor at either 3′ or 5′ or at both ends, which target a subset of 'simple sequence repeats' (SSRs) and amplify the region between two closely spaced and oppositely oriented SSRs (Fang et al., 1997; Fang and Roose, 1997; Moreno et al., 1998). **I**SSR technique permits the detection of polymorphisms in microsatellites and inter-microsatellites loci without previous knowledge of the DNA sequence (Moreno et al., 1998).

Some other microsatellites based on the same principle include the following:

(i) Randomly Amplified Microsatellite Polymorphism (RAMP): This is a micro satellite – based marker which shows a high degree of allellic polymorphism, but they are labor-intensive (Agarwal and Shrivastava, 2008). On the other hand RAPD markers are inexpensive but exhibit a low degree of polymorphism. To compensate for the weaknesses of these approaches, a technique termed as RAMP was developed (Wu et al, 1994). The technique involves a radiolabeled primer consisting of a 51 anchor and 31 repeats which is used to amplify genomic DNA in the presence or absence of RAPD primers. (Agarwal and Shrivastava, 2008).

(ii) The Sequence Characterized Amplified Region (SCAR): The SCARS are PCR-based markers that represent genomic DNA fragments at genetically defined loci that are identified by PCR amplification using sequence specific oligonucleotide primer (McDermoth et al, 1994).

(iii) Simple Primer Amplification Reaction (SPAR): SPAR uses the single SSR oligonucleotide principles.

(iv) Sequence – Related Amplified Polymorphism (SRAP): The aim of SRAP technique (Li and Quiros, 2001) is the amplification of open reading frames (ORFs). It is base on two-primer amplification using the AT- or GC- rich cores to amplify intragenic fragment for polymorphism detection (Agarwal and Shirvastava, 2008).

(v) Target region amplification polymorphism (TRAP): The TRAP technique (Hu and Vick, 2003) is a rapid and efficient PCR-based technique, which utilizes bioinformatics tools and expressed sequence tag (EST) database information to generate polymorphic markers, around targeted candidate gene sequences.

Type of DNA markers

	RFLP	PCR-based				
		RAPD	AFLP	SSR	STS	SNP
Principle	Restriction enzyme	Random priming	Selective PCR	SSR repeats	InDel length	DNA chip/ Hybridization
DNA required (μg)	10	0.02	0.5~1.0	0.02	0.02	0.02
Gel-based	Yes	Yes	Yes	Yes	Yes	Yes/No
Genomic abundance	High	Very high	Very high	Very high	Very high	Very high
Marker type	Codom.	Dom.	Dom.	Codom.	Codom.	Codom.
Reproducibility	Very high	Fair	Very high	Very high	Very high	Very high
Sequence informat. required	No	No	No	Yes	Yes	Yes
Ease of use	Labor intensive	Easy	Relat. difficult	Easy	Easy	Easy

Single locus and multi-locus markers:

Molecular markers may be multi-locus or single locus markers.

In the case of multi-locus markers many bands will be observed in a single analysis whereas for single locus systems, a maximum of two bands, one corresponding to each allele will be observed in a diploid genome.

In the case of RFLP's for mapping purposes, single locus markers are normally used. However, certain multi-locus RFLP's, using probes for repeated sequences, have been

developed. These markers are also often known as mini/micro satellites or Variable Number of Tandem Repeat (VNTR) markers, in which the polymorphism and the size of fragment observed depends on the number of tandem repeats which are present in each region of the genome. RAPD, DAF and AFLP are all multi-locus markers.

NGS based markers:

Next-generation sequencing (NGS) technology has dramatically revolutionized plant genomics. NGS technology combined with new software tools enables the discovery, validation, and assessment of genetic markers on a large scale. NGS technology has enabled the discovery and genotyping of markers at a very high density for comprehensive genome-wide association studies.

Application of molecular markers in crop improvement:

Molecular markers have evolved as potential tool lor a large number of applications ranging irom localization of a gene to improvement of plant varieties by marker assisted-Selection. With the advancement in the technology in the field of molecular marker our understanding in genetic analysis, and genomic has got significant impetus.

Fingerprinting of crop plants:

DNA fingerprinting refers to identify an individual unambiguously using multilocus DNA profiling. It can be done using hybridization markers, PCR based marker either locus specific amplification or by using random primers and sequencing. Huge number of scientific literatures is available in various crops in the context of DNA fingerprinting. Alec Jeffery and his associates were the first to develop method of DNA fingerprinting through simultaneous detection of highly variable DNA fragments by hybridizing multilocus probes with electrophoretically separated restriction fragments.

DNA fingerprinting has remarkable importance in plant vanety protection (PVP). And the utilities include identification of cultivars and genotypes; true to type plants at juveline stage (DUS testing) for

Seed purily mutants and chimeras; nucellar and zygotic embryos; somatic hybrids in fusion experiments and somaclonal variants etc.

Mapping and tagging of genes: Tools for MAS

Plant breeding is the science that aims at crop improvement, using the available variability. The outcome of crop improvement is selection of right kind of plant with right combination of genes/alleles. Conventional breeding takes lot of time for evolution, identification and introgression of novel genes. Molecular markers have accelerated conventional plant breeding. It is a powerful tool for identification of diverse line, mapping and tagging of genes. With the use of molecular markers it is now a routine to trace valuable alleles in a segregating population. These markers once mapped enable dissection of the complex traits into component genetic units more precisely, thus providing breeder with a new tool to manage this complex unit more efficiently in breeding programme.

There are several examples of gene mapping and utility in marker assisted-selection in various crops using various molecular markers. The very first genome map in plants was reported in Maize, followed by rice, *Arabidopsis* etc. using RFLP markers. Maps have since then been constructed for several other crops like potato, barley, banana, members of Brassicaceae, etc.

Phylogeny and evolution:

Molecular markers are powerful tools in phylogenetic and evolutionary studies. These studies strengthened the earlier studies made based on morphological and cytological evidences for establishing relationship between the wild relatives of species and their cultivated species. The comprehensive studies on genetic structure using molecular markers have revealed evolutionary forces that led the wild relatives to the present cultivable from of species. RFLP, DNA sequencing, and a number of PCR-based markers are being used extensively for reconstructing phylogenies of various species. The techniques are speculated to provide path-breaking information regarding the fine time scale on which closely related species have diverged and what sort of genetic variations are associated with species formation. Furthermore, these studies hold a great promise for revealing more about the pattern of genetic variation within the species. In connection to plant breeding, they are very much helpful in understanding the crop

evolution from wild progenitor and to classify them to appropriate groups. This would help in introgression of useful genes from wild progenitors into cultivated high yielding varieties ot crop species.

Diversity Analysis:

One of the important utilities of molecular markers is diversity analysis. Lines with similar morphological characters may substantially diverse from each other at DNA level and vice-versa. Diversity analysis can be done based on pedigree data, biochemical data, and more recently molecular (DNA-based) data. DNA based markers can unambiguously distinguish two different lines The revealed through molecular markers can be used to deduce genetic distance among the germplasm, breeding lines and population. There are several utilities of diversity analysis like (1) selection of parents for developing hybrids (2) selection of parents for developing mapping population (3) to study genetic inheritance of a trait (4) combining ability studies (5) understanding the environmental effect on geographically diverse lines (6) population genetic studies (7) identification of regions specific fixed allelism landraces.

The commonly used measures of genetic distance or genetic similarity (GS) using binary data are (1) Nei and Li's (1979) coefficient (GDNu), (2) Jaccard's (1908) (3) Sokal and Michener's (1958) simple matching coefficient (GDsm), and (4) Modified Rogers distance, (1972) (GDuR).

QTL mapping:

Some of the most difficult tasks of plant breeders relate to the improvement of traits that show a continuous range of values. The most common method of determining the association between marker and QTL is done by analyzing phenotypic observation of trait and scoring of molecular data by one-way analysis of variance and regression analysis. For each marker, presence of a specific fragment of DNA is considered a marker class, and all individuals possessing that marker class are considered to be positive for that class.

In chickpea, two RIL populations were used to construct a composite linkage map with the help of RAPD, ISSR, RGA, SSR and ASAP markers. Marker trait association was

observed among three yield related traits: double podding, seeds per pod and seed weight. Duran et al. (2002) developed a QTL map for plant height, pod dehiscence, number of shoots and seed diameter from inter-subspecific population of *Lens culinaris* ssp culinaris x *Lens culinaris* ssp *orientalis*.

9. Genetic Maps: Construction of linkage maps, high-density maps, QTL mapping, association mapping, integration of genetic maps with physical maps/chromosomes.

Genetic Maps:

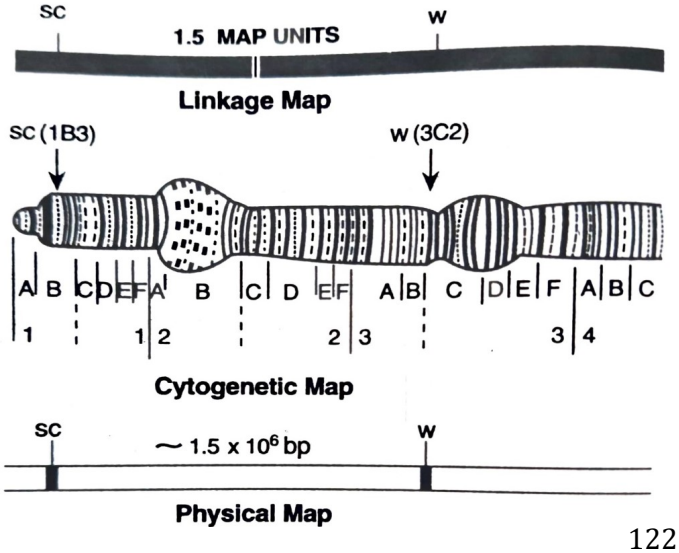
A genetic map is a schematic representation of the various genetic markers in the specific order in which way they are located in a chromosome as well as the relative distance between these markers. Three different strategies have been used to construct genetic maps. The strategies are:

Cytogenetic Map:

A cytogenetic map depicts the locations of various genes in a chromosome relative to specific microscopically visible landmarks in the chromosome.

Physical Map:

Provide physical distance between landmarks on the chromosome, ideally measured in nucleotide bases. Map is



created by fragmenting the DNA molecule using restriction enzymes and then looking for overlaps

Linkage map:

A **linkage map** depicts the order of genetic markers and the relative distances between them as measured in terms of **recombination frequencies** between the markers.

Linkage mapping:

- The greater the frequency of recombination (segregation) between two genetic markers, the further apart they are assumed to be.
- Conversely, the lower the frequency of recombination between the markers, the smaller the physical distance between them.
- Historically, the markers originally used were detectable phenotypes (enzyme production, eye colour) derived from coding DNA sequences.
- Eventually, confirmed or assumed noncoding DNA sequences such as microsatellites or those generating RFLPs have been used.

Recombination frequency:

Recombination frequency is the frequency with which a single chromosome crossover will take place between two genes during meiosis. It is a measure of genetic linkage and it is used in creation of genetic linkage maps. A **centimorgan (cM)** is a unit that describes a **recombination frequency of 1%**. Using cM as map units, the genetic distance between two loci can be measured, based upon their recombination frequency.

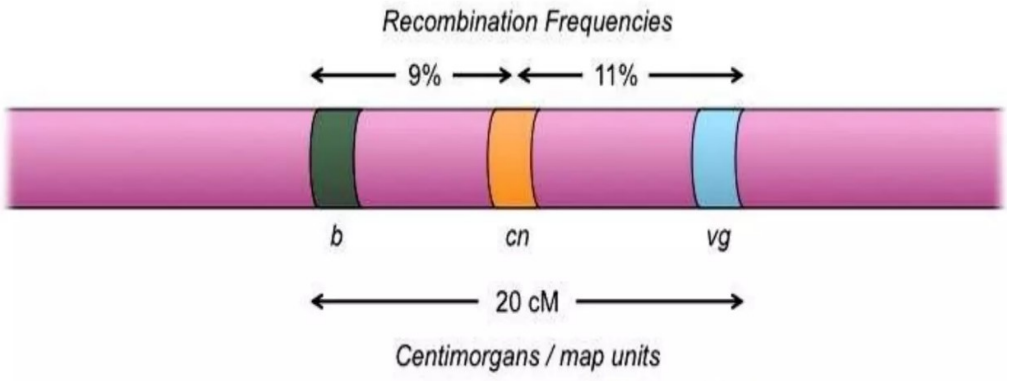
$$\text{Recombination frequency } (\theta) = \frac{\text{Number of recombinants}}{\text{Total number of progenies}} \times 100\%$$

Map unit or Centimorgan: A map unit or centimorgan is a unit of measure used to approximate the distance between genes. The Morgan unit is named in honour of **T. H. Morgan**.

1 Map unit or 1 cM = 1% Recombination frequency

Example:

- Distance between gene b & cn = 9cM (as Recombination frequency is 9%)
- Distance between gene cn & vg = 11cM (as Recombination frequency is 11%)
- Total Distance between gene b & gene vg= 9cM + 11cM = 20cM



Construction of a Linkage Map:

As **linkage map or genetic map** is the outcome of crossing over studies, it is also called as **cross over map**. The linkage mapping includes the following processes:

1. Determination of Linkage Groups (Mapping Populations).
2. Determination of Map Distance.
   - (a) Two-point test cross
   - (b) Three-point test cross
3. Determination of Gene Order.
4. Combining Map Segments.
5. Interference & Coincidence

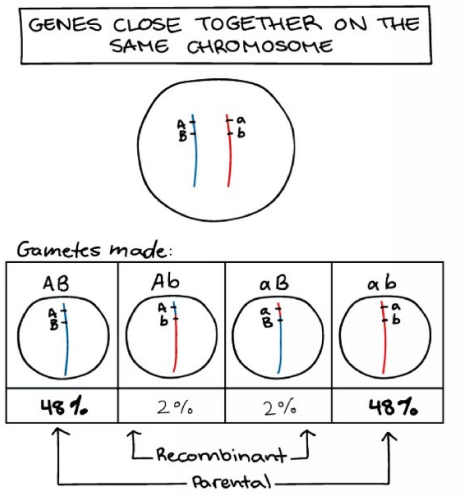
1. Determination of linkage groups:

Mapping population is a group of individuals used for gene mapping. Selection of appropriate mapping population is fundamental to the success of a gene mapping project.

- Before starting a gene mapping of the chromosome of a species, the exact number of chromosomes must be known.

- The total number of genes of that species by undergoing hybridization experiments in between wild & mutant strains has to be determined.
- By the same hybridization technique, it can also be determined that how many phenotypic traits remain always linked and consequently their genes during the course of inheritance.
- And thus the different linkage groups of a species can be determined.

2. Determination of map distance:



The intergene distance on the chromosome cannot be measured in the customary units employed in light microscopy. Geneticists use an arbitrary unit to measure the map units, to describe distance between linked genes. A map unit is equal to 1% crossover, i.e. It represents the linear distance along the chromosome for which a recombinant frequency of 1% is observed.

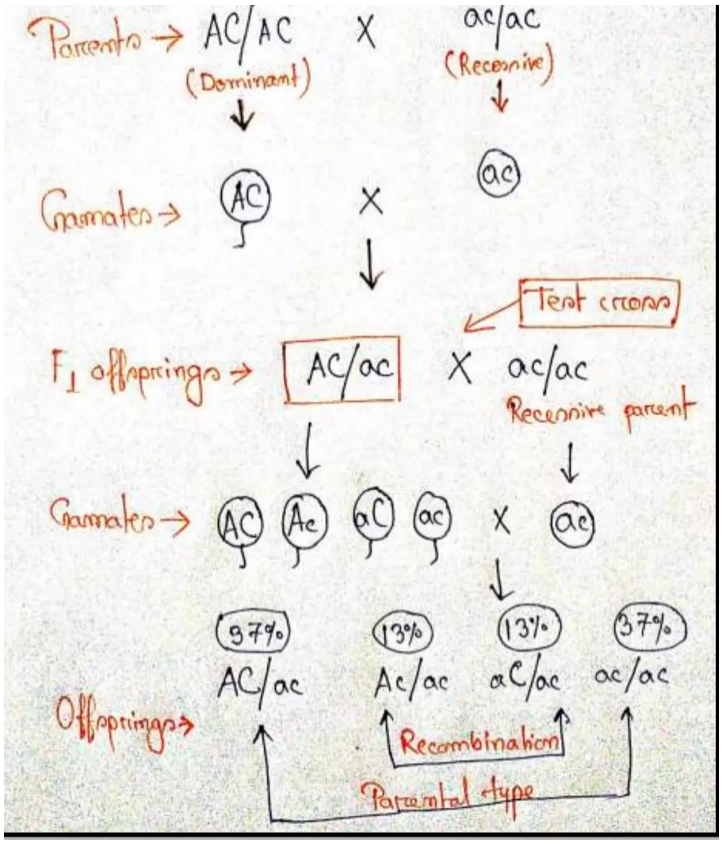
Example: In this cross the crossover of gamates are Ab(2%) and ab(2%) . Total crossover = 4% (aB%+Ab%) So, distance between A & B will be 4 cM or map unit.

(a) Two-point test cross:

The % of crossing over between two linked genes is calculated by a two-point test cross.

Example:

- In this dihybrid test cross the Ac/ac(13%) & aC/ac(13%) were produced from the crossover gametes from the dihybrid parents.
- Thus 26 % (13% + 13%) of the gamates are cross over types.
- So distance between the loci A & C is estimated to be 26 cM
- As double crossover does not occur between genes less then 5cM apart, so for genes farther apart 3-point test cross is used.



(b) Three-point test cross:

A three-point test cross gives us the linear order in which three genes should be present on chromosome. Example:

- In addition to the previous A & C genes, let us assume gene B, located in close proximity in the same linkage group.
- A trihybrid cross of ABC/ABC & abc/abc produced F_1 hybrid ABC/abc and its test cross with recessive parent abc/abc produced following offsprings:

Three Point test cross results (Btw. F1 hybrid ABC/abc & Recessive parent abc/abc)			
36% ABC/abc 36% abc/abc	9% Abc/abc 9% aBC/abc	4% ABc/abc 4% abC/abc	1% AbC/abc 1% aBc/abc
72% Parental Type	18% Single crossovers (Btw. A & B)-Region1	8% Single crossovers (Btw. B & C)-Region 2	2% Double crossover

To measure distance between two genes we add all double and single crossovers (CO)

Distance between A-B: 18% single C.O.+ 2% double C.O.= 20% = 20 cM

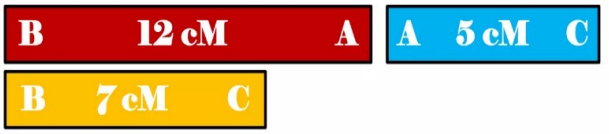
Distance between B-C: 8% single C.O.+2% double C.O.= 10% = 10 cM

Distance between A-C: A-B(20cM) + B-C(10cM) = 30 cM apart A-C

3. Determination of gene order: After determining the relative distance between genes of a linkage group, it becomes easy to place genes in their proper linear order.

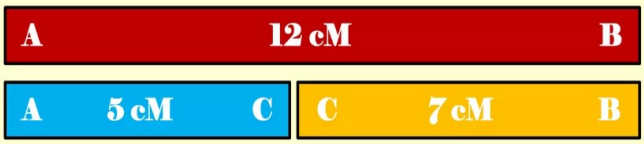
Example: If we suppose the distance between genes A-B=12cM, B-C=7cM, A-C= 5cM, we can determine the order of gene in the following manner

Case 1: Gene order = B-A-C
Incorrect: Distance between B-C are not equitable



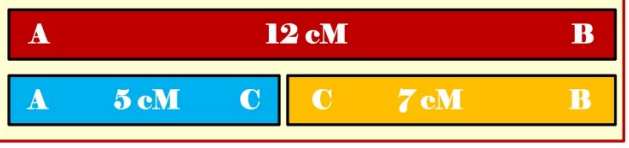
Case 2: Gene order = A-B-C
Incorrect: Distance between A-C are not equitable

Case 3: Gene order =**B-C-A**
Correct: Distance between A-B are equitable & **gene C** must be in centre.



4. Combining map segments: Finally, the different segments of maps of a complete chromosome are combined to form a complete genetic map. Example: We can combine the different segments of the previous example into a single map by using simple

Case 3: Gene order =**B-C-A**
Correct: Distance between A-B are equitable & **gene C** must be in centre.



As **A-C distance = 5 cM** & **B-C distance = 7 cM**; and by ordering the genes we know gene **C is in between A & B**, so we can combine the map putting **C in betwwen(5cM from A & 7cM from B)**

calculation.

5. Interference & Coincidence:

In higher organisms, one chiasma formation reduces the probability of another chiasma formation in an immediately adjacent region. The tendency of one crossover to interfere with the other is called **interference**. Centromere shows similar interference effect. The net result of this interference is, fewer double crossover then expected according to map distances. Interference is expressed in terms of coefficient of coincidence.

$$\text{Coefficient of coincidence} = \frac{\%\ \text{observed double crossovers}}{\%\ \text{expected double crossovers}}$$

$$\text{Coincidence} + \text{Interference} = 1.0$$

When interference is complete (1.0), no double crossover will be observed and coincidence becomes 0.

Biochemical markers in linkage mapping:

This has been particularly important with two types of organisms - microbes and humans. Microbes, such as bacteria and yeast, have very few visual characteristics so gene mapping with these organisms has to rely on biochemical phenotypes. A big advantage is that relevant genes have multiple alleles. For example- Gene called **HLA-DRB1** has at least **290 alleles** and **HLA-B** has over **400 alleles.**

DNA markers for linkage mapping:

Genes are very useful markers but they are by no means ideal. Mapped features that are not genes are called DNA markers. A DNA marker must have at least two alleles to be useful. DNA sequence features that satisfy this requirement are-

- Restriction Fragment Length Polymorphism (RFLPs)
- Simple Sequence Length Polymorphism (SSLPs)
- Single Nucleotide Polymorphism (SNPs)

Construction of Linkage Map using RFLP Markers:

Linkage maps are prepared using recombination frequencies between genes as a measure of distance between them. We can map RFLPs and other molecular markers much in the same way as we map other genetic markers, i.e., by making crosses between two parents differing for two or more RFLPs and scoring the progeny for these RFLP markers. Let us suppose that one RFLP is detected with probe #1and gives bands of 5 kbp and 6 kb in different strains. Similarly, the second RFLP is detected by probe # 2 and yields bands of 2 kb and 3 kb. The mapping of these two RFLP markers may be done as follow –

1. Two strains that are homozygous for the two RFLP markers, e.g., one shows bands of 5 kb and 3 kb, while the other shows 6 and 2 kb bands, are crossed together.
2. The F_1 so obtained shows all the four bands, viz., 6, 5, 3 and 2 kb bands. The F_1, is back- crossed to one of the parents, e.g., the parent having 6 and 2 kb bands.
3. The resulting back-cross progeny are scored for these RFLP bands. Each progeny will yield one of the four RFLP band patterns listed in Table. If the two RFLP markers were not linked, equal proportion of progeny will be present in these 4 categories. But in case of linkage, the proportion of parental progeny will be much higher than that of recombinant progeny.

In actual analysis of RFLP data, linkage is detected by a statistical test called the **lod** (Logarithm (base 10) of odds] **score method** developed by Morton in 1955. The theoretical basis of this method is quite complex, and computer programmes are used to analyze pooled data from a large number of pedigrees (human beings) or crosses. The programmes determine the probability that two markers exhibit a certain degree of linkage, i.e., are within a particular number of map units distance, and also the probability that the given data would have been obtained if the two markers were unlinked. The lod score is then estimated according to the following formula.

$$\text{LOD Score} = \text{Log}_{10} \frac{\text{Probability of certain degree of linkage}}{\text{Probability of independent assortment}}$$

If the lod score equals + 3 or is greater, the two RFLP markers are considered to be linked. Since a $\log_{10}$ value of 3 equals 1,000, a lod value of 3 suggests that there is a 1,000-fold greater probability that the two markers are linked than they are assorting independently. If a lod score indicates the two RFLP markers to be linked, the number of recombinant offspring is divided by the total number of Offspring to obtain the map distance in terms of map units or cM.

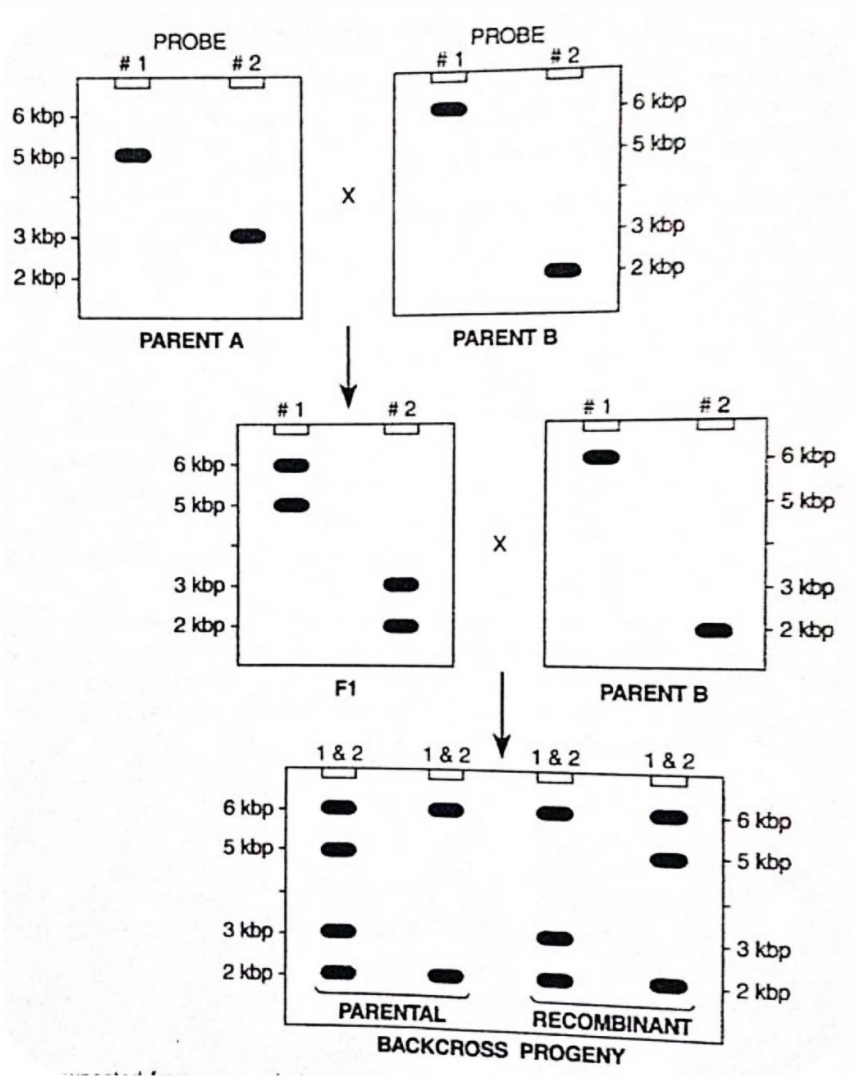


Fig.: Result expected from a cross between two parents differing for two RFLP markers.

RFLPs are quite common in virtually all species. Therefore, many RFLP markers can be analyzed 1or linkage relationships and a genetic map can be developed to depict the order and distances between Pairs of a large number of different RFLP markers; such a map is known as an **RFLP map**. RFLP maps have become increasingly useful in the genetic analysis of plants and animals where genomes are large and relatively few genes have been mapped. RELP mans can be used to locate functional genes. within the genome, and they are also useful in physical mapping of eukaryotic genomes. Functional genes are located in RFLP maps by analysing linkage relationships of these genes with several RFLP markers, using the same approach that is applied for mapping of RFLP markers.

For a successful linkage mapping with DNA markers, sufficient DNA sequence polymorphism must be present between the parents used for hybridization. Naturally outcrossing species tend to have high levels of DNA polymorphism, while the levels of DNA sequence variation are generally lower naturally inbreeding species. Sometimes, mapping in inbreeding species requires that parents be as distantly related as possible.

Several different kinds of populations can be used for linkage mapping of DNA markers. In the example described above (Fig.) a back-cross population was used. Other populations suitable for mapping are F_2, recombinant inbred lines (RILs) derived from a cross (easily developed by single seed descent procedure), and near-isogenic lines (NILs) developed by back-cross procedure. F_2 and back- cross populations are easy to construct, but they are ephemeral, i.e., cannot be maintained over a period of time, except by asexual propagation, and sterility in F_1 may limit some combinations of parents. Creation of RILs and NILS is time and labour-intensive (particularly in case of NILs), and regions of the genome may tend to remain heterozygous longer than theoretically expected. Further, obligate outcrossing species are much more difficult to map using RILs because of the difficulty in selfing these species.

High-density map:

High-density genetic map is a valuable tool for exploring novel genomic information, quantitative trait locus (QTL) mapping and gene discovery of economically agronomic

traits in plant species. A high-density genetic map is not only a key resource for studies on genome structure and genetic relationships but also provides the basis for quantitative trait locus (QTL) mapping and marker-assisted selection (MAS) based on the numbers of polymorphic markers.

High-density maps that integrate the genetic, cytological, and physical maps of chromosomes have been constructed for many chromosomes, including all of the human chromosomes. high-density genetic maps—are often constructed by using molecular markers such as restriction fragments of different lengths (restriction fragment-length polymorphisms, or RFLPs). STRs (short tandem repeats, microsatellites) have proven extremely valuable in constructing high-density maps of eukaryotic chromosomes.

QTL mapping:

Many agriculturally important traits such as yield, quality and some forms of disease resistance are controlled by many genes and are known as **"quantitative traits** or **polygenic** or **multifactorial** or **complex traits".**

- These traits show continuous variation in a population.
- These traits **do not** fall into **discrete classes**.
- They are measurable.

A **quantitative trait locus (QTL)** is a locus (section of DNA) that correlates with variation of a quantitative trait in the phenotype of a population of organisms. QTLs are mapped by identifying which molecular markers (such as SNPs or AFLPs) correlate with an observed trait. This is often an early step in identifying and sequencing the actual genes that cause the trait variation.

- The loci controlling quantitative traits are called **quantitative trait loci** or **QTL**.
- Term first coined by **Gelderman** in 1975.
- It is the region of the genome that is associated with an effect on a quantitative trait.
- It can be a single gene or cluster of linked genes that affect the trait.

QTLs have the following characteristics

- These traits are controlled by multiple genes, each segregating according to Mendel's laws.
- These traits can also be affected by the environment to varying degrees.
- Many genes control any given trait and Allelic variations are fully functional.
- Individual gene effects is small &The genes involved can be dominant,or codominant.
- The genes involved can be subject to epistasis or pleiotrophic effect.

QTL Mapping

The process of constructing linkage maps and conducting QTL analysis i.e. to identify genomic regions associated with traits is known as **QTL mapping**. Identify the location of polygenes or **QTL** by use of DNA markers. It involves testing DNA markers throughout the genome for the likelihood that they are associated with a QTL.

Principles

Genes and markers segregate via chromosome recombination during meiosis, thus allowing their analysis in the progeny. It detects the association between phenotype and genotype of markers. QTL analysis depends on the linkage disequilibrium. QTL analysis is usually undertaken in segregating mapping populations.

Objectives

The basic objective is to detect QTL, while minimizing the occurrence of false positives (Type I errors that is declaring an association between a marker and QTL when in fact one does not exist).

- To identify the regions of the genome that affects the trait of interest.
- To analyze the effect of the QTL on the trait.
- How much of the variation for the trait is caused by a specific region?
- What is the gene action associated with the QTL – additive effect? Dominant effect?
- Which allele is associated with the favorable effect?

Pre-requisites for QTL mapping

Availability of a good linkage map (this can be done at the same time the QTL mapping)

A segregating population derived from parents that differ for the trait(s) of interest, and which allow for replication of each segregant, so that phenotype can be measured with precision (such as RILs or DHs)

A good assay for the trait(s) of interest

Software available for analyses

Molecular Markers

Sophisticated Laboratory

Type of mapping population:

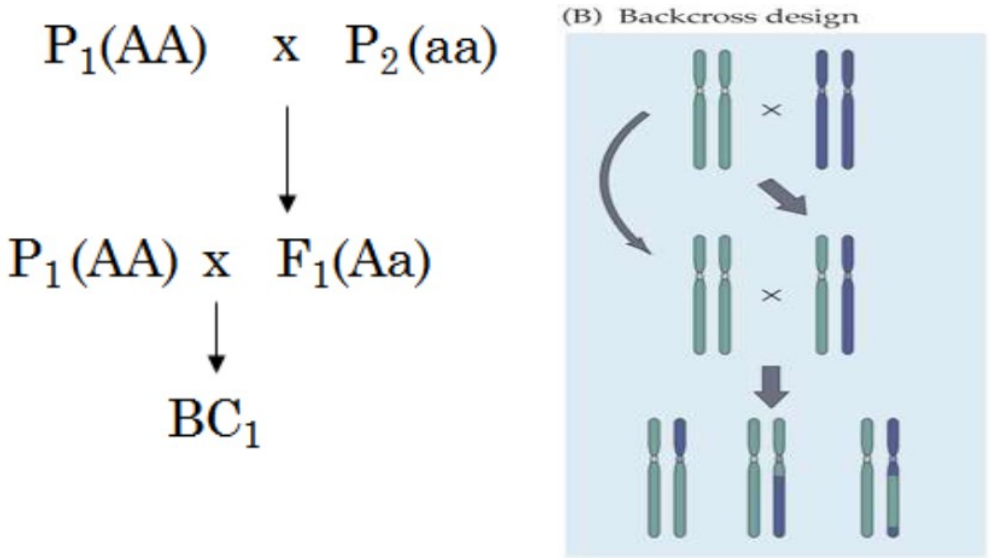
Backcross Populations

To analyze specific DNA fragments derived from parent A in the background of parent B, a hybrid F_1 plant is backcrossed to parent B. In this situation, parent A is the donor of DNA fragments and parent B is the recipient. The latter is also called the recurrent parent. During this process two goals are achieved: unlinked donor fragments are separated by segregation and linked donor fragments are minimized due to recombination with the recurrent parent. To reduce the number and size of donor fragments, backcrossing is repeated and, as a result, so-called advanced backcross lines are generated. With each round of backcrossing, the proportion of the donor genome is reduced by 50 %.

Advantages: It is easier to identify QTL as there are less epistatic and linkage drag effects; especially useful for crosses with wild species.

Disadvantages: Difficult or impossible in species that are highly heterozygous and outcrossing.

Use: Best when inbred lines are available.



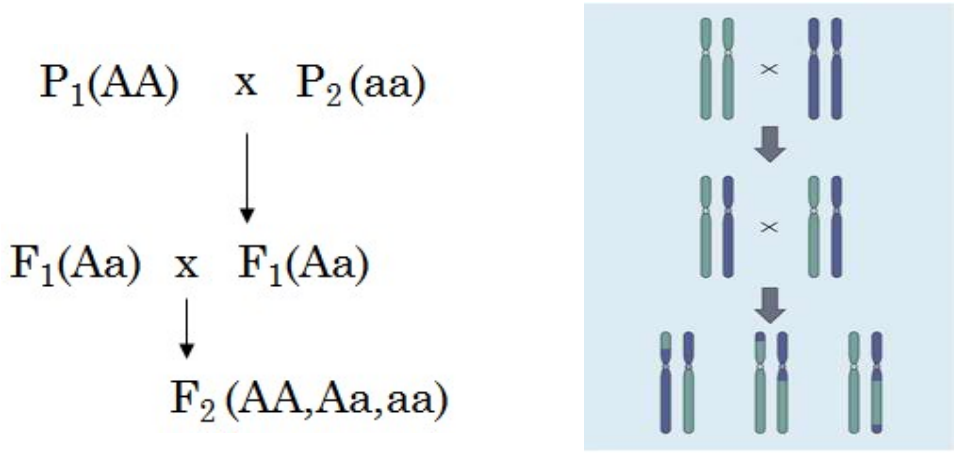
F_2 Populations

The simplest form of a mapping population is a collection of F_2 plants. This type of population was the basis for the Mendelian laws (1865) in which the foundations of classic genetics were laid. Two pure lines that result from natural or artificial inbreeding are selected as parents, parent 1 (P_1) and parent 2 (P_2). Alternatively, doubled haploid lines can be used to avoid any residual heterozygosity. If possible, the parental lines should be different in all traits to be studied. The degree of polymorphism can be assessed at the phenotypic level (e. g., morphology, disease resistance) or by molecular markers at the nucleic acid level. For inbreeding species such as soybean and the Brassicaceae, wide crosses between genetically distant parents help to increase polymorphism. However, it is required that the cross lead to fertile progeny. The progeny of such a cross is called the F_1 generation. If the parental lines are true homozygotes, all individuals of the F_1 generation will have the same genotype and have a similar phenotype. This is the content of Mendel's law of uniformity. An individual F_1 plant is then selfed to produce an F_2 population that segregates for the traits different between the parents. F_2 populations are the outcome of one meiosis, during which the

genetic material is recombined. The expected segregation ratio for each codominant marker is 1:2:1 (homozygous like P_1:heterozygous:homozygous like P_2).

Advantage: Fast and easy to construct

Disadvantage: F_3 families are still very heterozygous, so the precision of the estimates can be low (because of the high standard error); can't be replicated

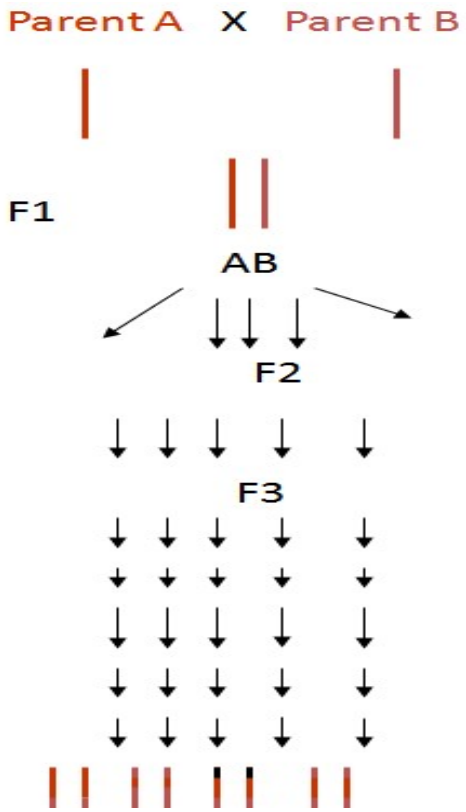


Recombinant Inbred Lines

Recombinant inbred lines (RILs) are the homozygous selfed or sib-mated progeny of the individuals of an F_2 population (Figures. The RIL concept for mapping genes was originally developed for mouse genetics. In animals, approximately 20 generations of sib mating are required to reach useful levels of homozygosity. In plants, RI lines are produced by selfing, unless the species is completely self-incompatible. Because in the selfing process one seed of each line is the source for the next generation, RILs are also called single-seed descent lines. Self-pollination allows the production of RILs in a relatively short number of generations. In fact, within six generations, almost complete homozygosity can be reached. Along each chromosome, blocks of alleles derived from either parent alternate. Because recombination can no longer change the genetic constitution of RILs, further segregation in the progeny of such lines is absent.

Advantages: fixed lines so can be replicated across many locations and/or years; can eliminate problem of background heterozygosity.

Disadvantages: Can take a long time to produce. (Some species are not amenable).



Doubled Haploid Lines

Doubled haploid lines contain two identical sets of chromosomes in each cell. They are completely homozygous, as only one allele is available for all genes. Doubled haploids can be produced from haploid lines. Haploid lines either occur spontaneously, as in the case of rape and maize, or are artificially induced. Haploid plants are smaller and less vital than diploids and are nearly sterile. It is possible to induce haploids by culturing immature anthers on special media. Haploid plants can later be regenerated from the haploid cells of the gametophyte. A second option is microspore culture. In cultivated barley it is possible to induce the generation of haploid embryos by using pollen from

the wild species *Hordeum bulbosum*. During the first cell divisions of the embryo, the chromosomes of *H. bulbosum* are eliminated, leaving the haploid chromosomal set derived from the egg cell. Occasionally in haploid plants the chromosome number doubles spontaneously, leading to doubled haploid (DH) plants.

AA	aa	aa	AA	aa	AA
BB	bb	BB	bb	bb	BB
cc	CC	cc	cc	cc	CC
dd	DD	dd	dd	DD	dd
ee	ee	EE	EE	ee	ee
FF	ff	FF	FF	FF	ff

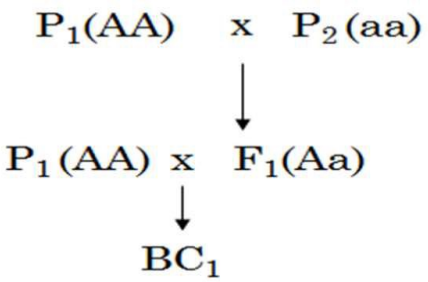
Advantages:

- ✓ Spontaneous chromosome doubling of Haploid microspores in in vitro culture
- ✓ Homozygosity achieved in a single step Plants.

Disadvantages: Less recombination between linked markers Not all systems are amenable to in vitro culture

Near Isogenic Lines (NILs):

NILs can be developed by repeated selfing or backcrossing of F1 with recurrent parent. Irrespective of dominant or codominant marker NILs segregate in 1:1 ratio.

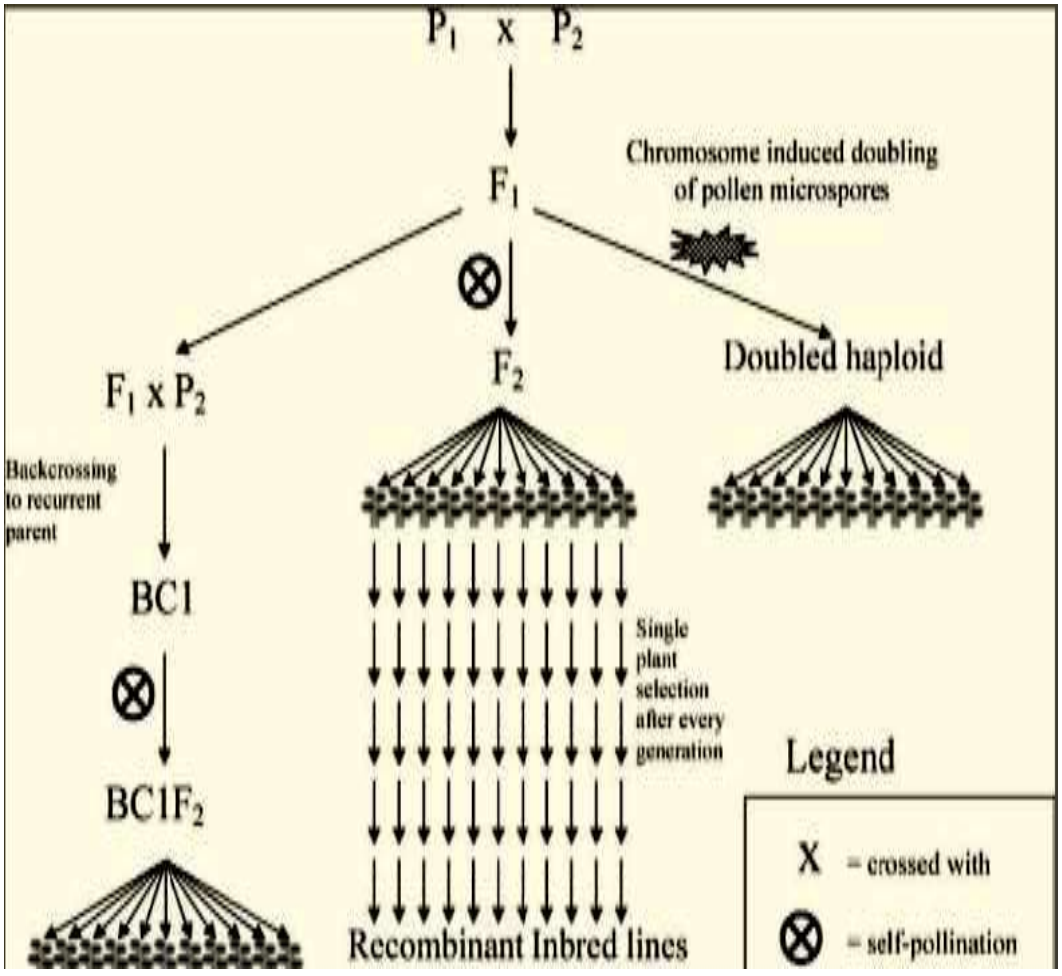


Advantage: Very precise and statistically strong, as background is constant; especially useful for validation experiments

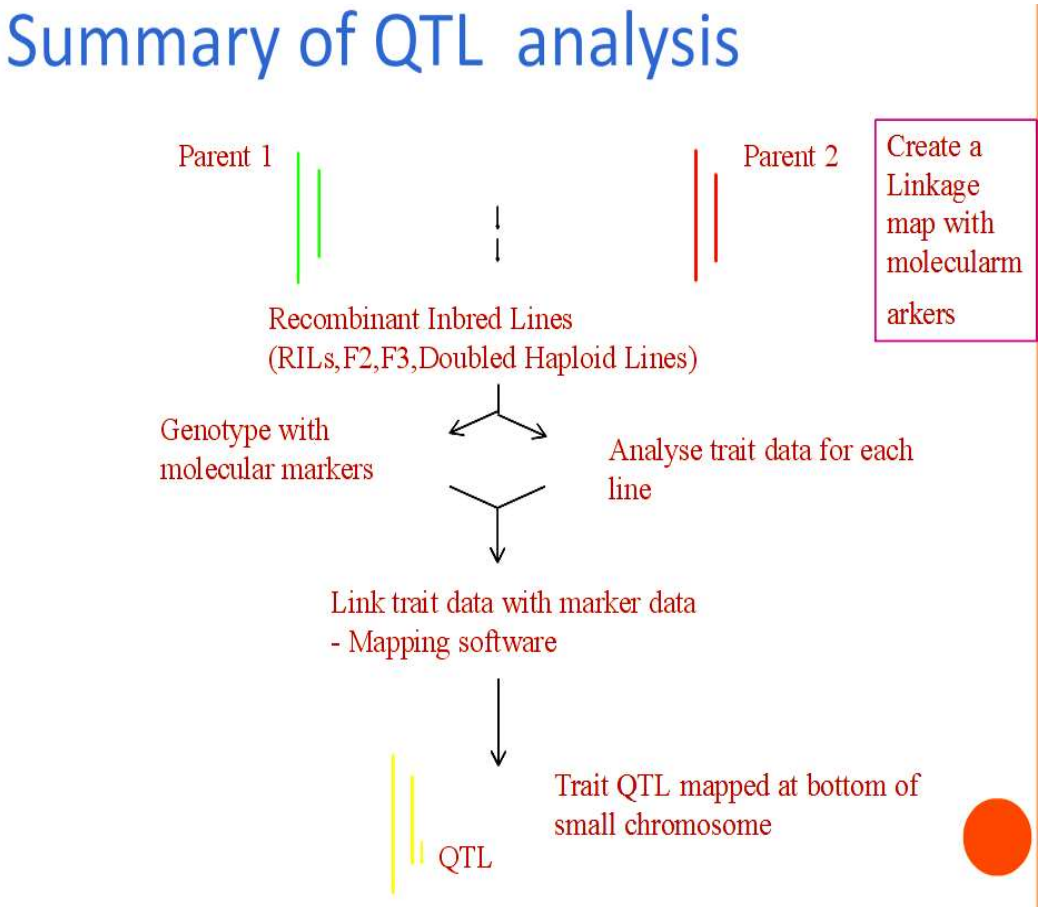
Disadvantage: Can take time to construct; only useful for specific target QTL

Steps involved in QTL Mapping:

- **Selection of parental lines**
  - Sufficient **polymorphism**
  - Parental lines are highly **contrasting phenotypically**
  - **Genetically divergent**
- **Selection of molecular markers (dominant/codominant)**
- **Making crosses**
- **Creation of mapping population**

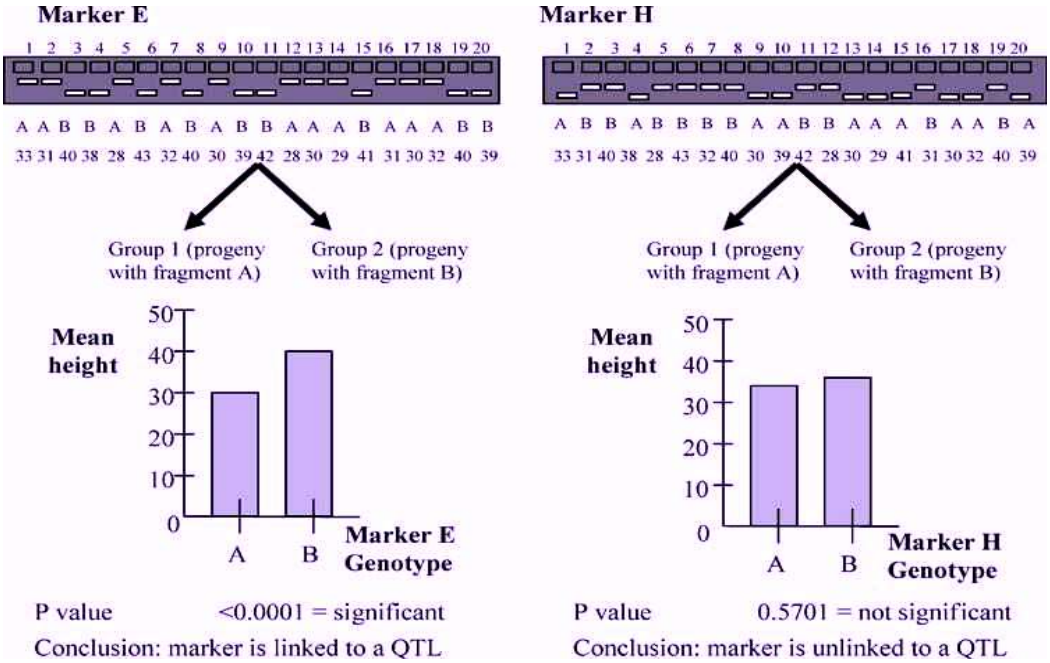


- **Phenotyping of the progenies**
- **Genotyping of the progenies**
- **Construction of linkage map**
    - Screening the mapping population using polymorphic molecular markers
    - Segregation patterns
    - Data is then analyzed using a statistical package such as **MAPMAKER** or **JOINMAP**
    - Assigning them to their linkage groups on the basis of **recombination** values
    - For practical purposes, in general recombination events considered to be less than 10 recombinations per 100 meiosis, or a map distance of less than 10 centiMorgans(cM).



QTL analysis

It is based on the principle of detecting an association between phenotype and the genotype of the markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured. A significant difference between phenotypic means of the groups, depending on the marker system and type of mapping population It is not easy to do this analysis manually and so with the help of a computer and a software it is done. The segregation data of both the phenotype and the genotype are collected and arranged in an excel sheet for QTL analysis using the appropriate software.



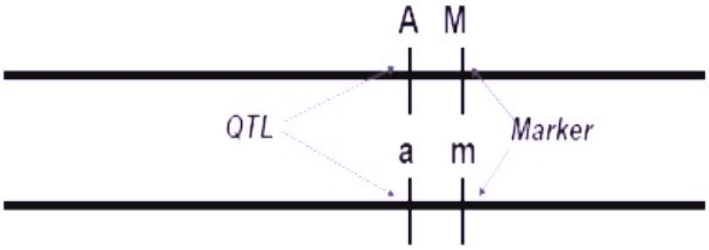
Methods to detect QTLs

- Single-Marker Analysis
- Simple Interval Mapping
- Composite Interval Mapping
- Multiple Interval Mapping

- Bayesian Interval Mapping

Single-Marker Analysis (SMA)

It is also known as single- point analysis. It is the simplest method for detecting QTLs associated with single markers.



This method does not require a complete linkage map and can be performed with basic statistical software programs. The statistical methods used for single-marker analysis **include t-tests, analysis of variance (ANOVA) and linear regression.** Linear regression is most commonly used because the coefficient of determination (R_2) from the marker explains the phenotypic variation arising from the QTL linked to the marker.

Limitations:

- Likelihood of QTL detection significantly decreases as the distance between the marker and QTL increases
- It cannot determine whether the markers are associated with one or more markers QTLs
- The effects of QTL are likely to be underestimated because they are confounded with recombination frequencies.

To overcome these limitations **the use of large number of segregating DNA markers covering the entire genome may minimize these problems.** QGene and MapManager QTX are commonly used computer programs to perform singlemarker analysis.

Simple Interval Mapping (SIM)

It was first proposed by **Lander** and **Bolstein**. It takes full advantages of the linkage map. This method evaluates the target association between the trait values and the

genotype of a hypothetical QTL (target QTL) at multiple analysis points between pair of adjacent marker loci (target interval). Presence of a putative QTL is estimated if the log of odds ratio exceeds a critical threshold. The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis. MapMaker/QTL and QGene are used to conduct SIM. The principle behind interval mapping is to test a model for the presence of a QTL at many positions between two mapped loci.

Statistical methods used for SIM

Maximum Likelihood Approach

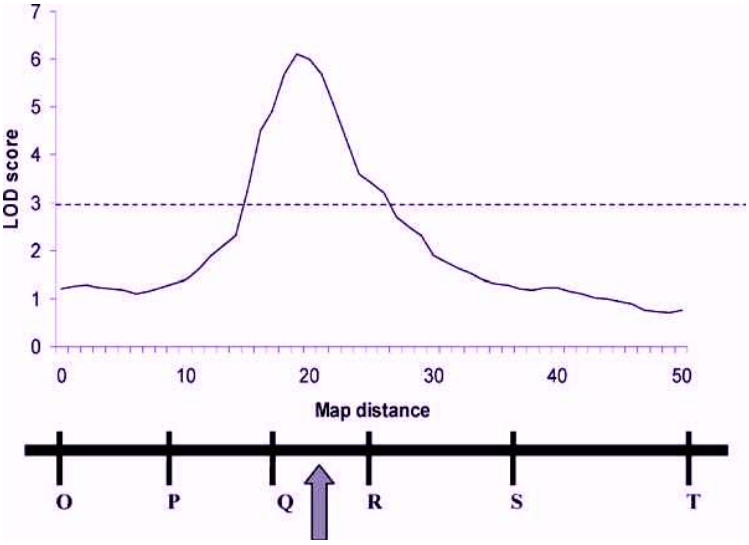
- It is assumed that a QTL is located between two markers, the two loci marker genotypes (i.e. AABB, AAbb, aaBB, aabb for DH progeny) each contain mixtures of QTL genotypes.
- Maximum likelihood involves searching for QTL parameters that give the best approximation for quantitative trait distribution that are observed for each marker class.
- Models are evaluated by comparing the likelihood of the observed distributions with and without finding QTL effect
- The map position of a QTL is determined as the maximum likelihood from the distribution of likelihood values.

Logarithm of the odds ratio (LOD score):

- Linkage between markers is usually calculated using odds ratio.
- This ratio is more conveniently expressed as the logarithm of the ratio, and is called a logarithm of odds (LOD) value or LOD score.
- LOD values of >3 are typically used to construct linkage maps.

$$\text{Odds ratio} = \frac{\text{probability of the data occurring with a QTL}}{\text{probability of the data occurring with no QTL}}$$

- LOD of 2 means that it is 100 times more likely that a QTL exists in the interval than that there is no QTL.
- LOD of 3 between two markers indicates that linkage is 1000 times more likely (i.e. 1000:1) than no linkage.
- LOD values may be lowered in order to detect a greater level of linkage or to place additional markers within maps constructed at higher LOD values.
- **The LOD score is a measure of the strength of evidence for the presence of a QTL at a particular location.**



Hypothetical output showing a LOD profile for chromosome 4. The dotted line represents the significance threshold determined by permutation tests. The output indicates that the most likely position for the QTL is near marker Q (indicated by an arrow). The best flanking markers for this QTL would be Q and R.

Interval Mapping by Regression

It is essentially the same as the method of basic QTL analysis (regression on coded marker genotypes) except that phenotypes are regressed on QTL genotypes.

Since QTL genotypes are unknown, they are replaced by probabilities estimated from the nearest flanking markers.

Softwares used: PLABQTL, QTL Cartographer, MapQTL

Composite Interval Mapping (CIM)

This method is developed by Jansen and Stam in 1994. It combines interval mapping for a single QTL in a given interval with multiple regression analysis on marker associated with other QTL. It is more precise and effective when linked QTLs are involved. It considers marker interval plus a few other well chosen single markers in each analysis, so that n-1 tests for interval – QTL associations are performed on a chromosome with n markers.

Advantages:

- Mapping of multiple QTLs can be accomplished by the search in one dimension.
- By using linked markers as cofactors, the test is not affected by QTL outside the region, thereby increasing the precision of QTL mapping.
- By eliminating much of the genetic variance by other QTL, the residual variance is reduced, thereby increasing the power of detection of QTL.

Problems

- The effects of additional QTL will contribute to sampling variation.
- If two QTL are linked their combined effects will cause biased estimates.

Multiple Interval Mapping (MIM)

It is also a modification of simple interval mapping. It utilizes multiple marker intervals simultaneously to fit multiple putative QTL directly in the model for mapping QTL. It provides information about number and position of QTL in the genome. It also determines interaction of significant QTLs and their contribution to the genetic variance. It is based on Cockerham's model for interpreting genetic parameters.

Bayesian Interval Mapping (BIM)

It provides a model for QTL mapping. It provides information about number and position of QTL and their effects. The BIM estimates should agree with MIM estimates

and should be similar to CIM estimates. It provides information posterior estimates of multiple QTL in the intervals. It can estimate QTL effect and position separately.

Comparison of methods of QTL Mapping

Particulars	Interval mapping	Composite Interval Mapping	Multiple Interval Mapping	Bayesian Interval Mapping
1. Markers used	Two markers	Markers used as cofactors	Multiple markers	Two markers
2. Information obtained about	Number and position of QTL	Number and position of QTL and interaction of QTLs	Number and position of QTL	Number and position of QTL and their effects
3. Designated as	SIM	SIM	MIM	BIM
4. Precision	High	Very high	Very high	Very high

Merits of QTL Mapping

- Where mutant approaches fail to detect genes with phenotypic functions , QTL mapping can help
- Good alternative when mutant screening is laborious and expensive e.g circadium rhythm screens
- Can identify New functional alleles of known function genes e.g.Flowering time QTL,EDI was the CRY2 gene
- Natural variation studies provide insight into the origins of plant evolution
- Identification of novel genes

Limitations

- Mainly identifies loci with large effects.
- Less strong ones can be hard to pursue.
- No. of QTLs detected, their position and effects are subjected to statistical error.

- Small additive effects / epistatic loci are not detected and may require further analyses.

Future Prospects

- Constant improvements of Molecular platforms
- New Types of genetic materials (e.g. introgression lines: small effect QTLs can be detected)
- Advances in Bioinformatics.

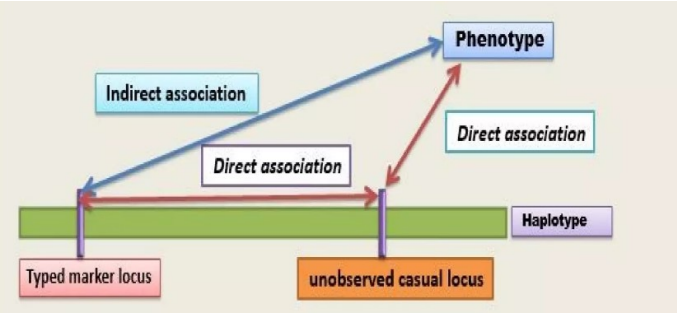
Association mapping:

Association mapping identifies quantitative trait loci (QTLs) by examining the marker-trait associations that can be attributed to the strength of linkage disequilibrium between markers and phenotype across a set of diverse.

Association mapping, also known as "**linkage disequilibrium mapping**", **is a method of mapping quantitative trait loci (QTLs) that takes advantage of linkage disequilibrium to link phenotypes to genotypes**. It offers greater precision in QTL location than family-based linkage analysis. It does not require family or pedigree information, can be applied to a range of experimental and non-experimental populations.

How it works?

Association studies are based on the assumption that a marker locus is 'sufficiently close' to a trait locus so that some marker allele would be 'travelling' along with the trait allele through many generations during recombination.

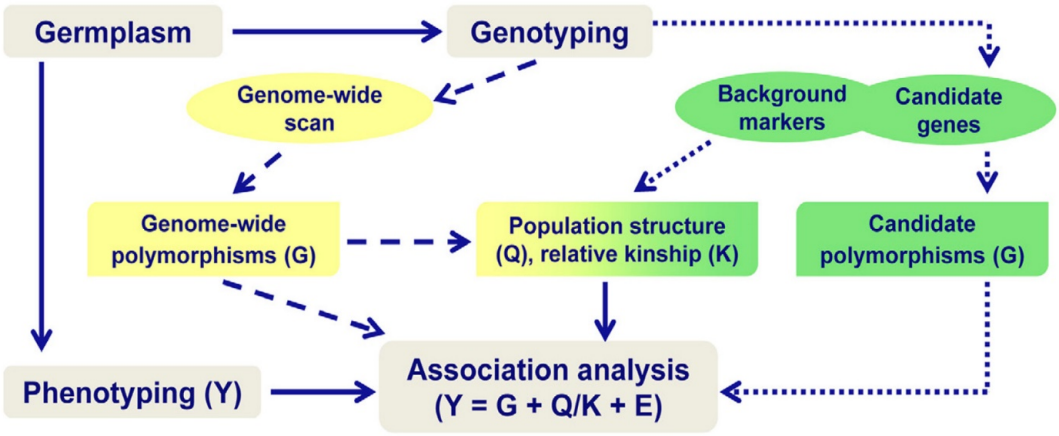


Types of Association mapping:

Based on the scale and focus of a particular study, association mapping generally falls into two broad categories:

1. Genome wide association mapping: Genome-wide association mapping or genome scan, which surveys genetic variation in the whole genome to find signals of association for various complex traits (Risch and Merikangas, 1996). Basically, entire genome is scanned using SNP or SSR rich marker, with saturated genome map leads to maximum opportunities for marker.

2. Candidate gene association mapping: Dissect out the genetic control of complex traits, based on the available results from genetic, biochemical, or physiology studies in model and non-model plant species (Mackay, 2001). Requires identification of SNPs between lines within specific genes.ait association and finding all possible across entire genome under study.



Genome-wide association mapping	Candidate-gene association mapping
It is a comprehensive approach to systematically search the genome for causal genetic variation. A large number of markers are tested for association with various complex traits, and prior information regarding candidate genes is not required. It works best for a research consortium with complementary expertise and adequate funding.	Candidate genes are selected based on prior knowledge from mutational analysis, biochemical pathway, or linkage analysis of the trait of interest. An independent set of random markers needs to be scored to infer genetic relationships. It is a low cost, hypothesis-driven, and trait-specific approach but will miss other unknown loci.

Steps in Association Mapping Association:

Mapping Population:

The choice of germplasm is crucial to the success of association analysis. Randomly mated populations represent a rather narrow group of germplasm, likely to lower resolution and harbour only a narrow range of alleles. Non-randomly mated germplasm is used; population structure needs to be controlled in the statistical analysis. Cluster analysis is done to know the variation in population and most diverse individuals are selected from each cluster to represent the individuals of that cluster.

Genotyping:

In association mapping studies, genotyping is required for inferences both on the population structure and relatedness as well as on marker-phenotype associations. With respect to the first task, the genotyping of a set of selectively neutral background markers distributed throughout the genome is required. Mostly multiallelic, reproducible, PCR-based markers are used. Microsatellites or simple sequence repeats (SSRs), and SNPs are more revealing than their dominant counterparts and, therefore, are more powerful. Due to higher genome density, lower mutation rate and wide distribution throughout the genome SNPs are rapidly becoming the marker of choice for complex trait.

Phenotyping:

Success of AM depends on accuracy and throughput of genotyping. Replications across multiple years in randomized plots and multiple locations and environments.

Field Design: - incomplete block design (Lattice), RBD (Eskridge, 2003).

Should be done on the basis of

- Diversity: - on the basis of phenotype and genotype
- Population structure: - Systematic difference in allele frequencies between. sub-population

Statistical analysis:

Structure of linkage disequilibrium (LD) for a specific locus will, reveal the association resolution possible at that locus. **TASSEL (http://www.maizegenetics.net)** is used to measure the extent of LD as squared allele frequency correlation estimates (R2, Weir, 1996) and measure the significance of R2. Eg. if LD decays within 1000 bp, then 1 or 2 markers per 1000 bp will be needed to identify associations. • Besides TASSEL there are many other types of software like DnaSP, Arlequin etc. used to calculate D‟ and R2.

Linkage Disequilibrium: Linkage disequilibrium means that we don't need to genotype the exact causal variant, but only a variant that is correlated with it.

LD refers to non-random association of alleles at different loci. It follows the fact that closely located genes are transmitted as a block, which only rarely breaks up in meiosis. Closely located genes often express linkage disequilibrium to each other: An example: Consider two independently segregating genes A and B with two alleles (A, a and B, b respectively) At equilibrium, the frequency of the AB should equal to the product of the allele frequencies of A and B,

PAB Pab =PAb PaB (1:1 ratio = no LD)

Any deviation from these values implies LD.

LD Quantification

1. $\mathbf{D = P_{AB} - P_A.P_B}$

   where… (P_{AB} - Gametic freq. ; P_A&P_B -allelic freq.)

1. $\mathbf{D' = \frac{D}{D_{max}}}$

   where… D_{max} = min(pA.pb, pa.pB) if D>0
   D_{max} = min(pA.pB, pa.pB) if D<0

- D‟ ranges from 0-1
  1) D= 0 …. no linkage
  2) D=1 …complete LD

3. **Correlation between two loci (r^2) =** $\frac{D^2}{P_A P_a P_B P_b}$

- r^2 ranges from 0-1
  1) $r^2 = 0$ ….complete linkage equilibrium
  2) $r^2 = 1$ ....complete linkage disequilibrium
  3) $r^2 \geq 0.33$ ....considered useful for LD mapping

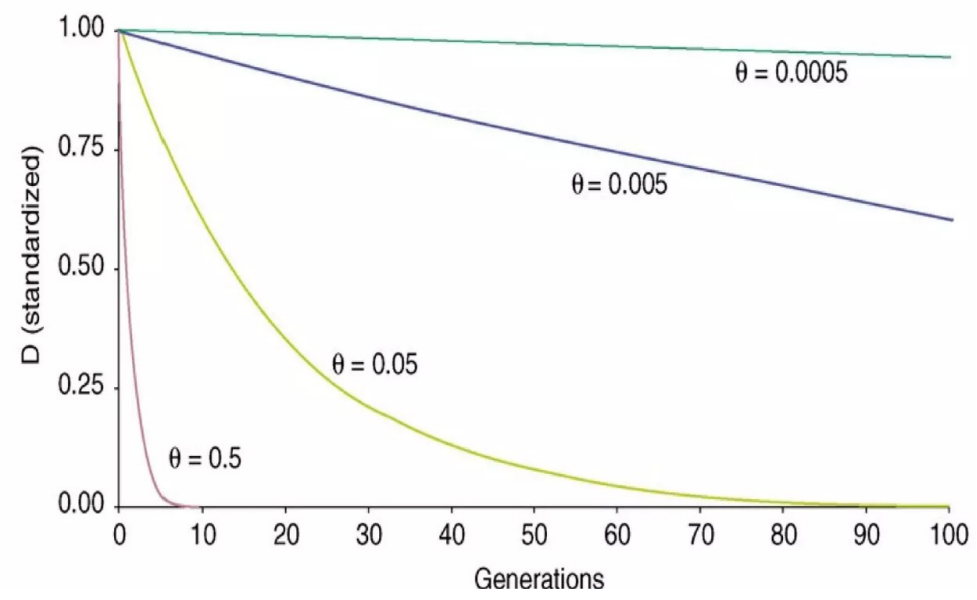


Fig.: LD Decay with time for four different recombination fractions (θ).

Application of association mapping:

- Much of the association mapping in crop plants is just emerging from the research phase and is beginning to be applied, especially in commercial breeding setting. •

- First attempt on candidate-gene association mapping study in plants (maize) resulted in the identification of DNA sequence polymorphisms within the D8 locus associated with flowering time (Thornsberry et al., 2001).
- Using same population, Whitt et al., 2002 associated the candidate gene su1 with sweetness taste, bt2, sh1 and sh2 with kernel composition, and Wilson et al., 2004 ae1 and sh2 with starch pasting properties.

Association mapping studies in plant species.

Plant species	Populations	Sample size	Background markers	Traits	References
Maize	Diverse inbred lines	92	141	Flowering time	(Thornsberry et al., 2001)
	Elite inbred lines	71	55	Flowering time	(Andersen et al., 2005)
	Diverse inbred lines and landraces	375 + 275	55	Flowering time	(Camus-Kulandaivelu et al., 2006)
	Diverse inbred lines	95	192	Flowering time	(Salvi, 2007)
	Diverse inbred lines	102	47	Kernel composition Starch pasting properties	(Wilson et al., 2004)
	Diverse inbred lines	86	141	Maysin synthesis	(Szalma et al., 2005)
	Elite inbred lines	75		Kernel color	(Palaisa et al., 2004)
	Diverse inbred lines	57		Sweet taste	(Tracy et al., 2006)
	Elite inbred lines	553	8950	Oleic acid content	(Belo et al., 2008)
	Diverse inbred lines	282	553	Carotenoid content	(Harjes et al., 2008)
Arabidopsis	Diverse ecotypes	95	104	Flowering time	(Olsen et al., 2004)
	Diverse ecotypes	95	2553	Disease resistance Flowering time	(Aranzana et al., 2005) (Zhao et al., 2007)
	Diverse accessions	96		Shoot branching	(Ehrenreich et al., 2007)
Sorghum	Diverse inbred lines	377	47	Community resource report	(Casa et al., 2008)
Wheat	Diverse cultivars	95	95	Kernel size, milling quality	(Breseghello and Sorrells, 2006b)
Barley	Diverse cultivars	148	139	Days to heading, leaf rust, yellow dwarf virus, rachilla hair length, lodicule size	(Kraakman et al., 2006)
Potato	Diverse cultivars	123	49	Late bright resistance	(Malosetti et al., 2007)
Pinus taeda	Unstructured natural population	32	21	Wood specific gravity, late wood	(Gonzalez-Martinez et al., 2006)
	Lines	435	288	Microfibril angle, cellulose content	(Gonzalez-Martinez et al., 2007)
Sugarcane	Diverse clones	154	2209	Disease resistance	(Wei et al., 2006)
Eucalyptus	Unstructured natural population	290	35	Microfibril angle	(Thumma and Nolan, 2005)
Perennial ryegrass	Diverse natural germplasms	26	589	Heading date	(Skøt et al., 2005)
	Diverse natural germplasms	96	506	Flowing time, water soluble carbohydrate	(Skøt et al., 2007)

Advantages:

- ✓ Saves time, effort, and cost needed for the development of specific mapping populations.
- ✓ The QTL-linked markers identified by AM can be directly used for MAS
- ✓ AM has high resolution
- ✓ AM would assess the entire range of diversity in the trait of interest

- ✓ Associated markers identified during AM can be used for either selection of parents for hybridization or for selection of desirable segregants

Disadvantages:

- The results from AM are affected by several factors like selection history, population structure, kinship, etc., may lead to false positive association
- Large number (hundreds of thousands or even millions) of markers would be required to adequately cover the entire genome.
- High quality phenotypic data required (Multiple environments with multi location)
- The rate of recombination is not uniform throughout the genome.

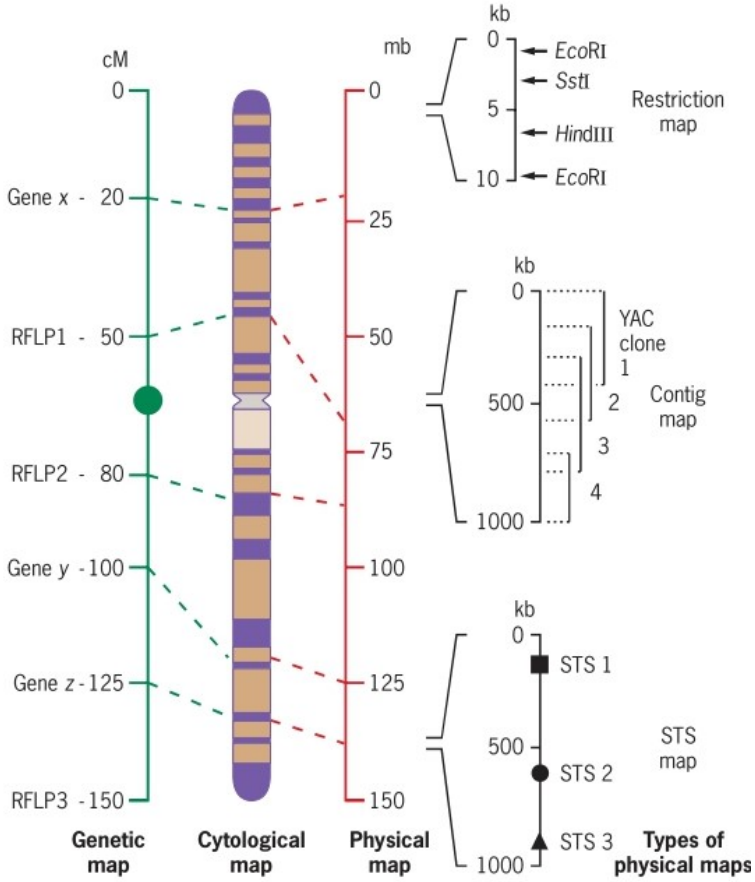
Integration of Genetic and Physical Maps

The ability of scientists to identify and isolate genes based on information about their location in the genome was one of the first major contributions of genomics research. In principle, this approach, called **positional cloning**, can be used to identify and clone any gene with a known phenotypic effect in any species. Positional cloning has been used extensively in many species, including humans.

Because the utility of positional cloning depends on the availability of detailed maps of the regions of the chromosomes where the genes of interest reside, major efforts have focused on developing detailed maps of the human genome and the genomes of important model organisms such as *D. melanogaster, C. elegans*, and *A. thaliana*. The goal of this research is to construct correlated genetic and physical maps with markers distributed at relatively short intervals throughout the genome. In the case of the human and Drosophila genomes, the genetic and physical maps can also be correlated with cytological maps (banding patterns) of the chromosomes.

The genetic maps are constructed from recombination frequencies, with 1 centiMorgan (cM) equal to the distance that yields an average frequency of recombination of 1 percent. Genetic maps with markers spaced at short intervals—high-density genetic maps—are often constructed by using molecular markers such as restriction fragments

of different lengths (restriction fragment-length polymorphisms, or RFLPs). Cytological maps are based on the banding patterns of chromosomes observed with the microscope after treatment with various stains. Physical maps, such as the restriction maps are based on the molecular distances— base pairs (bp), kilobases (kb, 1000 bp), and megabases (mb, 1 million bp)—separating sites on the giant DNA molecules present in chromosomes. Physical maps often contain the locations of overlapping genomic clones or contigs and unique nucleotide sequences called sequence-tagged sites, or STSs.



Physical maps of a chromosome can be correlated with the genetic and cytological maps in several ways. Genes that have been cloned can be positioned on the cytological map by in situ hybridization. Correlations between the genetic and physical maps can be established by locating clones of genetically mapped genes or RFLPs on the physical map. Markers that are mapped both genetically and physically are called **anchor markers**; they anchor the physical map to the genetic map and vice versa. Physical maps of chromosomes can also be correlated with genetic and cytological maps by using (1) PCR to amplify short—usually 200 to 500 bp—unique genomic DNA sequences, (2) Southern blots to relate these sequences to overlapping clones on physical maps, and (3) in situ hybridization to

determine their chromosomal locations (cytological map positions). These short, unique anchor sequences are called sequence-tagged sites (STSs). Another approach uses short cDNA sequences (DNA copies of mRNAs), or expressed-sequence tags (ESTs), as hybridization probes to anchor physical maps to RFLP maps (genetic maps) and cytological maps.

Physical distances do not correlate directly with genetic map distances because recombination frequencies are not always proportional to molecular distances. However, the two are often reasonably well correlated in euchromatic regions of chromosomes. In humans, 1 cM is equivalent, on average, to about 1 mb of DNA.

10. Molecular Breeding: Gene tagging, Marker Assisted Selection (MAS), Bulk Segregation Analysis (BSA), genomic selection, genome-wide association study (GWAS).

Gene tagging:

Gene tagging refers to the identification of existing DNA or the introduction of new DNA that can function as a tag or label for the gene of interest. Gene tagging is a most common method used today for selection against different biotic and abiotic stress resistances studies in crop plants. There are four different strategies used for gene tagging.

Types of gene tagging:

1. Marker based gene tagging
2. Transposon tagging
3. T-DNA tagging
4. Epitope tagging

Marker Assisted Selection (MAS):

Marker Assisted Selection (MAS) refers to indirect selection for a desired plant phenotype based on the banding pattern of linked molecular (DNA) markers. MAS is based on the concept that it is possible to infer the presence of a gene from the presence of a marker which is tightly linked to the gene of interest.

If the marker and the gene are located far apart then the possibility of their transmission together to the progeny individuals will be reduced due to double crossover recombination events.

Features of Marker Assisted Selection (MAS):

1. Pre-Requisites:

There are two pre-requisites for marker assisted selection. These are: (i) a tight linkage between molecular marker and gene of interest, and (ii) high heritability of the gene of interest.

2. Markers Used:

MAS makes use of various types of molecular markers. The most commonly used molecular markers include amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) or micro satellites, single nucleotide polymorphisms (SNP), etc. The use of molecular markers differs from species to species also.

3. Efficiency:

The relative efficiency of MAS is greatest for characters with low heritability, if a large fraction of the additive genetic variance is associated with the marker loci. In other words, MAS is useful when the heritability of the trait is low. Moreover, MAS is more efficient than purely phenotypic selection in quite large populations.

It has been found by some workers that MAS may become less efficient than phenotypic selection in the long term. This is because the rate of fixation of unfavourable alleles at

QTLs with small effects is higher under MAS than under phenotypic selection. It may be a consequence of the strong selection applied to QTLs with large effects under MAS in early generation. However, such problem comes after a long period.

4. Accuracy:

Molecular markers have very high accuracy. They are not affected by environmental conditions. MAS is a new breeding tool which is available to make more accurate and useful selections in breeding populations. MAS allows heritable traits to be linked to the DNA which is responsible for controlling that trait.

5. Speed of Progress:

MAS is a rapid method of crop improvement. For example, in conventional breeding when we transfer a recessive character through backcross, one selfing is required after every backcross for identification of recessive character. MAS permits identification of recessive alleles even in heterozygous condition and thus speeds up the progress of crop improvement work.

6. Traits Improved:

MAS can be used for improvement of both oligogenic and polygenic traits. In the past, MAS has been mostly used for the genetic improvement of oligogenic traits and little progress has been made with polygenic traits.

7. Material Developed:

MAS leads to development of non-transgenic genotypes or cultivars. In other words, MAS is used for development of non-transgenic cultivars. The transgenic cultivars face public resistance. On the other hand, cultivars developed by MAS are acceptable by consumers.

8. Cost:

MAS is very costly as compared to phenotypic selection. In MAS, the costly items include equipment's, consumables, infrastructure, labour and DNA extraction process. MAS requires sophisticated and well-equipped laboratory.

9. Application:

MAS is applicable for genetic improvement of plants as well as animals. In plants, it is equally applicable in both self-pollinated and cross-pollinated species.

Steps in Marker Assisted Selection (MAS):

In the marker aided selection, RFLP markers are widely used for genetic improvement of crop plants for various economic characters.

The marker aided selection consists of five important steps, viz:

(i) Selection of parents

(ii) Development of breeding population

(iii) Isolation of DNA from each plant

(iv) Scoring RFLPs

(v) Correlation with morphological traits

i) Selection of Parents:

Selection of suitable parents is an important step in marker aided selection. The parents should be such so that we can get usable level of polymorphism (variation) in the RFLP markers. In other words, parents with contrasting characters or divergent origin should be chosen. This will help in identification of DNA of both the parents and also their segments in F2 generation in various recombinations.

For selection of parents, we have to screen germplasm and select parents with distinct DNA. The parents that are used for MAS should be pure (homozygous). In self-pollinated species, plants are usually homozygous. In cross-pollinated species, inbred lines are used as parents.

ii) Development of Breeding Populations:

This is the second important step for application of marker aided selection. The selected parents are crossed to obtain F1 plants. F1plants between two pure-lines or inbred lines are homogeneous (alike phenotypically) but are heterozygous for all the RFLPs of two parents involved in the F1. The F2 progeny is required for the study of segregation

pattern of RFLPs. Generally, 50-100 F2 plants are sufficient for the study of segregation of RFLP markers.

iii) Isolation of DNA:

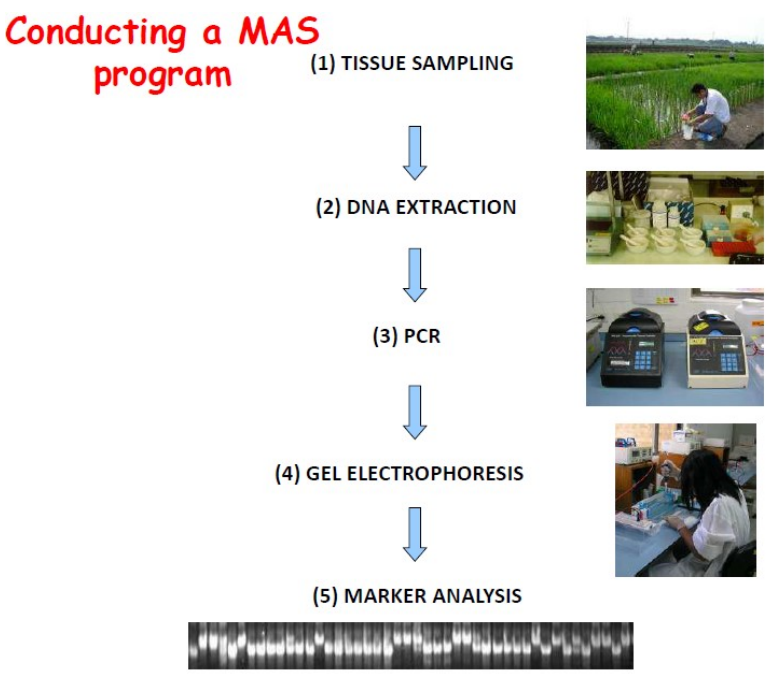
The third important step is isolation of DNA from breeding population. The main advantage of MAS is that DNA can be isolated even from the seedlings and we need not to wait for flowering or seed development stage. The DNA is isolated from each plant of F2population. Standard procedures are available for DNA isolation.

The isolated DNA is digested with specific restriction enzyme to obtain fragments of DNA. The DNA fragments of different sizes are separated by subjecting the digested DNA to agarose gel electrophoresis. The gel is stained with ethidium bromide and the variation in DNA fragments can be viewed in the ultraviolet light.

The DNA of chloroplasts, when digested with specific enzyme, produces about 40 fragments of different sizes. The nuclear DNA of higher plants, when digested with specific restriction enzymes, produces millions of fragments in a continuous range of sizes. It is a tedious job to identify individual DNA fragment in such cases.

iv) Scoring RFLPs:

The polymorphism in RFLPs between the parents and their involvement in the recombinants in F2 population is determined by using DNA probes. The labelled probes are used to find out the fragments having similarity.

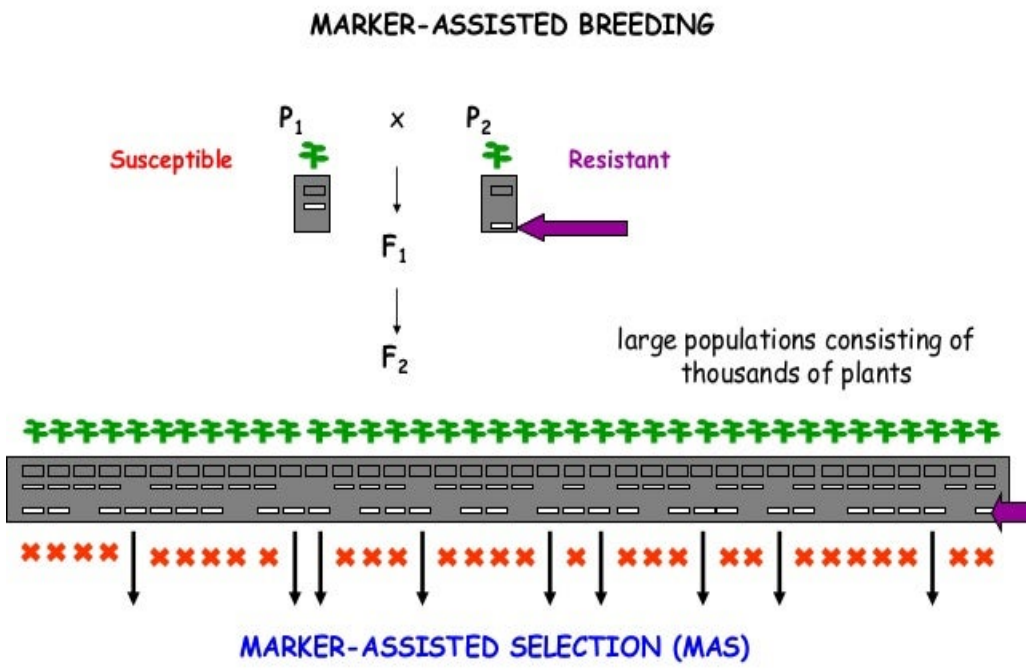


The probe will

hybridize only with those segments which are complementary in nature. Generally 32P is used for radioactive labelling of DNA probe. Now non-radioactive probe labelling techniques are also available. In this way RFLPs are determined.

v) Correlation with Morphological Traits:

The DNA marker (say RFLPs) are correlated with morphological markers and the indirect selection through molecular markers is confirmed. Once the correlation of molecular markers is established with morphological markers, MAS can be effectively used for genetic improvement of various economic traits.



Method whereby phenotypic selection is based on DNA markers

MAS schemes in plant breeding:

1. Marker-assisted backcrossing:

Marker-assisted backcrossing is the simplest form of MAS, in which the goal is to incorporate a major gene from an agronomically inferior source (the donor parent) into

an elite cultivar or breeding line (the recurrent parent). The desired outcome is a line containing only the major gene from the donor parent, with the recurrent parent genotype present everywhere else in the genome.

There are three levels of selection in which markers may be applied in backcross breeding.

- In the first level, markers may be used to screen for the target trait, which may be useful for traits that have laborious phenotypic screening procedures or recessive alleles.
- The second level of selection involves selecting backcross progeny with the target gene and tightly-linked flanking markers in order to minimize linkage drag. We refer to this as **recombinant selection**.
- The third level of MAB involves selecting backcross progeny (that have already been selected for the target trait) with background markers. In other words, markers can be used to select against the donor genome, which may accelerate the recovery of the recurrent parent genome.

2. Marker assisted pyramiding

Pyramiding is the process of simultaneously combining multiple genes/QTLs together into a single genotype. This is possible through conventional breeding but extremely difficult or impossible at early generations. Using conventional phenotypic selection, individual plants must be phenotypically screened for all traits tested. Therefore, it may be very difficult to assess plants from certain population types (e.g. F2) or for traits with destructive bioassays. DNA markers may facilitate selection because DNA marker assays are non-destructive and markers for multiple specific genes/QTLs can be tested using a single DNA sample without phenotyping. The most widespread application for pyramiding has been for combining multiple disease resistance genes in order to develop durable disease resistance.

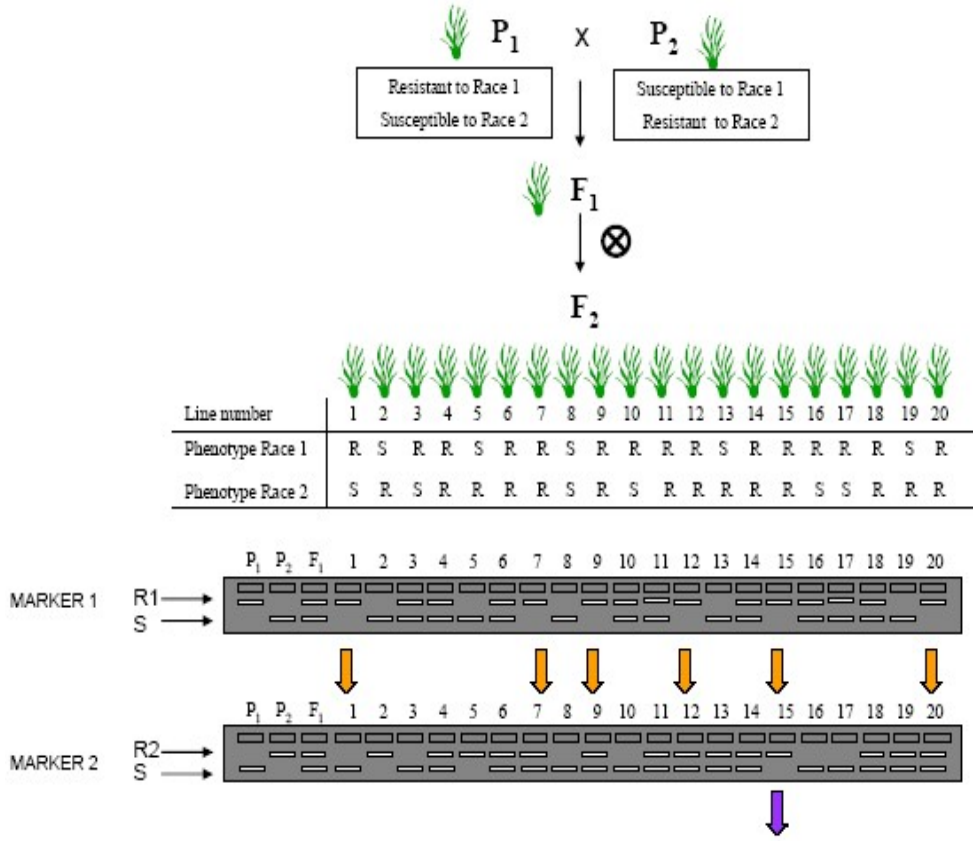


Fig.: Marker assisted pyramiding of two disease resistance genes.

3. Early generation marker assisted selection:

One of the most intuitive stages to use markers to select plants is at an early generation (especially F_2 or F_3). The main advantage is that many plants with unwanted gene combinations, especially those that lack essential disease resistance traits and plant height, can be simply discarded. This has important consequences in the later stages of the breeding program because the evaluation for other traits can be more efficiently and cheaply designed for fewer breeding lines.

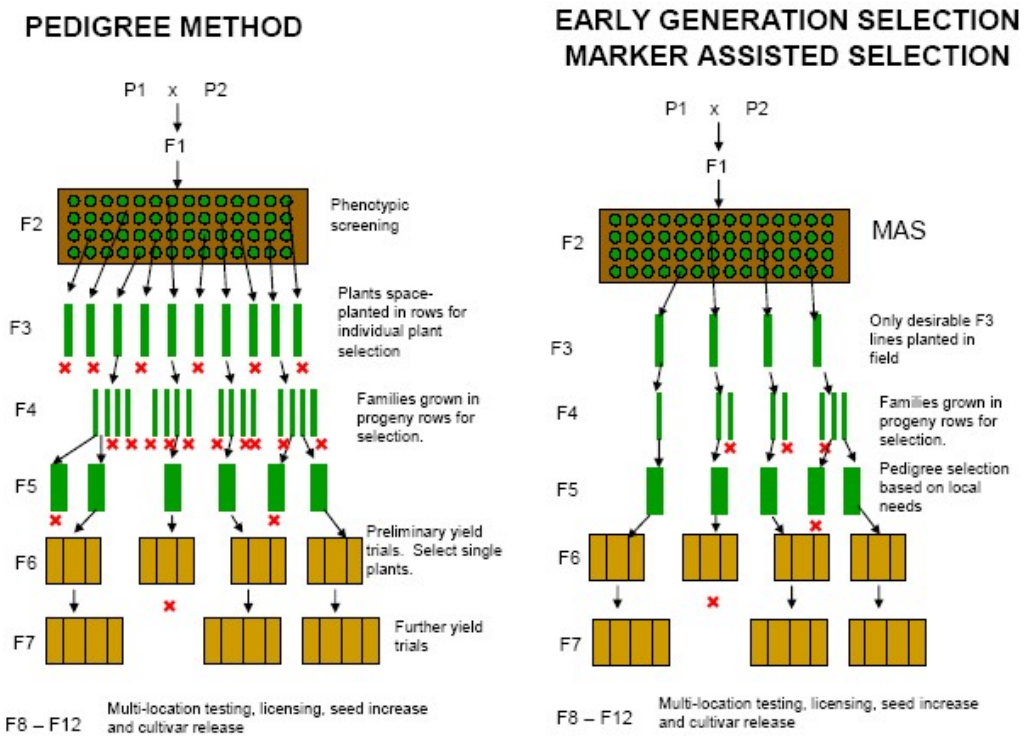


Fig.: Comparing conventional and marker-assisted backcrossing.

4. Combined approaches:

In some cases, a combination of phenotypic screening and MAS approach may be useful

1. To maximize genetic gain (when some QTLs have been unidentified from QTL mapping)
2. Level of recombination between marker and QTL (in other words marker is not 100% accurate)
3. To reduce population sizes for traits where marker genotyping is cheaper or easier than phenotypic screening

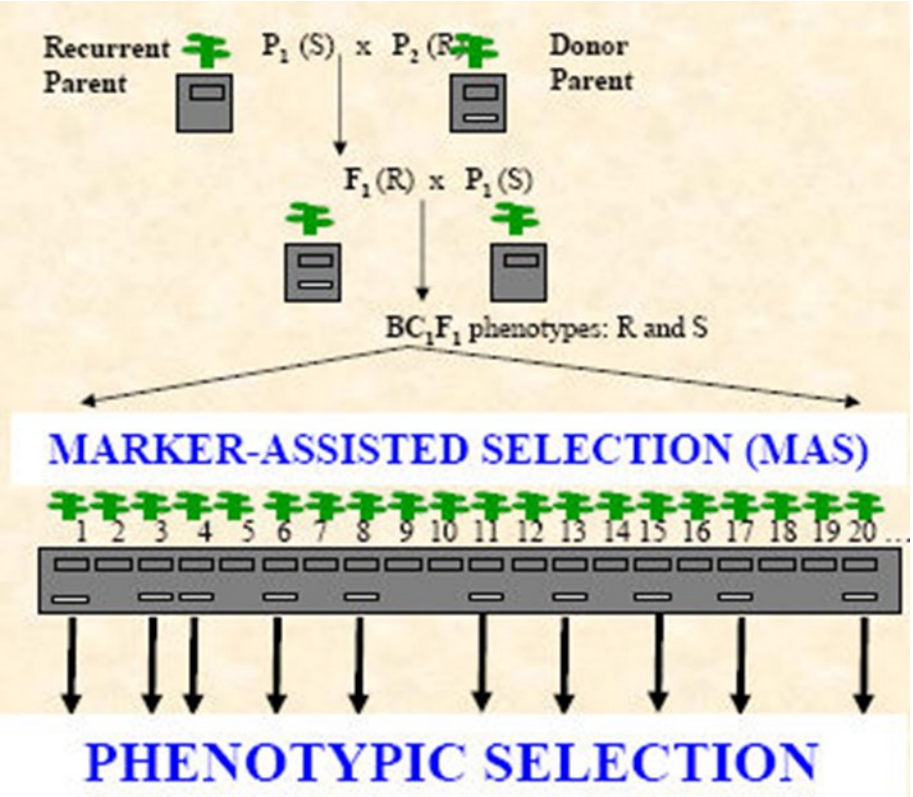


Fig.: Marker-directed phenotyping or tandem selection (can be used when markers are not 100% accurate or when phenotypic screening is more expensive compared to marker genotyping.

Applications of Marker Assisted Selection (MAS):

In crop improvement programmes MAS can be used in various ways. In other words, MAS has several useful applications in plant breeding.

Important applications of MAS in plant breeding are briefly presented below:

i. MAS is very effective, efficient and rapid method of transferring resistance to biotic and abiotic stresses in crop plants.

ii. It is useful in gene pyramiding for disease and insect resistance.

iii. It is being used for transfer of male sterility and photo period insensitivity into cultivated genotypes from different sources.

iv. MAS is being used for improvement of quality characters in different crops such as for protein quality in maize, fatty acid (linolenic acid) content in soybean and storage quality in vegetables and fruit crops.

v. MAS can be successfully used for transferring desirable transgene (such as Bt gene) from one cultivar to another.

vi. MAS is very effective in introgression of desirable genes from wild into cultivated genotypes.

vii. MAS is equally effective in genetic improvement of plants and animals.

viii. MAS is useful in genetic improvement of tree species where fruiting takes very long time (say 20 years) because for application of phenotypic selection we have to wait for such a long time.

ix. MAS has wide application for genetic improvement of oligogenic traits as compared to polygenic traits.

Achievements of Marker Assisted Selection (MAS):

MAS has been used for genetic improvement of different field crops such as maize, barley, rice, wheat, sorghum, soybean, chickpea, pea, sunflower, tomato, potato and some fruit crops for various economic characters. Some notable examples of the use of MAS:

i) Rice:

In rice MAS has been successfully used for developing cultivars resistant to bacterial blight and blast. For bacterial blight resistance four genes (Xa4, Xa5, Xa13 and Xa21) have been pyramided using STS (sequence tagged site) markers.

The pyramided lines showed higher level of resistance to bacterial blight pathogen. In Indonesia, two bacterial blight resistant varieties of rice viz Angke and Conde have been released through MAS. For blast resistance, three genes (Pil, Piz5 and Pita) have been pyramided in a susceptible rice variety Co 39 using RFLP and PCR based markers.

ii) Maize:

In maize, normal lines have been converted into quality protein maize (QPM) lines through MAS using opaque 2 recessive alleles. This work has been done at CIMMYT (International centre for wheat and maize improvement, Mexico).

Three SSR markers (Umc 1066, Phi 057 and Phi 112) present within opaque 2 gene have been used for this purpose. The MAS used for conversion of normal maize lines into QPM is simple, rapid and accurate.

TABLE 34.2. Characters being used for MAS in different crops

Crop	*Disease Resistance*	*Insect Resistance*	*Other Traits*
Rice	Bacterial blight	Brow plant hopper	Submergence tolerance
	Blast	Green leaf hopper	Salt tolerance
	Rice Tungro virus		Temperature sensitivity
	Gall midge		Male sterility
			Photoperiod sensitivity
			Semidwarf stature
			Shattering resistance
			Grain Aroma
			Amylose content
Maize	Northern corn blight	–	Cytoplasmic male sterility
			QPM
			Days to pollen shed
Wheat	Leaf rust	Hessian fly	Cyst rematode
	Powdery mildew		Root lesion nematode
	Loose smut		Earliness
			Vernalization requirement
			Coleoptile colour
			Flour colour
Sorghum	Head smut	–	Fertility restoration
Soybean	Cyst nematode	–	Linolenic acid
	Mosaic Virus		Content
Pea	Fusarium wilt	–	Nodulation ability
	Powdery mildew		
Tomato	Yellow leaf curl virus	–	–
	Black mold		
	Bacterial wilt		
Potato	Cyst nematode	–	–
	Potato virus ×		
	Late blight		
Sunflower	Downy mildew	–	–
Chick pea	–	–	Double podding
Barley	Powdery mildew	–	–
	Stripe rust	–	
Mung bean	Powdery mildew	–	–

iii) Soybean:

In soybean cyst nematodes pose serious problem and most of the varieties are susceptible to this parasite. The resistant gene (rhg 1) is available. In soybean, nematode resistant lines have been developed through MAS using SSR marker (Sat 309).

Advantages of Marker Assisted Selection (MAS):

MAS has several advantages over phenotypic selection and other breeding techniques.

i) Accuracy:

The accuracy of MAS, is very high because molecular markers are not affected by environmental conditions. It is very effective even with the characters having low heritability.

ii) Rapid Method:

MAS is a rapid method of crop improvement. It takes 3-5 years for developing a new cultivar against 10-15 years taken by the conventional method of breeding.

iii) Non-transgenic Product:

MAS leads to development of non-transgenic cultivars which are acceptable to everybody. In other words, it does not involve transgene. Hence there is no question of gene silencing.

iv) Identification of Recessive Alleles:

MAS permits identification of recessive alleles even in heterozygous condition and thus speeds up the progress of crop improvement programmes. In other words, it is equally effective for the genetic improvement of recessive characters.

v) Early Detection of Traits:

MAS permits early detection of traits that are expressed late in the life of plant. For example, characters such as grain or fruit quality, flower colour, male sterility, photoperiod sensitivity that express late in the life of a plant can be screened in the

seedling stage. In other words, DNA tested at seedling stage can through light about the trait which are expressed later on.

vi) Screening of Difficult Traits:

MAS permits screening traits that are extremely difficult expressive and time consuming to score phenotypically. For example, screening for traits such as root morphology and resistance to biotic (insects and diseases) and abiotic stresses (drought, salinity, heat, frost etc.) is very easy through MAS.

vii) Gene Pyramiding:

MAS is very effective method in accumulating multiple genes for resistance to specific pathogens and pests within the same cultivar. This process is called gene pyramiding. Maker assisted backcrossing is routinely applied in breeding programmes for gene introgression. MAS can provide an effective and efficient breeding tool for detecting, tracking, retaining, combining and pyramiding genes for disease resistance.

viii) Small Sample for Testing:

MAS requires only a small amount of plant tissue for DNA testing. In other words, MAS can be carried out with small breeding populations. Moreover, MAS can be applied at any stage of plant growth.

ix) Permits QTL Mapping:

MAS permits mapping or tagging of quantitative trait loci (QTL) which is not possible by conventional method.

x) Highly Reproducible:

The MAS is based on DNA fingerprinting technique and the results of DNA fingerprinting pattern are highly reliable and reproducible.

Limitations of Marker Assisted Selection (MAS):

- MAS is a costly method. It requires well equipped laboratory viz. expensive equipment's, glassware and chemicals.
- MAS requires well trained manpower for handling of sophisticated equipments, isolation of DNA molecule and study of DNA markers.
- The detection of various linked DNA markers (AFLP, RFLP, RAPD, SSR, SNP etc.) is a difficult, laborious and time-consuming task.
- MAS sometimes involves use of radioactive isotopes in labelling of DNA, which may lead to serious health hazards. This is a major disadvantage of RFLP based markers. The PCR, markers are safe in this regard.
- It has been reported that MAS may become less efficient than phenotypic selection in the long term.
- The use of MAS is more difficult for QTL because they have minor cumulative effects and are greatly influenced by environmental conditions and genetic background.

Bulked Segregant Analysis:

BSA (bulked segregant analysis) is a method for identifying genetic markers linked to a mutant phenotype. Geneticists can use this information to find genes that confer disease resistance or susceptibility. Forming two groups with opposing phenotypes for a trait of interest is the goal of this technique. Individuals in one group, for example, are disease resistant whereas those in the second group are not. The DNA of all individuals in each group is then pooled to create two bulked DNA samples.

These two bulked samples can then be analyzed using Restriction fragment length polymorphism (RFLP) or RAPD to detect similarities and differences in the genome's various loci. Except for loci associated with the mutation, both groups will have a random distribution of alleles in all of the genome's loci. A consistent difference between the two bulked samples on a locus most likely indicates that the locus is linked to the mutation of interest.

Individuals in the two testing groups are usually produced in animals by crossing two siblings who are heterozygous for the mutation of interest. It's necessary to use siblings to ensure that the alleles that cause the mutation are the same among the individuals.

To identify the genes associated with the trait of interest, there must be a minimum amount of heterozygosity in the various loci of the groups. Due to the inbred nature of most laboratory strains, outcrossing the homozygous mutated individual with a polymorphic strain is required to generate effective testing groups. To create testing groups, the offspring are crossed with each other.

Analysis Techniques for Bulked Segregant Analysis:

Southern blotting can be used to examine bulk DNA samples. RFLP, SNP and RAPD analysis require the use of restriction enzymes or PCR amplification on DNA. The restriction digest sites and the sequences on which PCR primers attach are the loci that are examined in these techniques. These sites are typically found all over the genome. Once linked loci have been identified, they can be mapped and linkage distances calculated.

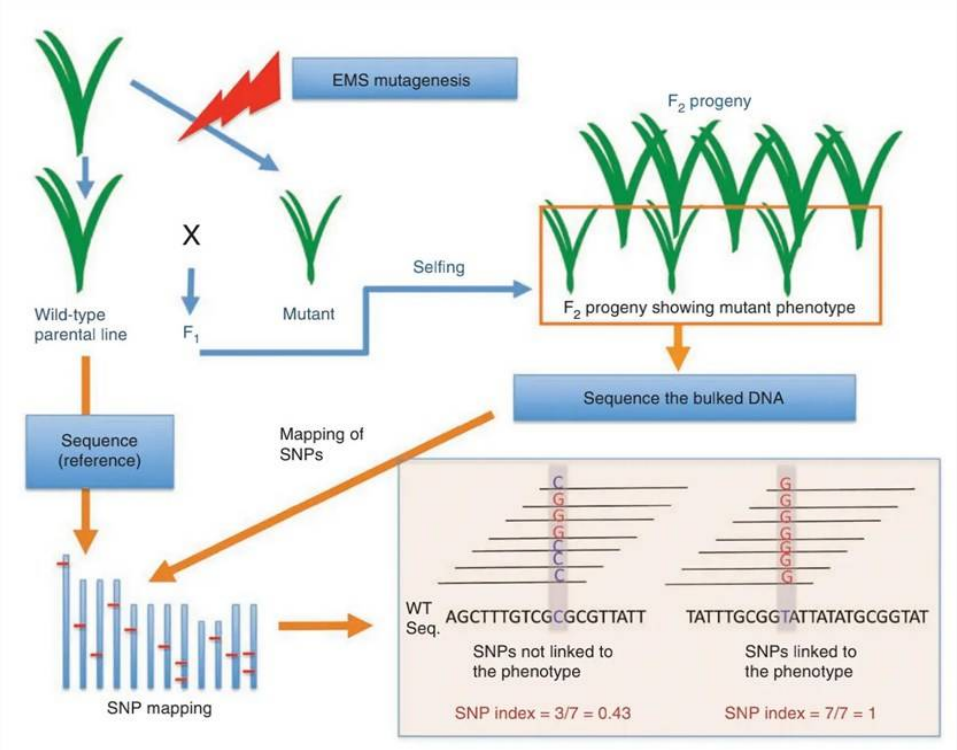


Fig.: Flowchart of the BSA Experiment.

BSA-seq Data Analysis:

To identify functional gene loci, a comparison at the SNP level between the two pooled and sequenced populations is essential. The most commonly employed method for this purpose is the SNP-index approach. Its underlying principle involves the statistical analysis of bases at each nucleotide position using the sequencing reads. A reference parent or an existing reference genome is typically selected as a reference. The counts of reads in the offspring pool that match or differ from the reference at a specific nucleotide position are tallied, and the ratio of differing reads to the total reads at that position is calculated, yielding the SNP-index. The SNP-index is determined within a sliding window, usually using a 1 Mb window size with a 10 kb increment for each slide. Additionally, Δ(SNP-index) is used to measure the difference in SNP indices between the two gene pools, effectively capturing variations at individual loci.

Advantages:

- Short experimental period
- Accurate mapping results
- Cost-effective

Common methods:

Quantitative Trait Loci Sequencing (QTL-Seq):

Bulked segregant analysis, as used in QTL-seq, is a powerful and efficient way to find agronomically important loci in crop plants. To find quantitative trait loci, QTL-seq was adapted from MutMap. This method locates a gene that has undergone natural mutations.

Mutmap:

The MutMap-Gap methodology identifies the mutation site in the reference genome's gap regions. This method locates a gene that has been artificially mutated.

Applications of Bulked Segregant Analysis:

Early applications almost exclusively used molecular markers and bulked segregant analysis to map genes with relatively large effects for agronomically important traits

like grain yield, drought tolerance, and heat tolerance in rice, water-stress tolerance in wheat, and salt tolerance in Egyptian cotton.

Many agronomical traits, including resistance genes to fungal pathogens, have been mapped using bulked segregant analysis (BSA) in several crops. It was suggested that the inheritance of ABS resistance in citrus is managed by a central recessive allele using BSA in diploid progenies.

Genomic selection:

Genome selection (GS) is a specialized form of MAS, in which information from genotype data on marker alleles covering the entire genome forms the basis of selection. The term GS was proposed by Meuwissen et al. (2001). The effects associated with all the marker loci, irrespective of whether the effects are significant or not, covering the entire genome are estimated. The marker effect estimates are used to calculate the **genomic estimated breeding values (GEBVs)** of different individuals/lines, which form the basis of selection.

Why to go for genomic selection?

- Marker-assisted selection (MAS) is well-suited for handling oligogenes and quantitative trait loci (QTLs) with large effects but not for minor QTLs.
- MARS attempts to take into account small effect QTLs by combining trait phenotype data with marker genotype data into a combined selection index.
- Based on markers showing significant association with the trait(s) and for this reason has been criticized as inefficient.
- The genomic selection (GS) scheme was to rectify the deficiency of MAS and MARS schemes. The GS scheme utilizes information from genome-wide marker data whether or not their associations with the concerned trait(s) are significant.

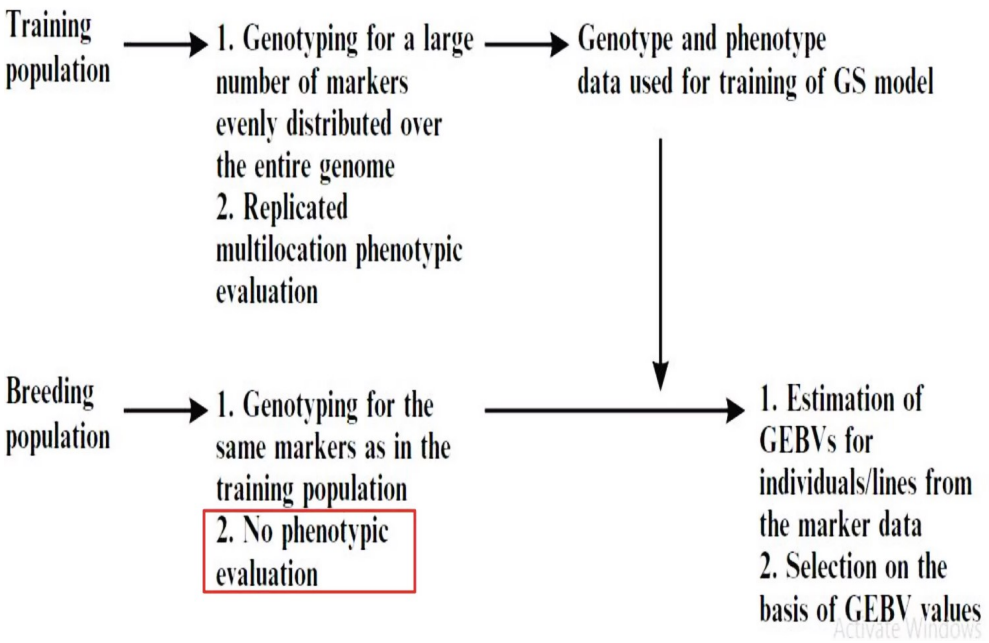
Genomic Estimated Breeding Values (GEBV):

The sum total of effects associated with all the marker alleles present in the individual and included in the GS model applied to the population under selection

- Calculated on a single individual basis

- ❖ Gene-assisted genomic selection: A GS model that uses information about prior known QTLs, the targeted QTLs were accumulated in much higher frequencies than when the standard ridge regression was used.

Schematic representation of genomic selection (GS) scheme:



Steps in genomic selection:

1. Creation of training population
2. Genotyping of training population
3. Phenotyping of training population
4. Model training
5. Genotyping of Breeding population
6. Calculation of GEBV values
7. Selection of superior lines /individuals

Training population (TP): used for training of the GS model and for obtaining estimates of the marker- associated effects needed for estimation of GEBVs of individuals/lines in the breeding population.

TP – Genotyping:

Markers like SNP, DArT, SSRs and GBS (Genotyping by sequencing) are widely used in GS. Dominant markers lower accuracy of GEBV prediction than co-dominant markers. Inexpensive, high-density genotypes. Dense marker coverage to maximize the number of QTL

TP – Phenotyping: Accurate, replicated and multi-location.

Breeding population:

The population subjected to GS for achieving the desired improvement and isolation of superior lines for use as new varieties/parents of new improved hybrids.

- Population with only genotypic data.
- Genotyping done for the same markers as in the training population.
- Breeding population derived from the parental lines that are present in the training population.

Statistical model development:

i. **Shrinkage models:** SR, RR-BLUP, G-BLUP
ii. **Dimension reduction methods**: Partial least square regression, Principal component regression, Least absolute shrinkage and selection operator (LASSO)
iii. **Variable selection models**: Bayes A & B, BayesCπ, BayesDπ
iv. **Kernel Regression and machine learning methods:** Support vector machine regression (SVM), Random Forest (RF).

Studies on GS in different crop species

Crop	Population size		Number of markers	GEBV accuracy[a]	GS model[b]	Reference
	Breeding population	Training population				
Maize	119	95	1,339	0.40–0.50	BLUP	Lorenzana and Bernardo (2009)
	349	28, 35, 70	160	0.59–0.72	BLUP	
A. thaliana	415	50–133	69	0.90–0.93	BLUP	
Barley	150	54, 96, 120	223	0.64–083	BLUP	
Maize	208	208	136	1.00	Several[c]	Piepho (2009)
Wheat	599	60	1,279	0.48–0.61	PM-RKHS[c]	Crossa et al. (2010)
Maize	300	270	1,148	0.42–0.79	LASSO	
Wheat	209	24, 48, 96	399	0.32–0.84	RR-BLUP	Heffner et al. (2010)
Wheat	174	24, 48, 96	574	0.41–0.73	RR-BLUP	
Maize	25 populations of 126–196	25–157 for each population	1,106	0.26–0.57	RR-BLUP	Guo et al. (2012)

Advantages of Genomic Selection:

- ✓ The marker effects are estimated from the training population and used directly for GS in the concerned breeding population, and QTL discovery, mapping, etc. are not required. 2. Both simulation and empirical studies reveal that GS produces greater gains per unit time than phenotypic selection.
- ✓ GS is able to predict the performance of breeding lines more accurately than that based on pedigree data, and GS seems to be an effective tool for improving the efficiency of rice breeding.
- ✓ The selection index approach integrates appropriately weighted data from multiple traits into an index that serves as the basis for simultaneous selection for the concerned traits.
- ✓ Combined selection index approach of GS increases the effectiveness of selection, particularly for low heritability traits

- ✓ GS would tend to reduce the rate of inbreeding and the loss of genetic variability in comparison to selection based on breeding values estimated from phenotype data without sacrificing selection gains
- ✓ Phenotyping for every selection cycle in the breeding population is not required. It reduces the length of breeding cycle, particularly in perennial species.
- ✓ Allow breeders to select parents for hybridization programs from among those lines that have not been evaluated in the target environment.
- ✓ GS can utilize information on marker genotype and trait phenotype accumulated over time in various evaluation programs covering a variety of environments and integrate the same in GEBV estimates of the various individuals/lines.
- ✓ GEBV estimates can be used for the selection of parents for hybridization programs and, possibly, for the development of hybrid varieties. These applications, however, must await validation of the concept in practice.

Disadvantages of Genomic Selection:

- GS has still not become popular with plant breeding community primarily due to insufficient evidence for its practical usefulness.
- The marker effects and GEBV estimates may change due to changes in gene frequencies and epistatic interactions. This would necessitate updating of the GS model with every breeding cycle.
- Most simulation models based on additive genetic variance. These models ignore epistatic effects, which does not seem to be realistic.
- Limited knowledge about the genetic architecture of quantitative traits limits our ability to develop appropriate models of GS to achieve the maximum prediction accuracy.
- The need for genotyping of a large number of marker loci in every generation of selection adds considerably to the cost.

GS *Vs* MAS

Feature	Genomic selection	MAS
Targeted QTLs	All QTLs affecting the trait	QTLs with significant and large effects
Basis of selection	GEBVs estimated from marker genotypes	Marker genotype
Number of markers used	Large number of genome-wide markers	Few markers linked to the targeted QTLs
QTL discovery, confirmation, and validation	Not required; QTL effects associated with the markers are estimated	Necessary for successful MAS
Model training	Necessary; based on a suitable training population	Not required
Phenotypic evaluation	Confined to the training population	During QTL discovery, confirmation, and validation
Overall objective of the breeding program	Improvement in the targeted quantitative traits	Introgression/accumulation of the targeted QTLs

Genome-Wide Association Study (GWAS):

GWAS is a method for the study of associations between a genome-wide set of single-nucleotide polymorphisms (SNPs) and desired phenotypic traits. The quantitative evaluation is based on linkage disequilibrium (LD) through genotyping and phenotyping of diverse individuals. Generally, a GWAS infers these associations through a hypothesis test with pertinent test statistics such as Pearson's x2 -test, Fisher's exact test, the F-test, or a regression model under a null hypothesis assumes no association.

GWAS is more used for identify significantly associated markers to assist agricultural breeding, therefore, the markers selected from GWAS are the key information for producing SNP chips for specific species. In contrast, genomic prediction calculates the estimated breeding values in order to rank the selection candidates in practical

breeding. The difference between GWAS and genomic prediction is that in GWAS, usually a single SNP is associated with the phenotype accordingly while in genomic prediction, all SNPs are simultaneously fitted in the model associating with the phenotype.

The process of GWAS in plants:

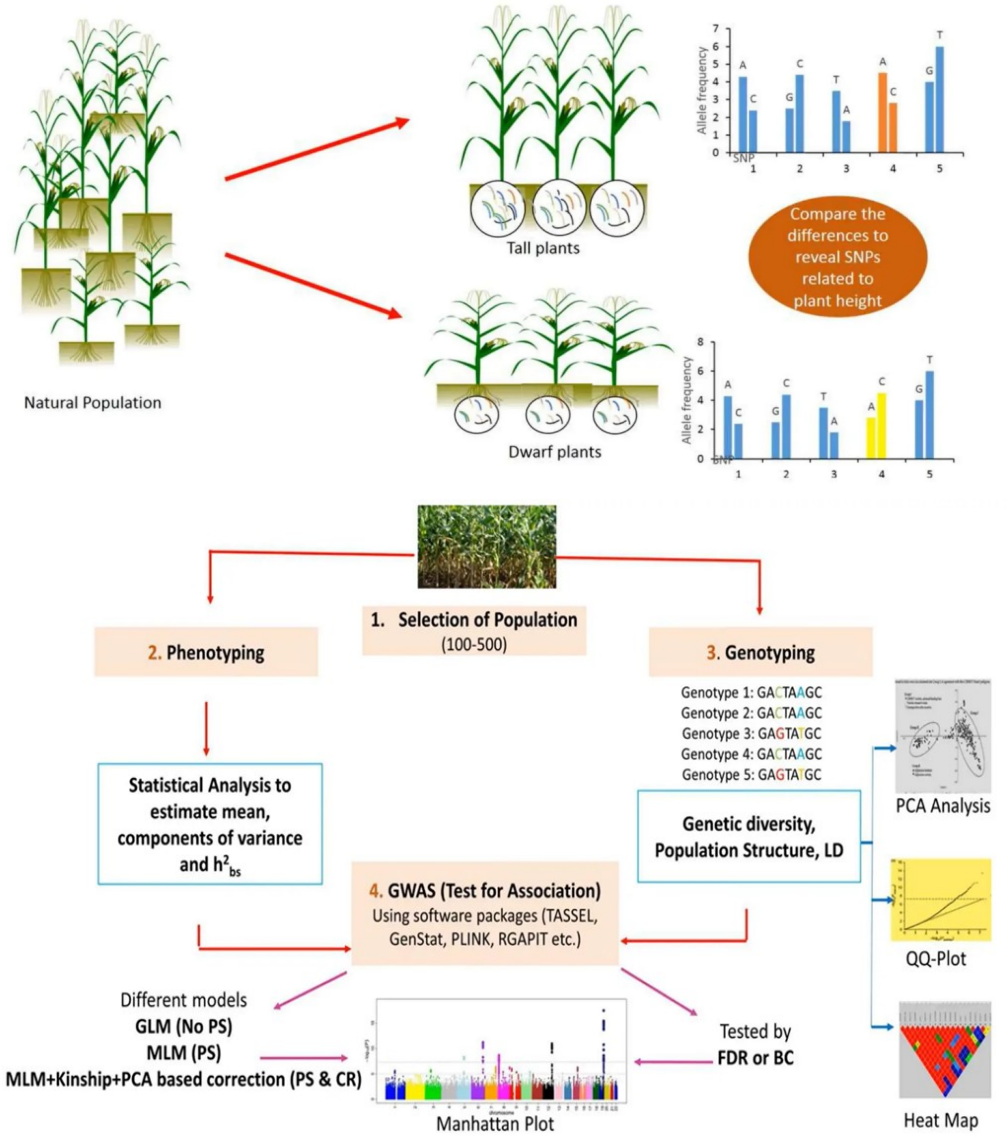


Fig.: Flowchart of the GWAS.

Application:

- Genome-wide association studies have investigated agriculturally important traits in many major crop species, including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), soybean [*Glycine max* (L.) Merr.], sorghum [*Sorghum bicolor* (L.) Moench], barley (*Hordeum vulgare* L.), cotton (*Gossypium hirsutum* L.), and numerous other crops beyond the model plant species *Arabidopsis*.
- Genome-wide association studies have identified genomic regions associated with many agronomic, physiological, and fitness traits including flowering time, plant height, kernel number, stress tolerance, and grain.
- Genome-wide association studies have also been used to study other types of phenotypes. Genome-wide association studies in rice have identified genes associated with geographical divergence and adaptation during domestication ty acid, amino acid, and nucleic acid metabolites. Data generated by high-throughput automated phenotyping have also been analyzed by GWAS. For example, GWAS in sorghum have detected significant associations for panicle architecture using automated feature extraction from images and for biomass traits using measurements taken by aerial drones.
- Genome-wide association studies are used both to detect novel associations with valuable traits and to validate loci identified by other methods. Genome-wide association studies may be conducted as stand-alone investigations, as a component of gene cloning studies, or as the foundational step in marker-assisted selection, among other uses. In turn, exploiting this information accelerates crop breeding. For example, loci identified by GWAS on provitamin A levels in maize grain were used as the basis of marker-assisted and genomic selection for this important nutritional trait.
- Genome-wide association studies have also been used to enable genetic engineering, as in the case of transgenic drought-tolerant maize developed after detection of ZmVPP1 by GWAS. As genome-editing technologies continue to

improve, particularly those based on CRISPR, the use of GWAS is expected to increase to identify target genes for editing.

- Genome-wide association studies were first developed in the context of human disease genetics and have led to the detection of thousands of genetic variants significantly associated with these diseases. New understanding gained from these GWAS has been clinically relevant, enabling the development of new therapeutic approaches for diseases ranging from schizophrenia to diabetes.

Advantages:

- Biological pathway of the trait does not have to be known.
- Discovering novel candidate genes.
- Encourages collaborative consortia.
- Rules out specific genetic association.
- Provides more robust data.
- Identifies the mutations explaining few percent of phenotypic variant.

Limitation:

- Results need replication in independent samples in different population.
- A large study of population is required, detects association not causation.
- Identifies specific location not complete gene.
- Focus on common variants and many associated variants are not causal.
- Detect any variant (>5%) in a population.
- Cost of each DNA sample and pooling them.
- Unavailability of funding agencies.
- Not predictive and explains less heritability.

11. Mutation Breeding: Utility and accomplishment of induced mutations. Management of M1 and M2generations, Factors influencing the mutation spectrum and the quality of mutants.

Mutation Breeding:

Mutation breeding is the process of exposing seeds to chemicals, radiation, or enzymes in order to generate mutants with desirable traits to be bred with other cultivars. Plants created using mutagenesis are sometimes called mutagenic plants or mutagenic seeds.

Utility and accomplishment of induced mutations:

Utility of induced mutations:

Induced macro- and micro-mutations can be utilized in one of the following two ways: (1) direct release as a variety, and (2) used as parents in hybridization prograrnmes. When mutant lines are used in hybridization programmes, the objective may be either to (2a) evolve a pure line variety having the mutant features or to (2b) produce a hybrid variety to exploit heterosis.

Direct Release as a Variety:

It is the simplest to isolate a desirable mutant, particularly a macro mutant, and release it as a new variety. There are many examples of such releases, particularly in seed propagated crops. For example, in Sweden, the early maturing barley mutant early-was released as Mari', which is early and lodging resistant. Similarly, the erectoides mutant, ert-k was released as Pallas' in 1960. However, direct releases of macromutations are limited by the associated undesirable effects due to other independent mutations and/or pleiotropic effects of the mutant alleles. These difficulties can be resolved in the following three ways: (1) isolation of a large number of similar mutants, from which the mutant, showing negligible or no side effect is selected, (2) backcrossing the mutants to the concerned parent varieties to get rid of the undesirable effects produced by other

mutational events, and (3) placing the mutant alleles in different genetic backgrounds to minimise/eliminate the undesirable pleiotropic effects.

Utilization in Hybridization Programmes:

Mutants, especially macro mutants, are often used in hybridization programmes to achieve one a following four objectives:

1. To remove undesirable associated effects generated by mutations independent of the concerned macromutation. The mutants are backcrossed to their parent varieties and in the segregating generations selection is made for side-effect-free mutant types.
2. When a macromutation produces genuine pleiotropic effects, these effects can be minimised by transferring the mutant allele into a suitable genetic background. For example, when barley erectoides mutant gene ertl6 derived (Derived from German Variety Hisa II) was transferred into the Ethiopean variety 'Bulch Gofa', the undesirable pleiotropic effects of the mutant allele were virtually eliminated.
3. Mutants may be crossed with other nonmutant varieties, or with other mutants to evolve new varieties. For example, barley mutant variety Mari (early-a^{8}) has been used as a parent in hybridization programmes to give rise to several varieties like Kristina, Mona, Eva, Salva and Stange. Another mutant barley variety, 'Pallas' (ert-k^{832}) has also been used in crosses to evolve several varieties. In India, jute variety JRO3690 was produced by crossing two low yielding mutants of C. olitorius. Simally, groundnut variety TG17 was developed by crossing two mutant groundnut strains, while variety TG19A was obtained from the cross of TG17 with another mutant strain TG1.
4. Mutants may be crossed with normal lines, mutant lines or their parental lines with a view to exploit heterosis. Monogenic heterosis due to the presence of specific mutant genes in the heterozygous condition has been reported in pea, tomato, oats and barley. Even mutants with extremely poor agronomic performance can give excellent F_1 generation; this phenomenon is called **Mutant heterosis**.

Accomplishment of Induced mutation:

The year 1969 is widely regarded as the year of transition from mainly fundamental investigations to practical mutation breeding. By the year 1989, a total of 522 mutant

varieties had been released world-wide. This number had gone up to 2,252 by the year 2000; of these, 585 varieties were released by direct multiplication of selected mutants, while 667 were developed through hybridization involving a mutant. Radiation was used to develop 64% of the varieties were due to gamma-rays, while 22% were induced by X-rays. The number of mutant varieties released till November, 2008 had gone up to 2.775: bulk these varieties were developed in Asia (1582) and in food crops 656 of the varieties. Among seed propagated crops, the largest number of varieties have been developed in rice, closely followed by barley and wheat, etc. Of these, China has developed the largest number of mutant varieties, followed by India, USSR and Japan.

The world's first variety developed from a mutagenesis programme was X-ray induced Cotton variety MA-9 released in 1948 in India; It had enhanced drought tolerance. Later, **Primax** white mustard (*Brassica hirta*) was released in 1950, and **Regina II** summer rape (B. campestris) in 1953. Both the varieties were developed at the Svalof station of the Swedish Seed Association. They represented a small but consistent increase in yield and oil content over the concerned parent varieties, and it took 10-13 years to develop them. Soon, mutants were released as varieties in pea (in 1955), barley (in 1958) and peanut (in 1900).

Mutagenesis work in India started in 1930s on a small scale, but received considerable attention during late 1950s and 1960s. The first mutant variety (MA-9: cotton) was developed in 1948, and till 2008 a total of 343 varieties had been released. Bulk of these varieties belong to ornamentals (119 varieties), followed by legumes (85 varieties) and cereals (74 varieties) Among food crops, rice tops the list (42 varieties), followed by groundnut 18 varieties mungbean (05 varieties), barely (13 varieties), etc.

Some of the mutant varieties developed in India are listed in Table. NP836 wheat is an awned mutant from the awnless wheat variety NP99. **Jagannath** rice is a crop gamma-ray induced semidwarf mutant from the tall cultivar TI41; it has an improved resistance to lodging, higher yield, and is more responsive to fertilizer application than the parent variety. Rice variety CRMI3, developed through mutagenesis, has reduced height and days to maturity, and increased yield as compared to the parent Mutant variety. Jute (C. olitorius) variety **JR03690** was produced by crossing two low yielding mutants.

Prabhavati variety of rice is an EMS-induced mutant from the tall, scented variety Ambemohar local. It is semidwarf, nonlodging, responsive to fertilizer application, and high yields than parent variety. It has medium-slender, translucent and grains. Variety **HUR 46005** is an EMS induced mutant from Dehradun Basmati; it is strongly scented and gives higher yields than the parent.

Table: A list of some mutant varieties developed in India

Crop	*Mutant variety*	*Parent variety*	*Mutagen/Remarks*
Pigeonpea (arhar; *C. cajan*)	TV1 (Trombay Vishakha-1)	T21	γ-rays
	TAT5	T21	γ-rays
	TAT10	T21	γ-rays
	TAT7	S8	γ-rays
Brassica juncea	Kranti	Varuna	γ-rays
	RLM198	RL18	—
	RLM514	RL18	—
	RLM619	RL18	—
Chickpea (*C. arietinum*)	BGM408 (Pusa 408; Ajay)	G130	Resistant to Ascochyta blight and wilt
	BGM413 (Pusa 413; Atul)	G130	—
	BGM417 (Pusa 417; Girnar)	BG203	—
	Pusa-547	—	Released in 2006
	RSG2	RS10	—
	RS11	RS10	—
Citronella java	RRL-JOR-3-1970	—	39% more oil
Cotton (*G. hirsutum*)	MCU7	1143EE	—
	MCU10	MCU4	γ-rays
Cotton (*G. herbacum*)	Indore-2	An American variety	—
	DB-3-12	Western 1	Spontaneous mutant
Cumin	Gujarat Cumin 2	—	—
East Indian lemongrass	SD-68	—	100% more oil
(*Cymbopogan flexuosus*)	RRL-38, RRL-57, RRL-59	—	—
	RRL (B)-14	—	63% more oil
Ginger	Suravi	—	—
Groundnut (*A. hypogaea*)	TG17	—	γ-rays
	TG19A*	TG1 × TG17 (TG1 is a mutant)	γ-rays
	TG1 (Vikram)	—	γ-rays
	TG13A*, TG14*, TG15*	—	γ-rays
	TG16*, TG18*, TG19*	—	γ-rays
	BP1, BP2, CO2	—	—
	TG3, TG4, TG7	—	γ-rays
Hyoscyamus niger	Aela	—	40% more biomass
Jute (*C. olitorius*)	JRO3690	Hybridization between two mutants	—
	TKJ40 (Mahadev)	—	γ-rays
	JRO412, JRO514	—	

Rice	Jagannath	T141	γ-rays
	CRM13-324	—	—
	Prabhavati	Ambemohar	EMS
	HUR-36	Mahsuri	γ-rays + EMS
	Gautam**	Rasi	EMS
	GEB24	Konamani	Spontaneous mutant
	Shyama	Kalimoonch	Spontaneous mutant
	Sattari, K84,	—	—
	Hybrid Mutant 25	—	—
	Parbhani 1, AU-1, Indira	—	—
	Biraj, CNM6, CNM20	—	—
	CLM25, CLM31, AU-4	—	—
	Mohan, Padmini, Rashmi	—	—
	PL56, IET60, IET5878	—	—
	HUR5-1	Lanjhi	EMS
	HUR46000	Dehradun Basmati	EMS
Sugarcane (*S. officinarum*)	Co8152	Co527	γ-rays
	Co8153	Co775	γ-rays
Tobacco (flue-cured)	Jayasri	—	Chemical mutagen
	Bhavya	—	Chemical mutagen
Turmeric	BSR-1	—	—
Urdbean (*V. mungo*)	CO4	CO1	MMS (0.02%)
	TAU-1	—	γ-rays
Wheat (*T. aestivum*)	Sharbati Sonara	Sonora-64	γ-rays
	NP836	NP799	γ-rays
	NP111	NP4	Spontaneous mutant
	Pusa Lerma	Lerma Rojo	γ-rays

Management of M_1 and M_2 generations

Management of M_1 population:

Planning for the M_1 generation:

Radio-Sensitivity:

The seeds before treatment are known as the M_0 generation. Radio-sensitivity testing should precede the bulk treatment to determine the optimum dose levels for mutation induction. Radio-sensitivity tests are normally performed in the greenhouse, but fields can also be used.

Control population:

A control (untreated) population should always be grown to serve three purposes:

a. provides a comparison of the treatment effects on germination, growth, survival, M_1 injury and sterility;

b. assesses the phenotypic variability of the parent genotype stock used to produce M_1 and;

c. provides a 're-purified' parent genotype as a back-up for initiating a new M_1 generation to be grown during the same season with the M_2 generated from the first M_1 if needed.

Mutagen and dose treatment:

It is advisable to use three doses of the chosen mutagens, which should be ± 20 per cent of the optimal dose found through the radio-sensitivity tests. Normally the selected doses to be applied on cereals cause between 30 to 50 per cent reduction in seedling growth in laboratory tests. In practice, at least two replicates should be made. When rather large numbers of seeds are treated, i.e. 5000 to 10 000 seeds per treatment, subdivision into several treatment replicates will improve the uniformity of the treatments.

Population size of M_1:

Assuming a 90 per cent probability of success in recovering a mutant occurring at a frequency of 1×10^{-3} per test unit (e.g. per spike, fruit, or branch), and that each plant grown is expected to produce three units, the number of seeds to be treated, if the M_l has 80 per cent survival rate, would be about 600 seeds. Thus, in the above example treating about 6000 seeds might yield the breeder as many as 10 mutations in the desired direction.

Sowing of M_1 seeds:

Considering the detrimental effects of mutagens on seed viability, the M_1 must be handled with more care than untreated controls. The M_1 should therefore be grown in benign conditions.

Greenhouse conditions:

If possible, the M_1 should be grown in a greenhouse where careful attention can be paid with respect to watering, fertiliser provision, lighting and temperature, weed, pest and disease control to maximise plant survival and production of the next generation. However, it should be noted that the use of greenhouses is relatively expensive compared to the field.

Field conditions:

If greenhouse conditions are not available or are not affordable, then field conditions can be used. It is particularly important to ascertain that the moisture and the physical conditions of the seed bed prepared for sowing the M_1 are optimal for seedling growth and development. A restriction in field cultivation is that it is restricted to the selected variety's cropping season.

Time of sowing M_1:

The M_1 material will develop optimally if sown during the season when the climate is best for early seedling and plant development and weed control is less of a problem. However, slightly later sowing (2 or 3 weeks) may help reduce tillering and may improve the conditions for isolation against cross-pollination.

Condition of the treated M_1 seeds:

Dry seeds are easier to plant with machinery or by hand, and a more uniform growth can be obtained without extra care. If adequately low in moisture, dry seeds can be stored for some time prior to sowing. Vacuum packing is the best option, if available, to store treated seeds for longer time in order to match with the normal growing season.

Density of sowing:

In general, the spacing of M_1 seeds within and between rows should be such as to restrict development to primary tillers; to 2 – 3 in cereals, and the primary branching in grain legumes and other dicotyledonous species. This can also be adjusted based on the space available, the number of treated M1 seeds, the expected survival rate based on the dose effect and the expected M2 population size.

Weed control

Normally, a relatively weed-free seed bed should be prepared just prior to planting of the M_1 seeds. Pre-emergence herbicides recommended for the area and crop may be used successfully. Contact types of post-emergence herbicides may be useful for weed control if the M_1 plot is too large or the weeds are too numerous for manual control. Systemic herbicides, such as 2, 4-D should normally not be used with cereals.

Isolation of M_1 material:

Generally, it can be assumed that some level of genetic heterogeneity is always present even in populations of parent material from self-pollinating plants. Therefore, several disrupting hazards may be expected, especially in field plantings, which may affect the certainty of the origin of the variability observed in a mutation breeding programme as presented below.

a. Outcrossing – distant pollen grains may be transmitted by wind or insects from varieties of the same species growing nearby; the extent of the contamination varies with crop variety, the treatment, the mating system of the plant species and the distance from the mutated crop field in relation to wind directions and potential sources of contamination. Appropriate selfing method, such as bagging to shield flowers before anthesis, should be applied at the right time to prevent outcrossing. In cross pollinated species, where male and female flowers are separated, selfing can be accomplished by hand pollination of the female flower. The female flower should be covered before opening to present cross pollination.

b. 'Volunteer' crop – M_1 should not be sown on land previously used for the same species this is particularly important for hand-seeded legumes.

c. Bird damage – the risk of loss of M_1 material to birds is often greater than with non-mutated plant materials because the range of maturity variation of in treated materials may be greater. In practice, the M_1 plots should be planted at some distance from bird populations. In some cases, an extra plot area might be sown on different dates to divert birds away from the mutagen treated plots.

d. When the M_1 population must be planted where bird damage is possible, the plants might be covered with bird-proof nylon or metal netting, which is relatively inexpensive or the selfing bag should be left until maturity to serve as protection from bird damage.

e. Soil borne toxicity, disease or in some cases, parasitic weeds, such as *Striga* spp. may cause complete loss of the M_1 population and therefore, extreme care should be exercised to avoid planting the M_1 seeds on soil having such problematic history.

Care during cultivation and data recording:
As stated previously, the M_1 population should receive optimum cultivation practices in either greenhouse or field cultivation for the selected crop, including supplemental irrigation, weed control by herbicide or by mechanical means, prevention of severe disease levels if necessary. In addition, records on the condition of the M_1 at various developmental stages are useful and need to be recorded.

A) Emergence – because the application of a mutagen commonly induces some delay in emergence for treated seeds, recording the estimates of emergence percentage at a time when the control population can be considered 50 – 90 percent emerged is useful. If emergence of treated populations is poor, either the treatments are too severe, or the cultural conditions are poor, and adjustments must be made for the next M1 planting.

B) Seedling survival – estimates of seedling survival recorded at the tillering or branching stages provide data on delayed effects of the treatments. If seedling survival of treated population is low relative to emergence, the mutagen treatments could be considered too severe for the cultural conditions.

C) M_1 chimera induction – even crude data on the occurrence of sectors of chlorophyll deficiency or other morphological changes in the appearance of M_1 plants may be useful data for estimating the effectiveness and intensity of the treatments.

D) Delayed development – the retarded growth of some mutagen-treated materials can often be estimated in relation to seedling establishment, the time of flowering or

maturation of the plants as well as by the variability in plant development within treatments.

E) Survival to maturity – estimates of the number of surviving plants in each treatment at the time of maturity provide information on the severity of the injury induced by the mutagen when compared with the number of seeds planted.

F) Sterility in M1 – useful estimates of M_1 sterility can be obtained in various ways, or detailed counts may be made on appropriate samples of the population. These estimates may sometimes be made by visual inspection, or via M_1 seed yield (weight) corrected for differences in survival relative to the control population.

Harvest of M_1:

Methods of harvesting the M_1 populations will depend on the pattern of ontogenetic development in the species, the methods of screening and the foreseen generation to be screened for desired mutants. In most instances, the genetic changes induced by mutagen treatments occur as chimeras in the somatic tissue of the M_1 plant, and the ontogenetic pattern is a prime factor affecting the expression of observed mutant-tissue chimeras in generative (reproductive) tissue. However, both the ontogenetic pattern of development and the seed yield of each M_1 inflorescence may have a bearing on the efficiency of the methods for analysing the M_l generation. A consideration of the relation of these factors to the methods of managing mutagen treated populations of different plant forms is presented below.

Tiller, branch or plant progeny methods: With monocot species like cereals and grasses the maximum potential for induced genetic variability is in the primary tillers, which arise from the already differentiated primordial meristem present in the treated seed embryos. Some secondary tillers may individually yield higher frequencies of M_2 mutants but the same mutations would, generally, be also present in the progeny from primary tillers. With dicots largely self-pollinated seed plants, like beans, peas, tomato, etc., the methods applicable to M_1 analyses may be similar to those used for cereals

except that each 'primary tiller' is equivalent here to a main branch on the M_1 plant, but in some studies even secondary branches might be analysed.

Single or multiple seeds bulk methods:

The single-seed bulk method, in some form, is applicable to both monocots and dicots and to all experimental situations. Its usefulness is based on the fact that the probability of occurrence of a single mutant offspring within the progeny of a fruit developed from mutated tissue is higher than the frequency of the particular mutation in the total population of plants (or branches) sampled.

Mass bulk methods:

Mass population management also is applicable if the land and resources for mechanization are less costly than the labour required for other operations. However, in this case, with both monocot and dicot species some mechanism to limit the seed yield on each M_1 plant should be devised.

Management of M_2 population

Selection of mutant traits is usually practiced for qualitative traits, in self-pollinated crop plants, in the M_2 generation as most of the mutants are – by then – recessive, the mutant phenotype can thus, only be seen in the M_2 generation at the earliest However, in cross-pollinated plants mutant genes are likely to be heterozygous in M_2 where further selfing should be practiced for producing M_3 progenies in which homozygous individuals for the mutant genes will segregate and selection can be applied.

Systems of handling M_2 populations

All methods for the isolation of mutant genotypes in sexually reproduced plants are based on the pedigree method, modified to account for the chimeric structure of the M_1 plants. Furthermore, the applicable methods are based on population genetics procedures since the induced frequency of any specific mutant gene or desired mutant phenotype is appreciably lower in the M_1 population than that of a specific gene introduced into an F_1 population by hybridization. In addition, because a mutated tissue in an M_1 plant may appear only in part of the spike, pod or fruit, the segregation ratio of

mutants in the progeny of seed units (pods, fruits, spikes, etc.) will usually be lower than in normally monogenic heterozygous material. The mutation breeder in such case must choose the method of screening most adaptable to his or her own circumstances.

1. M_1 population bulk - If the parental material is quite homogeneous, the tillering of M_1 well controlled by close spacing, and the M_1 grown in isolation, the bulk method of M_2 population management can be very efficient, especially for the selection of relatively divergent mutant phenotypes. Here, the whole M_1 population is harvested as one bulk and the M_2 population is grown out as a population of single plants that are screened for mutant phenotypes. The method is adaptable to mechanization at virtually all phases including the selection of variants, e.g. mechanical screening for seed size, weight, shape, etc. In some instances, particularly when mutants are better identifiable in M_3 than in M_2 a combination of methods may be desirable:

a. M_1 population bulk to M_2 single seed bulk to M_3 ear to row progenies;

b. M_1 population bulk to M_2 ear to row progenies.

2. M_1 ear to row bulk - This method, based on randomly harvested M1 ears, is similar to method 5 below, but differs in that the relation of the ears, branches, fruit, etc., to one another is not maintained, permitting a type of bulk processing comparable to that obtained with method 4 but requiring smaller M_2 progenies (perhaps 25 – 30) and adaptable to semi-bulk harvesting of units from M_1 plants.

3. M_1 single-seed or multiple-seeds bulk - This method involves selecting a single seed at random from each M_1 spike (or fruit, branch, etc.) of M_1 plant to constitute an M_2 population of single plants from the resultant bulk. M_2 single plants can be selected for mutant phenotypes that can be further progeny-tested in the M_3. Alternatively, single M_2 ears can be harvested for selecting within M_3 ear row progenies for new mutant phenotypes.

4. M_1 plant to row - In this method, all seeds or a sample of the seeds produced from a given M_1 plant are grown to produce the M_2 generation, which is then screened for mutant phenotypes. Successful use in cereals will depend on the screening efficiency,

targeted trait and land availability since generally a somewhat larger number of M_2 is needed. The overall cost for land, labour, etc., is intermediate between the spike progeny and the bulk methods.

5. M_1 ear, branch, pod, and fruit (within plant) to row. Here, each ear taken from the M_1 is processed as a separate entity and sown out as an ear-row progeny, which is then screened for mutant phenotypes. This method offers the greatest precision with regard to the origin of a mutant when the material treated is genetically homogeneous as regards the non-mutant allele, and when outcrossing is controlled. The method is, however, the costliest in terms of space, labour, equipment and materials.

Screening methods and mutant selection techniques:

Various screening methods to identify and select desired mutants have been proposed and tested. The efficiency of each method is dependent on factors more or less under the control of the breeder.

1. Visual methods of selection for identifying mutant phenotypes are common and can be very efficient. However, when breeding for specific traits it is important that the breeder ignores all other deviants. Unusual genetic variants may however, be of value for fundamental studies and in developing a mutant germplasm base for future breeding purpose. As a precautionary measure seeds from variants should be preserved. Visual selection is often the prime basis for selecting for disease resistance, earliness plant height, colour changes, non-shattering, adaptation to soil, climate, growing period, etc. The procedures involved are essentially the same as for variation introduced by cross breeding.

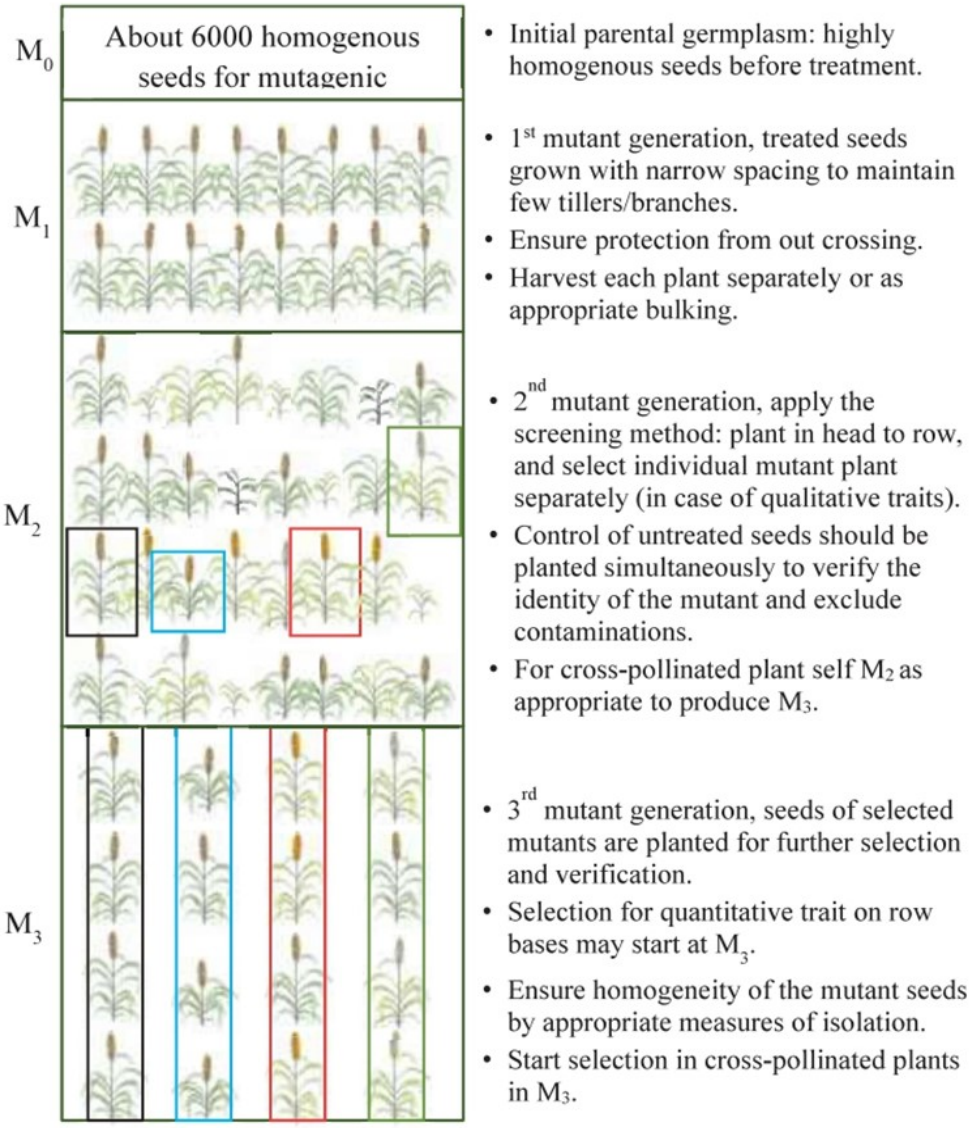


Fig.: Scheme for mutant population development, identification, selection and advancement of mutant from M_0 to M_3 generations.

2. Mechanical or physical methods of selection can also be used very efficiently in screening for seed size, shape, weight, density. etc., using appropriate sieving machinery as they are readily adaptable for processing of large quantities of seeds.

3. Other selection methods, such as chemical, biochemical, physiological, physio-chemical, and various specific methods may be needed for selecting certain types of mutants. However, virtually all employ visual parameter to expedite detection. Low alkaloid content mutants, for example, maybe sought using colorimetric tests on M_2 seeds or plants or even M_3 seeds; protein analyses by colorimetric, chromatographic or electrophoresis techniques may be conducted on individual seeds from M_1 plants, on seeds from M_2 plants or on the bulk from M_2 plant progenies, the efficiency depending on the degree to which such techniques can be mechanized.

4. Screening for abiotic stresses such as drought, salinity, heat etc., requires setting up of the selection pressure and maintaining a uniform stress over the M_2 and M_3 populations. Recent advances in hydroponics and laboratory techniques have led to the development of different screening methods for abiotic stresses for conventional breeding programmes that can readily be adapted to handle mutant population of larger size with greater efficiency to identify phenotypic mutants in M_2 or M_3 generations.

Factors influencing the mutation spectrum and the quality of mutants:

The success of the mutation breeding programme is measured mainly by the production of superior varieties, but also by the spectrum and quality of mutants induced, identified and recovered from a segregating mutant population. The factors which could limit the success in recovering the targeted mutant trait. These mainly include, the situations described below.

1. **Differences due to the genotype:**

Much evidence exists that genetic differences, even when they are as small as single gene differences, can induce significant changes in radio-sensitivity, which in turn influences not only the total rate but also the spectrum of recoverable mutations and the degree of background. Although nobody is able to predict the influence of a

particular genotype on the mutation spectrum, the choice of the parent material is a key factor of any programme in mutation breeding.

More definite information is available with regard to the influence of the ploidy level on the mutation spectrum. In diploid species the great majority of mutations occur in single recessive genes. However, deviation from the normal 3:1 ratio due to deficiency of recessives has been very frequently observed. Dominant vital mutations hardly occur, in fact that they are mostly lethal or semi-lethal in the homozygous condition, in contrast to diploid organisms, as the dose required to produce them is unlikely to result in viable plants. Many genes are re-duplicated in polyploids, which increases their ability to bear a high mutational load, including gross chromosome aberrations, with no apparent negative effects. This results in the more frequent discovery of dominant and semi-dominant mutations amongst such species.

Phenotypic buffering is another property of polyploids that restricts mutability of many characters, especially those essential for the whole life of the plant: e.g. the process of chlorophyll formation. Thus, chlorophyll mutations decrease with the increasing level of ploidy; however, the total rate of mutation increases. For example, in *Triticum* spp. the total mutation rate was about three times higher in hexaploid wheat than in the tetra and diploid genotypes.

Thus, it seems that genetic variability in the background of a genotype is an important factor. According to several authors those characters that showed greater variability in the background could be improved more easily and give better expectation of mutant improvement. Heterozygosity as a genotypic property can also influence the type and frequency of mutation. Many polyploids are less sensitive to chromosome aberrations if they are in heterozygous condition.

2. Type of mutagen and dose:

The difference in mutation spectrum among different sources of irradiation is obvious in the spectrum of induced flower colour changes following mutagen treatment. For instance, densely ionizing radiations such as different sources of ion beam produce relatively more chlorophyll mutations of the albina, striata, and xantha type, whereas

the frequency of the viridis type is highest following gamma-ray treatment. Thus, the chance of selecting desired mutants might be considerably increased by broadening the choice of mutagens.

Another problem in the mutant quality is the number of mutation events that occur in the same meristematic cell at the time of treatment that are transmitted to later generations. The number of desirable events is far less than the undesirable ones and consequently, the number of mutant plants that carry only desirable changes will further decrease if more than one mutation per cell is induced. Several measures can be taken to avoid this undesirable result. **Firstly,** one should not apply too high a dose of any mutagen. **Secondly,** one should seriously consider that super-mutagens, which give mutation rates of at least 50 percent on the basis of plant or spike progenies, may not be at all advantageous for mutation-breeding purposes. **Thirdly,** if high mutation rates have been induced, they should be allowed to segregate, and selection for useful types should be conducted in M_3 or later generations. However, one should realize that the latter technique will not eliminate those

3. Pleiotropy and linkage:

Generally, it seems to be nearly impossible to find a mutation in an organism that results in only one single divergent phenotype compared with its initial wild genotype. For instance, mutations resulting in pale green plants also result in reduction in general plant growth and delayed maturity and, in most cases a group of distinct variants can be observed and this group as a whole is transferred from one mutant generation to the next showing mostly a 3:1 segregation ratio. Theoretically, there are three possible interpretations for this behaviour:

a. a single mutant gene is responsible for the whole complex of deviating characters;

b. a tiny portion of a chromosome has been lost containing several genes;

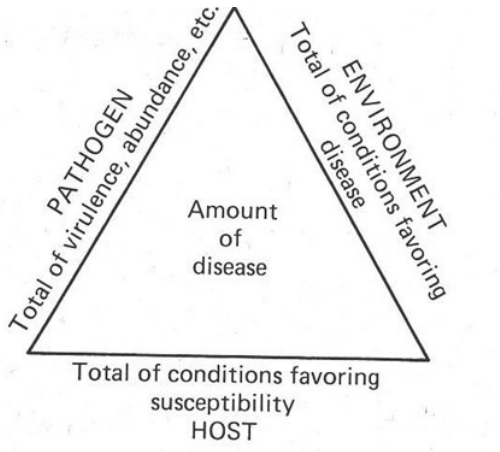
c. and several closely linked or neighbouring genes have mutated.

Monohybrid segregation will occur in all these cases but only the first one is a true example of a pleiotropic gene action. The other two events simulate a pleiotropic effect of one gene, although several genes are lost or altered. It is practically impossible, in

most cases, to state which of these possibilities is realized; therefore, the term 'pleiotropic gene action' is commonly used in the literature for the whole group of these phenomena.

12. Breeding for Disease Resistance: Pathogenicity vs. Virulence, Physiological races and differential hosts, Models for plant pathogen recognition, Flor's hypothesis, Vertical and Horizontal resistance.

Plant disease resistance protects plants from pathogens in two ways: by pre-formed structures and chemicals, and by infection-induced responses of the immune system. Relative to a susceptible plant, **disease resistance** is the reduction of pathogen growth on or in the plant (and hence a reduction of disease), while the term **disease tolerance** describes plants that exhibit little disease damage despite substantial pathogen levels. Disease outcome is determined by the three-way interaction of the pathogen, the plant and the environmental conditions (an interaction known as the disease triangle).



Development of any disease is affected by (1) host and (2) pathogen genotypes, and (3) the environment. Both contact and infection stages are greatly affected by the environment; disease escape is the consequence of this effect. Host genotype greatly influences the establishment and development phases. Some cases of resistance prevent establishment, while others reduce symptom development and spore production. The pathogen genotype determines if it will be able to produce disease symptoms in a given host.

Defense activating compounds can move cell-to-cell and systematically through the plant's vascular system. However, plants do not have circulating immune cells, so most cell types exhibit a broad suite of antimicrobial defenses. Although obvious *qualitative* differences in disease resistance can be observed when multiple specimens are compared (allowing classification as “resistant” or “susceptible” after infection by the same pathogen strain at similar inoculum levels in similar environments), a gradation of *quantitative* differences in disease resistance is more typically observed between plant strains or genotypes. Plants consistently resist certain pathogens but succumb to others; resistance is usually specific to certain pathogen species or pathogen strains.

Host plant resistance

A physiological deviation from the normal functioning of the organism (i.e., the crop plant) caused by pathogenic organisms is a disease and may be caused by fungi, bacteria or viruses. The inherent ability of an organism (i.e., the crop plant) to resist or withstand the pathogen is called resistance. Disease resistance commonly met with in the plant kingdom relative in nature, total immunity being too rare. Its hereditary transmission from parent to offspring is essentially “Mendalian”, but often polygenic.

The earliest demonstration of the behaviour of “disease-resistance” as a character transmissible from parent to off-spring in the “Mendelian” fashion was given by Biffen (1905) in his work on yellow rust of wheat. Since then, intensive work has been done on this aspect which has proved the value of applying genetical principles in developing disease-resistant varieties of plants for effective control of diseases.

Resistant varieties can be the simplest, practical, effective and economical method of plant disease control. The use of resistant varieties cannot only ensure protection against diseases but also save the time, energy and money spent on other measures of control. In addition to these advantages, resistant varieties, if evolved, can be the only practical method of control of such diseases as viruses, phytoplasmas wilts, and rusts etc. in which chemical control is very expensive and impractical. In crops of low cash value, chemical and other methods of control are often too expensive to be applied. In such crops development of varieties resistant to important diseases can be an acceptable recommendation for the farmer. **Pathogenicity** is the ability of a pathogen to attack a host. Pathogenicity includes virulence and aggressiveness. Virulent strains of pathogen cause much severe symptoms of the disease and they carry the virulence gene that enables it to attack a particular host genotype.

Virulence is due to the action of one or a few genes. An aggressive strain of a pathogen causes severe disease on all the host genotypes which they are able to attack and aggressiveness is polygenically inherited. Host – Pathogen relationship A disease is the result of an interaction of genes governing resistance in the host with those governing pathogenicity in the pathogen. The resistance of a crop to a physiological race of the pathogen depends not only on the genotype of the host for resistance, but also upon the genotype of the pathogen for virulence or aggressiveness. Flor (1942) proposed the gene-for-gene hypothesis, according to which, for every gene for resistance in the host, there is a corresponding gene for pathogenicity in the pathogen.

It means that there are atleast two alleles at a locus controlling resistance/susceptibility in the host (R-r) and two alleles at a corresponding locus in the pathogen (V-v) controlling virulence / aggressiveness. Out of the four possible interactions between these alleles, only one combination leads to the expression of resistance. The demonstration of gene-for-gene relationship requires genetic studies of both the host and the pathogen.

Pathogen

VI v1 + Pathogen can infect; the host is R1 -+ susceptible r1 + + -

The demonstration of gene-for-gene relationship requires genetic studies of both the host and the pathogen.

Physiological Races and Pathotypes:

Physiological races are strains of a single pathogen species, which differ from each other in their ability to attack different varieties of its host species. The varieties of a host species used to identify physiological races of a pathogen are called **host testers or differential hosts**. The genes for resistance present in the host testers are ordinarily not known.

But when pathogen isolates are classified on the basis of their virulence to host varieties each of which carries a single distinct gene for resistance, they are referred to as **pathotypes**. Pathotypes are identified by **ideal differential hosts**, each of which carries a single and distinct gene for resistance.

A set of 12 ideal differentials is available for pathotype classification of the pathogen for late blight of potatoes (*Phytophthora infestans*). Pathotype differentiation is common in air-borne fungi and viruses. Some pathotype differentiation occurs in soil-borne fungi, bacteria, nematodes and insects.

Table: Pathotype classification based on a set of 12 differentiation hosts, each carrying a single distinct resistance gene (R_1 – R_{12}) to *Phytophthora infestans*, the fungus causing potato late blight.

Resistance gene present in the differential host	**Pathotype of potato late blight fungus**											
	$P_{(1)}$	$P_{(2)}$	$P_{(3)}$	$P_{(4)}$	$P_{(5)}$	$P_{(6)}$	$P_{(7)}$	$P_{(8)}$	$P_{(9)}$	$P_{(10)}$	$P_{(11)}$	$P_{(12)}$
R_1	S											
R_2		S										
R_3			S									

R_4				S								
R_5					S							
R_6						S						
R_7							S					
R_8								S				
R_9									S			
R_{10}										S		
R_{11}											S	
R_{12}												S

Disease Resistance

The response of different lines of a host to different strains of a pathogen may be classified as follows: (1) susceptible, (2) immune, (3) resistant and (4) tolerant.

(1) Susceptible Reaction: There is profuse disease development and spore production presumably unchecked by host genotype.

(2) Immune Reaction: The host plants do not show any symptom of the disease. Generally it is produced by a **hypersensitive reaction** by the host, i.e., a group of cells surrounding the point of infection dies and usually the pathogen also dies along with the host cells. This type of reaction is found in case of obligate parasites or biotrophic pathogens. In some cases, it may result from a prevention of the pathogen from reaching the appropriate part of its host.

(3) Resistance: Disease symptom production and pathogen reproduction both occur. But both are much less in magnitude than those in the case of susceptible response. It is believed to be involved nutritional factors and in some cases chemical growth inhibitors may be involved.

(4) Tolerance: In this case, disease symptoms and pathogen reproduction are comparable to those in susceptible response. But the reduction in yield and/or biomass is sihnificantly lower. Plant breeders always select for tolerance whenever yield is the basis election.

Genetics of Disease Resistance

Disease resistance shows the following three distinct modes of inheritance: (1) oligogenic, (2) polygenic and (3) cytoplasmic.

(1) Oligogenic Resistance: In such cases, one or few oligogenes/major genes govern resistance and resistance is generally dominant to the susceptible reaction. Oligogenes usually produce immune reaction, which is race-specific, i.e., it is synonym to vertical resistance. More than 30 different resistance genes are known for stem rust of wheat.

(2) Polygenic Resistance: In polygenic inheritance, resistance is determined by several genes. Each gene produces a small effect, which is mainly additive in nature, but they also have non-additive effects. There is, in addition, a large environmental effect. As a result, host reaction shows a continuous variation. This type of resistance is race-nonspecific and synonymous to horizontal resistance. The mechanism of resistance involves resistance to infection, slow growth of pathogen, and reduced spore production. This type of resistance has never been shown to be eroded or broken down.

In some cases, a single oligogene, e.g., Lr34 in case of leaf rust of wheat and 2 or other genes may act in additive manner to produce, called **slow rusting** or **patial resistance**. A similar situation is known for wheat stem rust resistance gene Sr34 and some additional genes having effects.

Accurate measurement of polygenic resistance is based on an evaluation of disease severity. This assessment is not easy mainly because disease severity is affected by several factors, including inoculum pressure and interplant interference. Polygenic resistance is assessed as (i) percentage of tissue covered with the pathogen, (ii) area of tissue showing disease symptoms, e.g., discoloration, or (iii) the severity of symptoms, e.g., leaf rolling, molting etc., depending mainly on the host-pathogen system.

This type or disease reaction. i.e., severity of the disease, is thought to generally have the following components: infection frequency, latent and incubation periods, lesion size, spore production and infectious period. Many workers feel that selection for the component traits may be more desirable than that based on disease reaction itself.

(3) Cytoplasmic Resistance: In a few cases, disease resistance is specified by plasmagenes located in the cytoplasm. The only good example of this type is the resistance in maize to *Helminthosporium* leaf blight; it is specified by mitochondrial DNA.

Vertical resistance (VR) and horizontal resistance(HR)

Van der Plank (1960) has discussed the whole issue of disease resistance in a different perspective. He calls the unstable and often complete type of resistance as vertical resistance and the more stable but somewhat incomplete resistance as horizontal resistance. If resistance to some races of a pathogen is more than to other races, it is called **Vertical resistance**. It is also called Perpendicular resistance, Physiological resistance, seedling resistance, hypersensitivity, race specific resistance or qualitative resistance. As it is conditioned by one or a few genes, it is called major gene or monogenic of oligogenic resistance.

Resistance to more than one race of the pathogen or to many or all races of the pathogen is called **Horizontal Resistance**. It is non-specific resistance governed by polygenes. It is severally termed as non-specific, general, polygenic, minor gene, mature plant, adult, quantitative resistance, partial or field resistance or tolerance. HR causes reduction in the number and rate of sporulation of the pathogen on the host and slows down the infection rate. HR includes tolerance slow development of disease, escape and exclusion mechanisms besides hypersensitive reaction. The difference between vertical resistance and horizontal resistance are given in table.

Differences between vertical and horizontal disease resistance

Feature	Vertical resistance	Horizonal resistance
Pathotype-specificity	Race specific	Race nonspecific
Nature of gene action	Oligogenic	Polygenic; rarely oligogenic
Response to pathogen	Usually, hypersensitive	Resistant response
Phenotypic expression	Qualititative	Quantitative
Stage of expression	Seedling to maturity	Expression increases as plant matures (Adult plant)
Selection and evaluation	Relatively easy	Relatively difficult
Risk of 'boom and burst'	Present (rarely durable)	Absent (durable)
Suitable for: a. Host b.Pathogen	Annuals but not perennials Immobile pathogen, e.g., Soil pathogens, but for mobile air-borne, pathogens	Both annuals and perennials All pathogens
Need for specific deployment of resistant varieties	Critical for success with mobile pathogens	None
Need for other control measures	Likely	Much less likely
Host-pathogen interaction *	Present	Absent
Efficiency	Highly efficient against specific races	Variable, but operates against all races

Vertical resistance to specific races is generally governed by a single (monogenic) dominant gene or by a few dominant genes. Some of these genes may be multiple alleles as in leaf rust gene, Lr2 that accords resistance to *Puccinia recondite tritici*. In that locus,

four genes designated as Lr2a, Lr2b, Lr2c and Lr2d are present and are tightly linked. Each of these genes accord resistance to a different spectrum of races and hence can be differentiaited from one another. Such multiple alleles exist on Sr9 locus of wheat for *P. graminis tritici* and gene Pi-k in rice for resistance to *Pyriculariva grisea*. The tight linkage between the multiple alleles permits an efficient transfer of all these genes in one attempt.

'Horizontal resistance' (HR) reduces the rate of disease spread and is evenly spread against all races of the pathogen. The low terminal disease severity in HR is assumed to result from polygenic resistance. Morphological features such as size of stomata, stomatal density per unit area, hairiness, waxiness and several others influence the degree of resistance expressed. Partial resistance, dilatory resistance, lasting resistance are some other terms coined for denoting horizontal resistance.

The phenomenon of slow rusting manifested as lesser number of pustules per unit leaf area, smaller size of uredosori and increased latent period in some wheat cultivars is a typical example of this type of resistance. Although it is preferable to use varieties that have both vertical and horizontal resistance, most of the resistant varieties carry only one or few (2 or 3) major genes of vertical resistance. If varieties are resisitant only to some of the races of pathogen and if the pathogen is airborne, then new races evolve easily, as happens with cereal rusts, the powdery mildlew and *Phytophthora infestans*. Appearance of new races leads to breakdown of resistance of the popular, ruling genotype. As a result, varieties with vertical resistance need to be replaced at frequent intervals.

Boom and burst cycle

In varietial improvement programmes, it is easy to incorporate the monogenic vertical resistance genes. But the success of exploiting the monogenic host resistance invariably does not last long. Whenever a single gene-based resistant variety is widely adopted, the impact would be the arrival of new matching pathotypes.

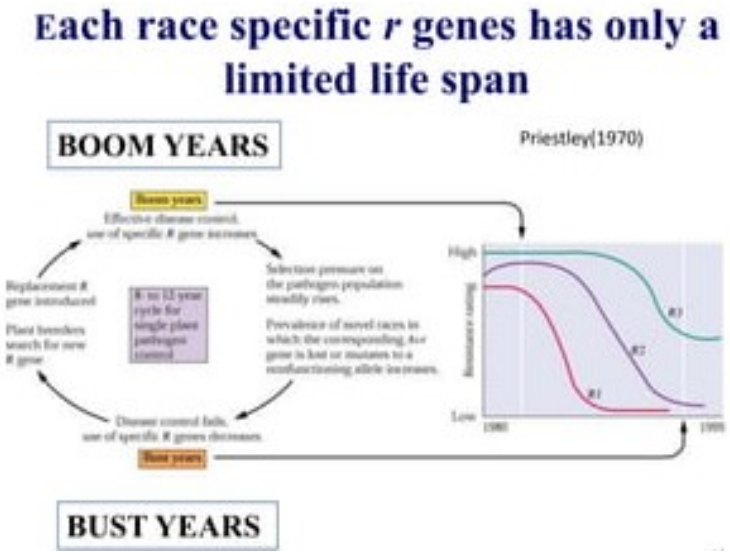
These pathotypes soon build up in population to create epidemics and eventually the variety is withdrawn. This phenomenon is generally called "boom and burst". The avoid

the implications of boom and burst phenomenon, use of durable host resistance is advocated in several crops. Durable resistance remains effective even though it may be widely grown ovoer a long period of time, in an environment that favours the disease. For example, oat variety, Red Rust Proof is still resistant against crown rust even after a hundred years. Wheat varieties, Thatcher and Lee have withstood stem rust for 55 and 30 years, respectively. Cappelle Desprez expresses at adult stage, a moderate resistance to yellow rust and this has been maintained for the last 20 years.

Two of the genes like Lr34 for resistance of leaf rust and Sr2 for resistance to stem rust have been recognized for durability. Wheat cultivars such as HD2189, HP1102, DL153-2, DL803-3 and DL802-2, which possess Lr34 with other gene combinations, have a good degree of resistance and hae become popular with growers. So far, there is not precise way available to identify the genetic components that are associated with durable resistance. Nor does dissociation of genes for virulence totally explain the basis of varietal durability, though it is likely to be the most plausible reason Boom and burst cycle-a characteristic of vertical resistance Resistance to virus and virus vectors Resistane to plant pathogenic viruses is generally oligogenic in nature

For example, the host pathogen reaction to the barley yellow dwarf virus (BYDV) is controlled by detectable single gene. The discovery of Yd2 gene in Ethiopial barley further confirms that against some of the viral diseases, vertical resistance is very much functional. Antiobiotics is the most common phemomenon where the host plant metabolites interfere with the normal life and growth of the insects following feeding activity.

Invariably, the adult body weight, fecundity and various facets of multiplication of the insects are adversely affected. The number of life cycles completed in a given period of time is also less. Therefore, in plants that exhibit antibiosis towards crop maturity, there is marked reduction in the level of pest infestation (virus vector population) and host damage. Mechanism of disease resistance or Nature of disease resistance Disease resistance is governed by several in built mechanisms of the host, plants against infection by the pathogen. They are disease escape, disease endurance or tolerance and true resistance.



a. Disease escape

It is a prevention mechanism that causes the host to escape pathogenic infection. Early or late maturity of the crop may prevent physical contact of the pathogen with the host. Mechanical and anatomical barriers such as thick cuticle, waxy bloom on leaves and stem, stomatal regulation prevent penetration of spores. Ergot, a fungal disease of inflorescence in cereals caused by *Claviceps purpurea* does not affect varieties of wheat and barley in which the flowers remain closed until pollination occurs. Erect leaves of barley avoid deposition of spores of *Erysiphe graminis* tritici in contrast to prostrate leaves. Early maturing varieties of groundnut escape early leaf spot infection (*Cercospora arachidicola*) and early varieties of wheat escape rust and loose smut infection.

A change in planting season has also been successfully employed as a measure of securing escape, e.g., the leaf rust of sugarcane (Puccinia sacchari) in the canal areas of Bombay severely affects cane when planted in June, but is of minor importance or absent in crops sown in October. Disease escape confers pseudo-resistance.

b. Disease endurance

The host after being infected by the pathogen tolerates the infection and suffers less damage. It does not result in any substantial decrease in yield. This is brought about by influence of external factors. It is a well-known phenomenon that plants fertilized with phosphatic and potash manures are more tolerant to disease; this is the case in wheat against rust infection. Rice crops fertilized by silicates are "resistant" to blast (*Pyricularia oryzae*) in Japan. Wheat crops fertilized by potash and phosphatic manures are highly tolerant to mildew and rust infection. The fertilizers act indirectly to arrest vegetative growth and promote early maturity, better straw and strengthening tissues to protect the plant which form a bulwark against pathogenic invasion.

c. True resistance

It is the ability of the host plant to resist or withstand the attack of a pathogen. True resistance is inheritable and much less subject to environmental influence. It is specific in character. The basis of resistance may be morphological, functional, structural or protoplasmic. Functional nature of resistance is determined by opening of the stomata, time of opening of flowers and time of maturity, rate of cork formation and cambial activity.

Structural characters include the proportion of strengthening tissues, fibre content, and nature of middle lamella, corky layers, number and structure of stomata and lenticels and their sizes. Protoplasmic factors controlling resistance are related to cell contents and include acids, tannins, anthocyanins, chemical constituents and their proportion, antibiotic activity and hypersensitivity present in the plant cells and in addition biological antagonism of the protoplasm of the host and the pathogen. True resistance, however, is of a specific character and is determined by the defence equipment and activities of the plant itself against the parasitic invasion and is therefore not subject to any appreciable modifications by external factors.

R genes and R proteins

Plants have evolved R genes (resistance genes) whose products mediate resistance to specific virus, bacteria, and Oomycetes fungus, nematode or insect strains. R gene products are proteins that allow recognition of specific pathogen effectors, either

through direct binding or by recognition of the effector's alteration of a host protein. Many R genes encode NB-LRR proteins (proteins with nucleotide-binding and leucine-rich repeat domains, also known as NLR proteins or STAND proteins, among other names). Most plant immune systems carry a repertoire of 100-600 different R gene homologs. Individual R genes have been demonstrated to mediate resistance to specific virus, bacteria, oomycete, fungus, nematode or insect strains. R gene products control a broad set of disease resistance responses whose induction is often sufficient to stop further pathogen growth/spread.

Studied R genes usually confer specificity for particular strains of a pathogen species (those that express the recognized effector). As first noted by Harold Flor in his mid-20th century formulation of the gene-for-gene relationship, a plant R gene has specificity for a pathogen avirulence gene (Avr gene). Avirulence genes are now known to encode effectors. The pathogen Avr gene must have matched specificity with the R gene for that R gene to confer resistance, suggesting a receptor/ligand interaction for Avr and R genes. Alternatively, an effector can modify its host cellular target (or a molecular decoy of that target), and the R gene product (NLR protein) activates defenses when it detects the modified form of the host target or decoy.

Gene-for-Gene Relationship:

The gene-for-gene hypothesis of host and pathogen relationship was proposed in 1953 by Flor on the basis of his work on flax rust resistance. This hypothesis has been valid for almost all host-pathogen relationships and it is universally accepted. According to **Flor hypothesis**, for every resistant gene present in the host, pathogen has a corresponding virulence gene. When pathogen has the virulence gene corresponding to the resistance gene present in the host, the pathogen produces disease, i.e., the host response is susceptible. But when the pathogen does not have the virulence gene corresponding to a resistance gene present in the host, disease is not produced, i.e., host response is resistant. Flor showed that the inheritance of both resistance in the host and parasite ability to cause disease is controlled by pairs of matching genes. One is a plant gene called the resistance (R) gene. The other is a parasite gene called the avirulence

(Avr) gene. Plants producing a specific R gene product are resistant towards a pathogen that produces the corresponding Avr gene product. Gene-for-gene relationships are a widespread and very important aspect of plant disease resistance. An example can be seen with *Lactuca serriola*.

RR = homozygous resistant ; Rr = heterozygous resistant; rr = homozygous susceptible
AVR AVR = homozygous avirulent; AVR avr = heterozygous avirulent; avr avr = homozygous virulent

		HOST GENOTYPE	
		RR or Rr	rr
PATHOGEN GENOTYPE	AVR avr or AVR AVR	Disease-resistant	Susceptible to disease
	avr avr	Susceptible to disease	Susceptible to disease

Gene-for-gene hypothesis Multifactor Interactions

Avirulence/virulence	Resistance/susceptibility			
	R1 R2	r1 R2	R1r2	r1r2
A1A2	-	-	-	+
a1A2	-	-	+	+
A1a2	-	+	-	+
a1a2	+	+	+	+

where, – = Resistance
+ = Susceptible

(Agrios 2007)

Resistance genes

Classes of resistance gene

There are several different classes of R Genes. The major classes are the NBS-LRR genes and the cell surface pattern recognition receptors (PRR). The protein products of the NBS-LRR R genes contain a nucleotide binding site (NBS) and a leucine rich repeat (LRR). The protein products of the PRRs contain extracellular, juxtamembrane, transmembrane and intracellular non-RD kinase domains.

Within the NBS-LRR class of R genes are two subclasses:

- One subclass has an amino-terminal Toll/Interleukin 1 receptor homology region (TIR). This includes the *N* resistance gene of tobacco against tobacco mosaic virus (TMV).
- The other subclass does not contain a TIR and instead has a leucine zipper region at its amino terminal.

The protein products encoded by this class of resistance gene are located within the plant cell cytoplasm.

The PRR class of R genes includes the rice XA21 resistance gene that recognizes the ax21 peptide and the Arabidopsis FLS2 peptide that recognizes the flg22 peptide from flagellin here are other classes of R genes, such as the extracellular LRR class of R genes; examples include rice Xa21D for resistance against *Xanthomonas* and the *cf* genes of tomato that confer resistance against *Cladosporium fulvum.*

The *Pseudomonas* tomato resistance gene (Pto) belongs to a class of its own. It encodes a Ser/Thr kinase but has no LRR. It requires the presence of a linked NBS-LRR gene, *prf*, for activity.

Specificity of resistance genes

R gene specificity (recognising certain Avr gene products) is believed to be conferred by the leucine rich repeats. LRRs are multiple, serial repeats of a motif of roughly 24 amino acids in length, with leucines or other hydrophobic residues at regular intervals. Some may also contain regularly spaced prolines and arginines.

LRRs are involved in protein-protein interactions, and the greatest variation amongst resistance genes occurs in the LRR domain. LRR swapping experiments between resistance genes in flax rust resulted in the specificity of the resistance gene for the avirulence gene changing.

Recessive resistance genes

Most resistance genes are autosomal dominant but there are some, most notably the *mlo* gene in barley, in which monogenic resistance is conferred by recessive alleles *mlo* protects barley against nearly all pathovars of powdery mildew.

Avirulence genes

The term "avirulence gene" remains useful as a broad term that indicates a gene that encodes any determinant of the specificity of the interaction with the host. Thus, this term can encompass some conserved microbial signatures (also called pathogen or microbe associated molecular patterns (PAMPs or MAMPs)) and pathogen effectors (e.g. bacterial type III effectors and oomycete effectors) as well as any genes that control variation in the activity of those molecules.

There is no common structure between avirulence gene products. Because there would be no evolutionary advantage to a pathogen keeping a protein that only serves to have it recognized by the plant, it is believed that the products of Avr genes play an important role in virulence in genetically susceptible hosts.

Example: AvrPto is a small triple-helix protein that, like several other effectors, is targeted to the plasma membrane by N-myristoylation. AvrPto is an inhibitor of PRR kinase domains. PRRs signal plants to induce immunity when PAMPs are detected. The ability to target receptor kinases is required for the virulence function of AvrPto in plants. However, Pto is a resistant gene that can detect AvrPto and induce immunity as well. AvrPto is an ancient effector that is conserved in many *P. syringae* strains, whereas Pto R gene is only found in a few wild tomato species. This suggests recent evolution of the Pto R gene and the pressure to evolve to target AvrPto, turning a virulence effector to an avirulence effector.

Unlike the MAMP or PAMP class of avr genes that are recognized by the host PRRs, the targets of bacterial effector avr proteins appear to be proteins involved in plant innate immunity signaling, as homologues of Avr genes in animal pathogens have been shown to do this. For example, the AvrBs3 family of proteins possess DNA binding domains, nuclear localisation signals and acidic activation domains and are believed to function by altering host cell transcription.

Biotrophy and gene for gene systems:

All the parasites in which gene for gene relationship has been proved are essentially biotrophic or biotrophs at least for some time after start of infection.

- (*Xanthomonas campestris* pv. *malvacearum*, *Phytophthora infestans*, *Venturia inaequalis* (Vander Plank, 1978).
- The genes-for-gene systems thus involve biotrophy.
- But the converse is not necessarily true. For example, *Plamodiophora brassicae* , the cause of club root of crucifers, is biotrophic but no evidence has yet been presented in the literature to suggest that host-pathogen interaction in them is based on a gene for gene systems.

According to Van der Plank (1978), specificity in gene for gene relationships lies in susceptibility. He explains it with the help of interactions of five host and five pathogens attacking them specifically.

Suppose there are five host varieties with five different R genes; R1, R2, R3----------R5. A plant with resistance gene R1 is attacked by a pathogen having virulence gene v1 and not to pathogen without this particular resistance gene irrespective of how many the virulence genes it may have.

Table. The diagonal check for specificity in a gene-for gene relationship

Pathogen	Plant				
	R_1R_1[b]	R_2R_2	R_3R_3	R_4R_4	R_5R_5
v_1v_1	S	R	R	R	R
v_2v_2	R	S	R	R	R
v_3v_3	R	R	S	R	R
v_4v_4	R	R	R	S	R
v_5v_5	R	R	R	R	S

a. Plant reaction when resistance gene R_1, R_2, R_3, R_4, R_5 at
interact with virulence genes v_1, v_2, v_3, v_4, v_5 at five loci
pathogen

b. Resistance is assumed to be dominant and RR can be r
by Rr. Virulence is assumed to be recessive. However, re
resistance and dominant virulence are also known.

R= resistant S= susceptible

Vander Plank (1978) elaborated protein for proteins hypothesis as a biochemical explanation of gene for gene interaction.

The protein for protein hypothesis states that in **gene for gene diseases the mutual recognition of host and pathogen is not by the genes themselves but by their coded proteins**.

Vander Plank (1978) hypothesized that in susceptibility the pathogen excretes a protein (virulence for product) into the host cell which copolymenizes with a complementary host protein (resistance gene product). This co-polymerization interferes with one auto regulation of the host gene that codes for the protein and by so doing turns the gene on to produce more protein.

In resistance, the protein specified by the gene for avirulence in the pathogen and excreted into the host does not polymerize with the protein coded for by the gene for resistance. It is not recognized by the host at all.

From a practical point of view, gene for gene relationship can be used to study the following:

1. The source of pathogenic variability in pathogens
2. The mutability of resistance and virulence genes
3. Why host resistance is expressed under one set of conditions and not others
4. Prediction of putative genotypes
5. Race nomenclature
6. Genetic dissection of complex loci
7. Cataloguing and storing of R genes in the form of plant seeds or cuttings and V genes in the form of pathogen strains
8. Management and deployment of resistance genes in space and tome
9. Detection of linkage and allelic relationship
10. Geographic distribution of R and V genes
11. Synthesis of multilines and multigene cultivars.

Vertifolia effect:

The vertifolia effect was discovered by Van der Plank (1963) who named it after a potato cultivar of this name, in which the effect was very pronounced. The vertifolia effect is a loss of horizontal resistance which occurs during breeding for vertical resistance. Its meaning was later extended to include the loss of horizontal resistance that occurs during breeding under the protection of pesticides.

The level of horizontal resistance can only be assessed by the level of parasitism. Clearly, if there is no parasitism because of a functioning vertical resistance, or a pesticide, the level of horizontal resistance cannot be assessed. Because individual plants with a high level of horizontal resistance are rather rare in a mixed screening population, the chances are that individuals with a relatively low level of horizontal resistance will then be selected on the basis of their other attributes. The loss is

usually quite small in a single breeding cycle but, after many cycles, it can become very serious indeed.

The prime example of the vertifolia effect is the loss of horizontal resistance to potato blight (*Phytophthora infestans*) that has continued ever since both the discovery of Bordeaux mixture in the late nineteenth century, and the discovery of vertical resistance in the twentieth century. A loss of horizontal resistance to cotton pests has continued ever since the discovery of DDT in the 1940s.

The vertifolia effect is a very modern phenomenon. Its overall consequences are seen in the high levels of horizontal resistance in heritage cultivars, when they are compared to modern cultivars. This is the main reason why heritage cultivars are so valued by organic farmers.

One of the main objectives of most amateur plant breeders will be to restore the horizontal resistances that were lost to the vertifolia effect.

Methods of breeding for disease resistance

The methods of breeding varieties resistant to diseases do not differ greatly from those adopted for other characters. The following methods are used:

1. Introduction,
2. Selection,
3. Hybridization followed by selection,
4. Back cross method,
5. Induced mutagenesis,
6. Development of multilines and
7. Tissue culture techniques

1. Introduction

It is a very simple and inexpensive method. Varieties resistant to a particular disease elsewhere may be thoroughly tested in the regions in which they are proposed to be introduced. Their yield performance and disease resistance should be confirmed by large scale cultivation. It is possible that a variety resistant in one region need not be

resistant in another region due to variation in the physiological race of the pathogen or due to a much different agroclimatic condition in the new location.

Introductions have served as a useful method of disease control. For example, Ridley wheat introduced from Australia has been useful as a rust resistant variety. Manila, a rice variety introduced in Karnataka from the Philippines, has tolerance to blast, bacterial leaf blight and sheath blight. Intan, a Javanica type rice variety introduced in Karnataka from Indonesia is highly resistant to blast. Munal, a rice variety introduced in West Bengal from the U.S.A. is tolerant to blast, bacterial leaf blight and leaf folder (pest). Some of IRRI rice varieties such as IR 20, IR.24, IR.28, IR.34, IR.36 and IR .50 possess resistance to one or more diseases. Early varieties of groundnut introduced from U.S.A. have been resistant to leaf spot (*Cercosora arachidicola*).

Kalyan Sona and Sonalika wheat varieties originated from the segregating materials introduced from CIMMYT, Mexico and were rust resistant. Introduftions also serve as sources of resistance in breeding programmes. For example, African pearlmillet (*P. americanum*) introductions have been used for developing downy mildew resistant male sterile lines (Tift 23A cytoplams) for use in hybrid pearlmillet production. This is an important development in the hybrid pearlmillet programmes since the original male sterile lines Tift 23A and 23D2A were extremely susceptible to downy mildew. The introduction of Co.475 variety of sugarcane in Mumbai has conquered red rot but brought in leaf rust and whip smut to the fore.

2. Selection

This is better method than introduction and has more chances of success in obtaining disease-resistant plants. The work of selection is carried out either in the naturally infected fields under field conditions or under artificially inoculated conditions. The resistance in such individuals will occur in nature by mutation. To ensure the resistant character of a plant, large population of crop plant may be exposed to the attack of pathogen under artificial conditions and the non-infected plants may be chosen. Suvarnamodan rice of Kerala is a pureline of ARC. 11775 and is highly tolerant to blast.

Sugandh of Bihar is a selection from Basmati rice of Orissa tolerant to bacterial leaf blight. Rice varieties Sudha (Bihar), Sabita, Nalini (West Bengal), Patel 85 (Madhya Pradesh), Janaki (Bihar), Improved White Ponni (Tamil Nadu), Ambika (Maharashtra), are some of rice selections resistant to one or more diseases. MCU 1 cotton, a selection from Co 4, is resistant to Kufri Red; a potato selection from Darjeeling Red Round is a disease resistant variety.

3. Hybridization

When selection of resistant varieties is not feasible, resistant varieties may be evolved by crossing the susceptible popular variety with resistant wild variety where in the resistant gene or genes transferred into the genetic make up of susceptible variety. Very often the F1 from crosses may be resistant but carry the other undesirable qualities of the resistant parent. The bad qualities are removed by several back crossing of F1 with the susceptible parent may ultimately yield a resistant progeny with good agronomic characteristics.

Under certain circumstances pedigree or bulk method of selection is followed to obtain a resistant variety. In this method, the crosses are made till F2 population is got. Selections are made in F2 generation for superior genetic traits including disease resistance. By continued selfing, selections are made through F3 to F5 or F6 generations and the best variety is selected. This method is suited for small grains and beans but unsuited to fruits and vegetables.

4. Back cross method

Back cross method is widely used to transfer disease resistance from wild species. Wild species are rice sources of disease resistance. Interspecific hybridization is made to transfer the gene or genes for resistance to the cultivated species. Resistance to grassy stunt virus from *Oryza nivara* to *O.sativa*, late blight resistance from *Solanum demissum* to cultivated potato, rust resistance from durum to *aestivum* wheat are some of the examples involving interspecific hybridization. Depending upon the number of genes governing resistance and the nature of the gene, whether dominant or recessive, the procedure varies. The number of back crosses to the cultivated species may be five to

six. Once the backcross progeny resembles the cultivated parent, then they are selfed and segregating progeny screened for disease resistance.

5. Induced mutagenesis

While following mutation breeding for disease resistance, a large number of mutation progeny should be produced and screened under artificial epiphytotic condition to select resistant plants. MCU10 cotton, a resistant variety to bacterial blight was evolved in Tamil Nadu by subjecting seeds of a susceptible variety CO4 to gamma rays followed by rigorous screening and selection

6. Development of multilines

The concept of multilines was first suggested by Jensen (1952) and developed by Borlaug (1959) for evolving multiline varieties to resist stem rust in wheat. A multiline variety is a composite of genetically similar lines, except that each line possesses a different gene for resistance to the pathogen. Lines that are genetically similar, except for one gene, are called isoline. It is assumed that gene for resistance in each isoline contributes resistance to a separate physiological race or group of races. Genes for disease resistance are transferred by backcrossing from donor varieties to a common disease susceptible, but agronomically superior, recurrent parent. Isolines are generated differing only in the gene for disease resistance. The isolines are composited to synthesize a multiline variety. The isolines are maintained for resynthesizing the multiline whenever needed. A multiline variety is composed of a mixture of resistant and susceptible genotypes and provides a buffering effect against rapid development of disease. It will provide resistance or tolerance to a broad spectrum of races of a pathogen. If new races of the pathogen are identified at a later stage, additional isolines resistant to the newly arisen races may be constituted and incorporated.

Care should be taken to see that there is uniformity for height, maturity and other features in the multiline. Though multilines provide stability of yield due to reduction of damage by pathogens, the limitations of multiline varieties are that the yield level of multiline varieties is limited to that of the recurrent parent, 4 to 5 years are required to stabilize isogenic lines and the pathogen may produce new races at a faster rate than

the development of a multiline. Multiline varieties have been developed for resistance to stem rust and stripe rust of wheat and crown rust of oats. The first multiline variety in wheat, 'Miramar 60' was developed and released in Columbia to combat the attack of yellow rust. 'Miramar 63' and 'Miramar 65' were resistant to stem rust and stripe rust. 'Yoqui 50', 'Crew' and 'Tumult' are a few other wheat multilines. Kalyan sona and Sonalika-based multilines of wheat resistant to different races of rust have been developed in India.

7. Tissue culture technique

Tissue culture techniques to produce somaclonal variation for disease are developed in different crops. Somaclonal variations for disease resistance are reported in *Zea mays* for *Drechslera maydis* race T-toxin resistance, in *Brassica napus* for resistance/tolerance to Phoma lingam, early and late blight resistance in potato, *Pseudomonas* and *Alternaria* resistance in tobacco, besides smut and rust disease resistance in sugarcane.

13. Back Cross Method of Breeding: Significance and limitations; multiline concept.

Back Cross Method

A cross between F1 hybrid and one of its parents is known as a backcross. It is proposed by Harian and Pope in 1922, as a method breeding for small grains and is employed in improvement of both hybrid.

In this method two plants are selected and crossed and hybrid successively backcross to one of their parents. As a result the grain hybrid backcross progeny becomes increasingly similar to that of the parents to which it identical with the parent used for backcrossing. In this method the desirable variety which are lacking in some

characteristics known as a recurrent or recipient parent, while the undesirable variety on wild variety processing only one or two desirable characteristics known as donor parent or non recurrent parent. The objectives of this method are to improve one or two specific defects of high yielding variety.

Pre-requisite for back cross breeding

For the successful development of a new variety, following requirements must be fulfilled.

- A suitable recurrent parent must be available which lack in one or two characters.
- A suitable donor parent must be available which passes the characters be transfer in highly intense form.
- The character to be transferred much has high heritability.
- A sufficient number of backcrossed should be made so that the genotype of the recurrent parent is recovered in full.

Application of the Backcross Method:

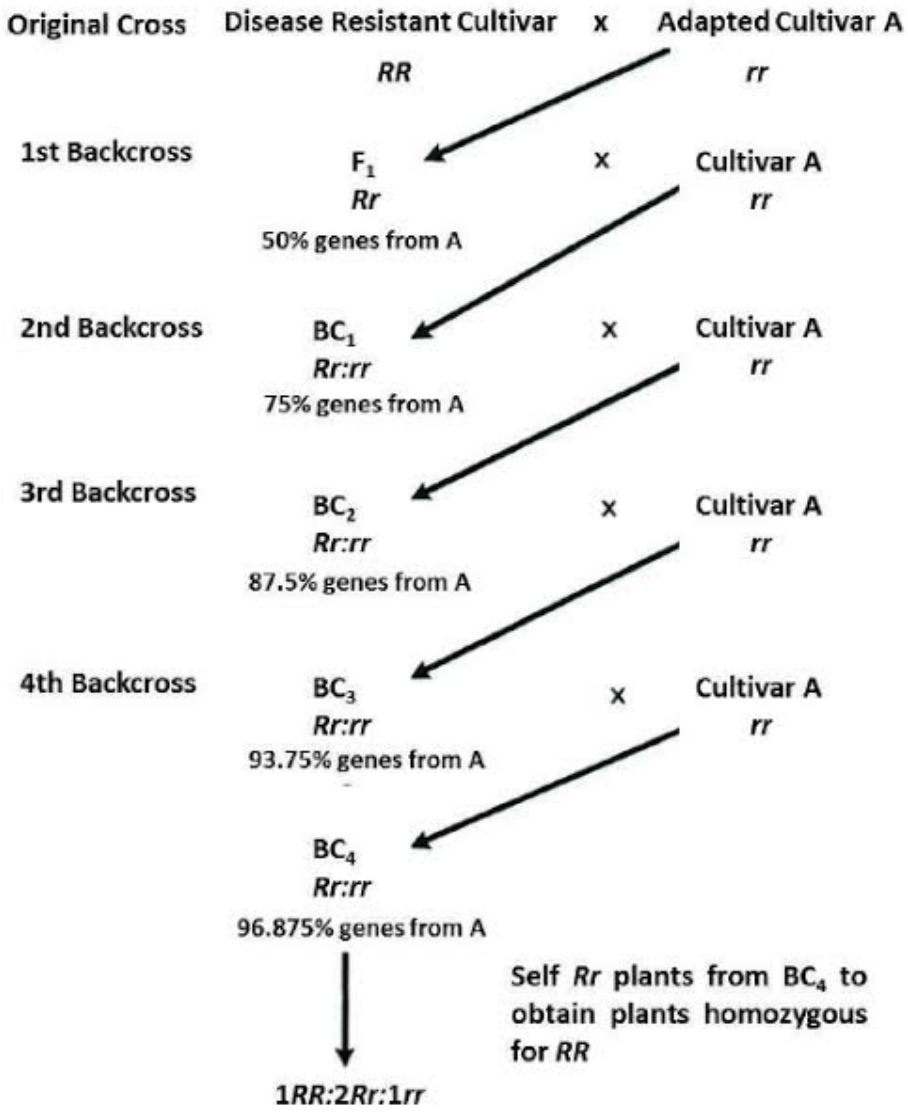
This method is commonly used for the transfer of disease resistant from one variety to another. But is also suitable for the transfer of quantitative characters and is applied is both self and cross pollinated crops.

1) **Intervarietal transfer of simply inherit characters** such as disease resistance, seed colour, plant height etc.
2) **Intervarietal transfer or quantitative characters**. Such as earliness, seed size, seed shape may be transferred from one variety to another belonging to same species.
3) **Interspecific transfer of simply inherited characters** i.e disease resistance from related species to cultivated species. Ex. Transfer of leaf and stem rust resistance from *Triticum monococum* to *Triticum aestivum*.
4) **Transfer of cytoplasm.** This is employed to transfer male sterility. The female parent will be having the sterile cytoplasm and recurrent parent will be used as male parent.

5) **Transgressive segregation** – Backcross method may be modified to produce Transgressive segregants.
6) **Production of isogenic line**
7) **Germplasm conversion**. E.g., Production of photo insensitive line from photo sensitive germplasm through back crossing. This was done in the case of *Sorghum*. Popularly known as conversion programme.

Genetic Consequences of Back Crossing:

- It results in rapid increase in homozygosity and frequency of homozygote.
- The repeated backcrossing results in increase in frequency of desirable genotype thus the genotype of progeny become increasingly similar to recurrent parent.
- The gene under transfer must be maintained by selection in the back cross generation.
- There would be opportunity in each backcross generation for crossing over to occur between the gene being transferred and tightly linked genes.
- Selection of recurrent parent type.



Selection of parents:

Backcross method of breeding change the genotype of recurrent parent only for the gene(s) under a transfer to correct the specific defect of the recurrent parent. But some unexpected changes in one or more character may also occur due to gene tightly linked with the gene being transferred. Therefore, the recurrent parent must be the most popular variety of the area, which has high yielding ability, desirable quality and high adaptability. In each crop, one or two varieties dominant, and they are vary popular

with the farmers. Such a variety may have one or two defects, e.g., susceptibility to disease or undesirable seed size or color, which may be removed by this method. The non-recurrent parent is selected for high intensity of the character that is to be improved in the recurrent parent, and yielding ability and other feature of the parent are not important. The intensity of the character should preferably be more than that desired in the recurrent parent because the intensity may decline during the transfer and in the new genetic background of the recurrent parent.

Transfer of a Dominant Gene –

- Suppose that a high yielding variety and widely adapted wheat variety A is susceptible to stem rust. Another variety B is resistant to stem rust and this resistance is dominant to susceptibility.
- Therefore, variety A is taken as Recurrent parent and variety B is used as Non-recurrent parent.
- Procedure is simple for dominant gene transfer than recessive gene transfer.

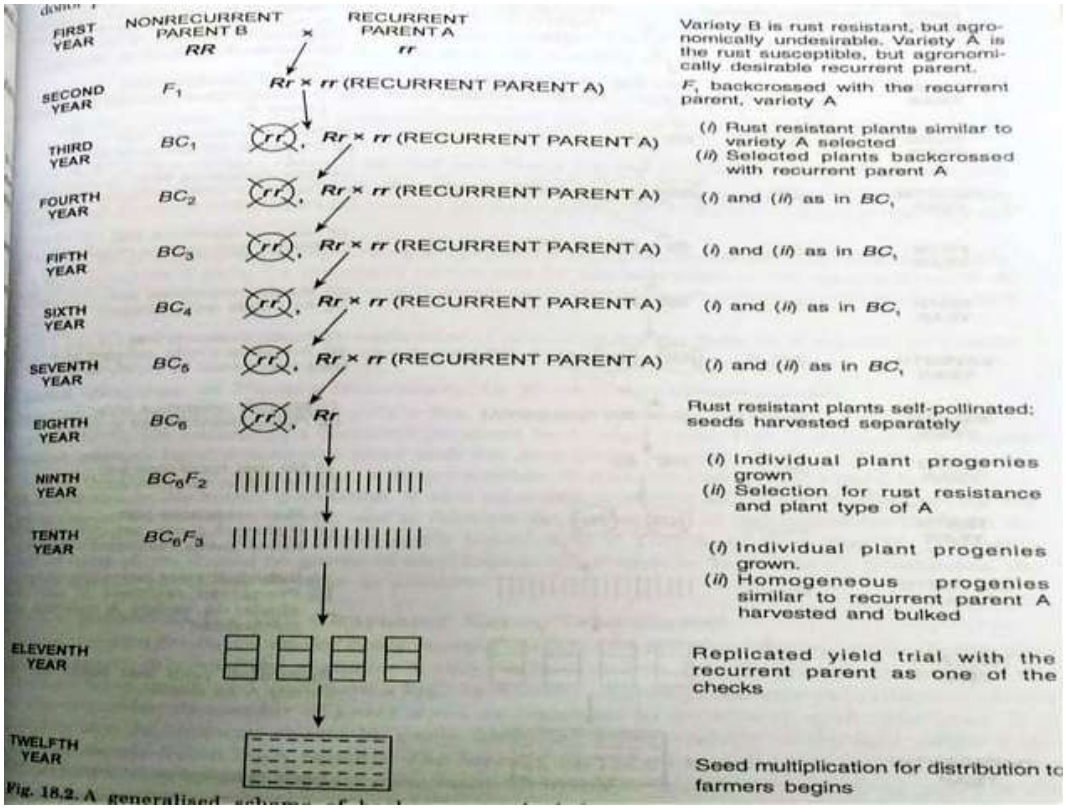
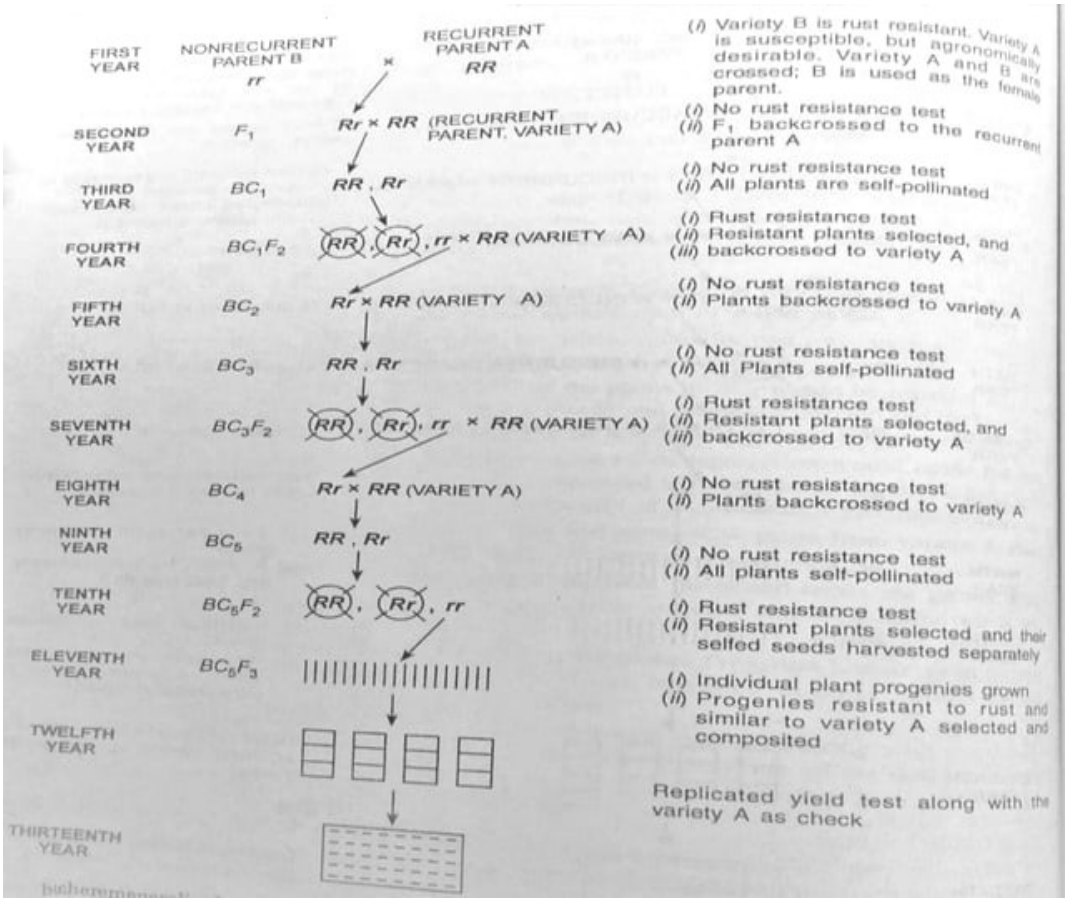


Fig. 18.2. A generalised

Transfer of a Recessive Gene –

- When rust resistance is due to recessive gene, all the backcross cannot be made one after the other. After the first backcross and after every two subsequent backcrosses, F_2 generation must be grown to identify rust resistant plants.
- The F_1 and the backcross progenies are not inoculated with rust because they will be uniformly susceptible so, only the F_2 populations are tested for rust resistance.
- Here, variety B is rust resistance (rr) and it is Non-recurrent parent, whereas variety A is agronomically desitrable and it is as Recurrent parent.



Merits of Backcross Method:

1. The method reduces the amount of field testing needed, as the new cultivar will be adapted to the same area as the original cultivar.
2. Backcross breeding is repeatable. If the same parents are used, the same backcrossed cultivar can be recovered.
3. It is a conservative method, not permitting new recombination to occur.
4. It is useful for introgressing specific genes from wide crosses.
5. It is applicable to breeding both self-pollinated and cross-pollinated species.

Demerits of Backcross Method:

1. Backcrossing is not effective for transferring quantitative traits. The trait should be highly heritable and readily identifiable in each generation. However, the application of molecular markers is helping to change the application of backcrossing to improving quantitative traits.
2. The presence of undesirable linkages may prevent the cultivar being improved from attaining the performance of the original recurrent parent.
3. Recessive traits are more time consuming to transfer.
4. Hybridization required for each backcross; which is often difficult, time taking and costly.

Achievements:

- ✓ Rust resistance has been transferred to Kalyan Sona from several diverse sources, e.g., Robin, HS19, Bluebird etc.
- ✓ Tift23A was used in backcross programme with resistant lines from India and Africa to develop downy mildew resistant male sterile lines, such as MS521, MS541, etc.
- ✓ *G. herbaceum* varieties Vijapla, Vijay, Digvijay and Kalyan are some of the cotton varieties developed by this method.

Multiline varieties

Multiline variety is a mixture of several pure lines of similar phenotype(height, seed color flowering time, maturity time and various other agronomic characteristics) but have different genes for the character under consideration the disease resistance means these are isogenic lines. At the same time they do not reduce the yielding ability of each other when grown in mixture (i.e. compatible).

What is Multiline Breeding?

Procedure followed to develop multiline variety is called as multiline breeding.

Examples of Multiline Variety

Wheat variety Kalyan sona is the suitable example to explain the concept. This variety was originally resistant to brown rust. Later on became susceptible to new races of pathogen. Several pure lines with different resistance genes are produced through backcross breeding using one recipient or recurrent parent. The donor parents are the one with different genes for the disease resistance, every donor parent is used in separate back cross program. Because of this each line receives different gene for disease resistance according to the type of pathogen. Five to ten of such lines with different alleles for disease resistance are mixed to develop multiline variety. The lines to be mixed are determined by the races of the pathogen relevant to the area considered.

MLKS11 (8 closely related lines)

KML7404 (9 closely related lines)

Production steps for Multiline Variety

- Selection of recurrent parent
- Selection of donor parent
- Transfer of resistance
- Mixing of isolines

Advantages of Multiline Variety

1. At the time of disease outbreak, only one or few lines of the mixture get attacked, others remain resistant. So the loss to the farmer is comparatively less.
2. Multiline varieties are more adaptive to environmental changes than individual pure line.

Disadvantages of Multiline Variety

1. Demerits of the multiline breeding are given below.
2. Races of pathogen change as time goes on, so farmer has to change seeds every few years which contains seeds of lines resistant to new pathogen races.
3. No improvement in yield or other characters
4. Takes more time to develop new variety, in the due course of time new pathogen races may evolve.
5. Costly
6. All the lines constituting multiline variety may get attacked by the new race of pathogen.
7. Not suitable for cross pollinated crops.

Achievements

Multiline variety appears to be a useful approach to control diseases like rusts where new races are continuously produced. In India, three multiline varieties have been released in wheat (*Triticum aestivum*). Kalyan Sona, one of the most populat varieties in the late 60s, was used as the recurrent parent to produce these varieties. Variety 'KSML 3' consists of 8 lines having rust resistance genes from Robin, Ghanate, K1, Rend, Gabato, Bluebird, Tobari etc. Multiline 'MLKS 11' is also a mixture of 8 lines the resistance is derived from E 6254, E 6056, E 5868, Frecor, HS 19, E 4894 etc. The third variety KML 7406 has 9 lines deriving rust resistance from different sources.

14. Heritability: Understanding, Components of phenotypic variance, Broad-sense and narrowsense heritability.

Heritabilityis a statistic used in the fields of breeding and genetics that estimates the degree of variation in a phenotypic trait in a population that is due to genetic variation between individuals in that population. Heritability is estimated by comparing individual phenotypic variation among related individuals in a population. Heritability is an important concept in quantitative genetics, particularly in selective breeding and behavior genetics.

Heritability, amount of phenotypic (observable) variation in a population that is attributable to individual genetic differences. Heritability, in a general sense, is the ratio of variation due to differences between genotypes to the total phenotypic variation for a character or trait in a population. The concept typically is applied in behaviour genetics and quantitative genetics, where heritability estimates are calculated by using either correlation and regression methods or analysis of variance (ANOVA) methods.

- Heritibility is the ratio ofgenotypic varience to the phrnotypic variance.
- Heritibility denotes the proportion of phenotypic variance that is due to genotype, i.e., heritable.
- It is generally expressed in percent (%).
- It is a good index of transmission of characters from parents to their offspring.

Heritability is expressed as $H^2=Vg/Vp$, where H is the heritability estimate, Vg the variation in genotype, and Vp the variation in phenotype. Heritability estimates range in value from 0 to 1. If H = 1, then all variation in a population is due to differences or variation between genotypes (i.e., there is no environmentally caused variation). If H= 0, there is no genetic variation; in this case all variation in the population comes from differences in the environments experienced by individuals.

Heritability is the ratio of the genetic variance over the phenotypic variance.

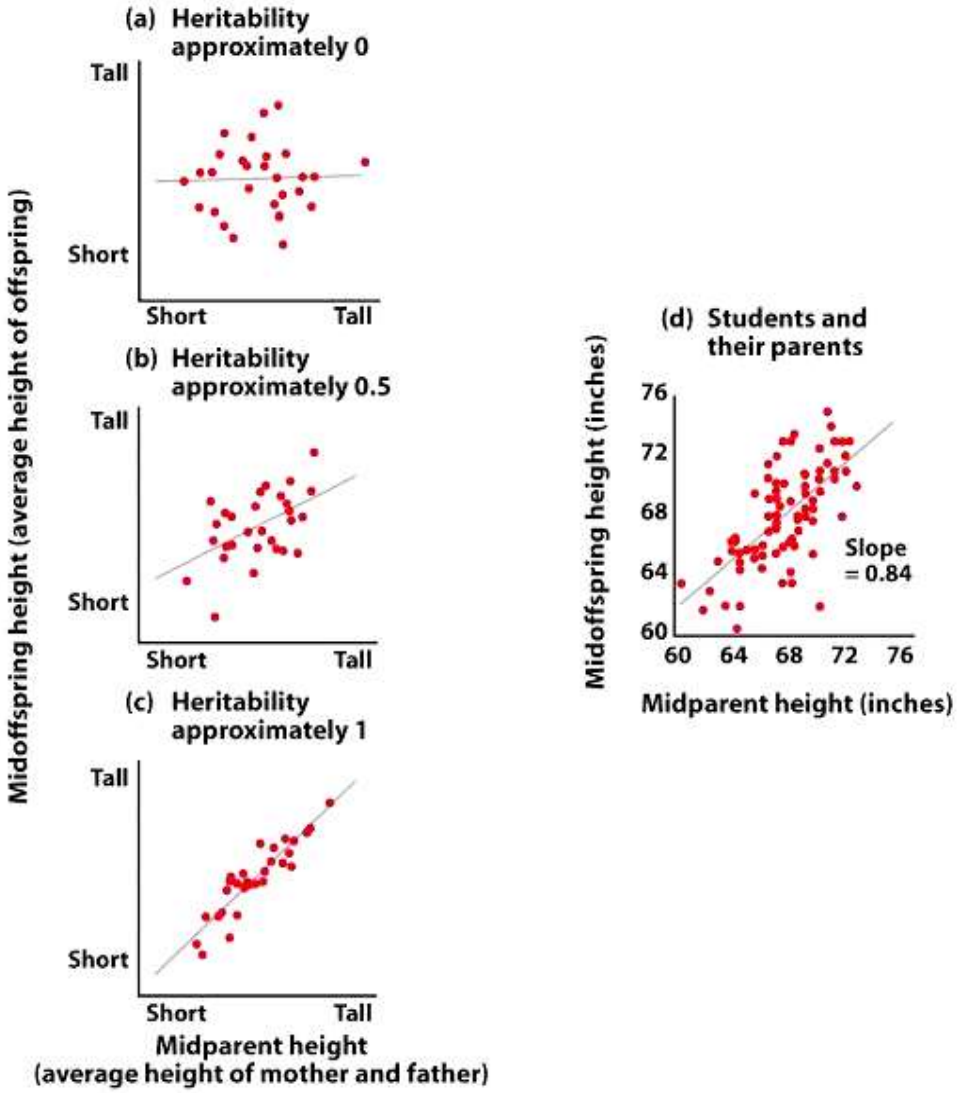


Fig.: Heritibility in between parents and offsprings.

Depending upon the components of variance used as numerator in the calculation, there are 2 definites of heritability.

1. **Broad sense heritability**
2. **Narrow sense heritibility**

Broad sense heritability:

Broad sense heritability includes all components of genetic variance. Often it is informative to calculate the proportion of the total phenotypic variance that is due to genetic differences among individuals in a population. This proportion is called the broad-sense heritability, symbolized H^2. In terms of Fisher's variance components,

$$H^2 = V_g/V_T$$
$$= V_g/(V_g + V_e)$$

The total phenotypic variance may be decomposed:

V_p = total phenotypic variance

V_g = total genetic variance

V_e= environmental variance

- Broad sense heritability (h_2) separates genotypic from environmentally induced variance: $h_2 = V_g/V_p$
- It can be estimated from both parental as well assegragating populations.
- It expresses the extent to which the phenotype is determined by the genotype, so called degree of genetic determination.
- It is most useful in clonal or highly selfing species in which genotypes are passed from parents to offsprings more or less intact.
- It is useful in selection of superior libes from homozygous lines.

The symbol for the broad-sense heritability, H^2, is written with the exponent 2 to remind us that this statistic is calculated from variances, which are squared quantities. Because of the way it is calculated, the broad-sense heritability must lie between 0 and 1. If it is close to 0, little of the observed variability in the population is attributable to genetic differences among individuals. If it is close to 1, most of the observed variability is attributable to genetic differences. The broad-sense heritability therefore summarizes the relative contributions of genetic and environmental factors to the observed variability in a population. However, it is important to note that this statistic is population-specific. For a given trait, different populations may have different values of

the broad-sense heritability. Thus, the broad-sense heritability of one population cannot automatically be assumed to represent the broad-sense heritability of another population. In the F^2 wheat population, $H^2 = 11.98/14.26 = 0.84$. This result tells us that in this population 84% of the observed variability in wheat maturation time is due to genetic differences among individuals. However, it does not tell us what these differences are. The genetic variance upon which the broad-sense heritability depends includes all the factors that cause genotypes to have different phenotypes: the effects of individual alleles, the dominance relationships between alleles, and the epistatic interactions among different genes. In the next two sections, we will see that by breaking out these components of genetic variability and by focusing on the component that involves the effects of individual alleles, we can predict the phenotypes of offspring from the phenotypes of their parents.

Narrow sense heritability includes only the additive genetic variance and it is this form of heritability that usually of interest. The ability to make predictions in quantitative genetics depends on the amount of genetic variation that is due to the effects of individual alleles. Genetic variation that is due to the effects of dominance and epistasis has little predictive power.

- It is the ratio of additive or fixable genetic variance to the total or phenotypic variance.
- Also known as degree of genetic resemblance.
- It plays an important role in the selection process in plant breeding.
- For estimation of narrow sense heritability, crosses have to be made in a definite fashion.
- It is estimated from additive genetic variance.
- It is useful for plant breeding in selection of elite types from segregating populations.

It is calculated with the help of following formula

Where V_a or V_d = additive genetic variance

V_P = total phenotypic variance

To see how dominance limits the ability to make predictions, consider the ABO blood types in humans. This trait is determined strictly by the genotype; environmental variation has essentially no effect on the phenotype.

However, because of dominance, two individuals with the same phenotype can have different genotypes. For example, a person with type A blood could be either I A I A or I A i. If two people with type A blood produce a child, we cannot predict precisely what phenotype the child will have. It could be either type A or type O, depending on the genotypes of the parents; however, we know that it will not have type B or type AB blood. Thus, although we can make some kind of prediction about the child's phenotype, dominance prevents us from making a precise prediction. Our ability to make predictions about an offspring's phenotype is improved in situations where the genotypes are not confused by dominance. Consider, for example, the inheritance of flower color in the snapdragon, *Antirrhinum majus*. Flowers in this plant are white, red, or pink, depending on the genotype. As with the ABO blood types, variation in flower color has essentially no environmental component; all the variance is the result of genetic differences. However, for the flower color trait, the genotype of an individual is not obscured by the complete dominance of one allele over the other. A plant with two w alleles has white flowers, a plant with one w allele and one W allele has pink flowers, and a plant with two W alleles has red flowers. In this system, the phenotype depends simply on the number of W alleles present; each W allele intensifies the color by a fixed amount. Thus, we can say that the color-determining alleles contribute to the phenotype in a strictly additive fashion. This kind of allele action improves our ability to make predictions in crosses between different plants. A mating between two red plants produces only red offspring; a mating between two white plants produces only white offspring; and a mating between red and white plants produces only pink offspring. The only uncertainty is in a cross involving heterozygotes, and in this case the uncertainty is due to Mendelian segregation, not to dominance.

Quantitative geneticists distinguish between genetic variance that is due to alleles that act additively (such as those in the flower color example just discussed) and

genetic variance that is due to dominance. These different variance components are symbolized as:

V_a = additive genetic variance

V_d = dominance variance

In addition, geneticists define a third variance component that measures variation due to epistatic interactions between alleles of different genes:

V_i = epistatic variance

Epistatic interactions, like dominance, are of little help in predicting phenotypes. Altogether, these three variance components constitute the total genetic variance:

$$V_g = V_a + V_d + V_i$$

If we recall that $V_T = V_g + V_e$, we can express the total phenotypic variance as the sum of four components:

$$V_T = Va + V_d + V_i + V_e$$

Of these four variance components, only the additive genetic variance, V_a, is useful in predicting the phenotypes of offspring from the phenotypes of their parents. This variance, as a fraction of the total phenotypic variance, is called the narrow-sense heritability, symbolized h^2. Thus,

$$h_2 = V_a/V_T$$

Like the broad-sense heritability, h^2 lies between 0 and 1. The closer it is to one, the greater is the proportion of the total phenotypic variance that is additive genetic variance, and the greater is our ability to predict an offspring's phenotype. Human stature is highly heritable, but litter size in pigs is not. Thus, if we knew the parental phenotypes, we would be better able to predict the height of a human's offspring than the litter size of a pig's offspring.

It is also referred to as **resemblance between relatives**.

Because individual components of variance are not directly measurable, it is necessary to use comparative measurements of phenotype to determine the contribution of individual variance components.

For example:

- By measuring a specific trait such as height in individual organisms from several populations, one could determine the range of height measurements for that species.
- Individuals from different populations could then be grown in a common garden and measured at the same point of maturity as the original organisms. The common garden would eliminate the environmental variance experienced between the different populations.
- Therefore, the difference between the phenotype variance of the wild populations and that of the common garden would give an estimate of the total genetic variance.

Factor affecting heritability:

- **Type of genetic material:** the magnitude of heritability is largely governed by the amount of genetic variance present inapopulation for the character under study
- **Sample size:** Large sample is necessary for accurate estimates
- **Sampling methods:** 2 sampling methods, Random and Biased. The random sampling methods provide true estimates of geneticvariance andhence of heritability
- **Layout or conduct of experiment:** Increasing theplotsizeand no. of replications we can reduce experimental error and get reliable estimates
- **Method of calculation: heritability is estimated by several methods**
- **Effect of linkage:** highfrequencyofcouplingphase(AB/ab) causes upward bias in estimates of additive and dominance variances
- **Excess of repulsion phase linkage (Ab/aB)** leads to upward bias indominance variance and downward bias in additivevariances

Gentic advance:

Improvement in the mean genotypic value of selected plants over the parental population is known as genetic advance. It is the measure of genetic gain under

selection. This success of genetic advance under selection depends upon three factors (Allard, 1960).

- Genetic variability: greater the amount of genetic variability in base populations higher the genetic advance
- Heritability: the G. A. is highwith characters having high heritability
- Selection intensity: the proportion of individuals selected for the study is called selection intensity; high selection intensity gives better results

Selection differential:

It is the difference between the mean phenotypiv value of selected population and mean phenotype of original population

This is the measure of the selection intensity and denoted by K.

$K = Xs - Xo$

Where, Xs = mean of phenotypic value of selected plants

Xo = mean of phenotypic value of parental population

Genetic gain:

The difference between the mean of phenotypic value of the progeny of selected plants and original parental population is known as genetic gain.

It is denoted by R.

$R = Xp - Xo$

Where, Xp = mean of phenotypic value of selected plants

Xo = mean of phenotypic value of base population

Merits of hreritability:

- ✓ It is useful in predicting the effectiveness of selection.
- ✓ It is also helpful for deciding breeding methods to be followed, for effective selection.
- ✓ It gives an idea about the response of various characters to selection pressure.
- ✓ It is useful in predicting the performance under different degree of intensity of selection.

- ✓ It helps for construction of selection index.
- ✓ Estimates of heritability serve as a useful guide to the breeder, to appreciate the proportion of variation, which is due to genotypic or additive effects.

Limitations of heritability:

- It does not indicat the degree to which a characteristic is genetically modified
- Pure brred no polydactyly rabbits: still polydactyly can happen
- An individual does not have heritability
- Narrow sense heritability of 0.6 in population does not indicate that an individual's cjharacters is 60% additive
- There is no universal heritability of a characteristic
- Two populations will have different heritability due to environment
- Even when heritability is high, environmental factors may influence a characteristic
- Human height
- Heritability indates nothing about the nature of population differences in a characteristic.

15. Design of Experiments: general principles of field trials, randomized blocks, latin square, split plot designs, layout of breeding experiment.

Design of experiment means how to design an experiment in the sense that how the observations or measurements should be obtained to answer a query in a valid, efficient and economical way. The designing of the experiment and the analysis of obtained data are inseparable. If the experiment is designed properly keeping in mind the question, then the data generated is valid and proper analysis of data provides the valid statistical

inferences. If the experiment is not well designed, the validity of the statistical inferences is questionable and may be invalid.

One of the main objectives of designing an experiment is how to verify the hypothesis in an efficient and economical way. In the contest of the null hypothesis of equality of several means of normal populations having the same variances, the analysis of variance technique can be used. Note that such techniques are based on certain statistical assumptions. If these assumptions are violated, the outcome of the test of a hypothesis then may also be faulty and the analysis of data may be meaningless. So the main question is how to obtain the data such that the assumptions are met and the data is readily available for the application of tools like analysis of variance. The designing of such a mechanism to obtain such data is achieved by the design of the experiment. After obtaining the sufficient experimental unit, the treatments are allocated to the experimental units in a random fashion. Design of experiment provides a method by which the treatments are placed at random on the experimental units in such a way that the responses are estimated with the utmost precision possible.

Principles of experimental design for field trial:

There are three basic principles of design which were developed by Sir Ronald A. Fisher. (i) Randomization (ii) Replication (iii) Local control

(i) Randomization: The principle of randomization involves the allocation of treatment to experimental units at random to avoid any bias in the experiment resulting from the influence of some extraneous unknown factor that may affect the experiment. In the development of analysis of variance, we assume that the errors are random and independent. In turn, the observations also become random. The principle of randomization ensures this.

The random assignment of experimental units to treatments results in the following outcomes.

a) It eliminates systematic bias.

b) It is needed to obtain a representative sample from the population.

c) It helps in distributing the unknown variation due to confounded variables throughout the experiment and breaks the confounding influence.

Randomization forms a basis of a valid experiment but replication is also needed for the validity of the experiment.

If the randomization process is such that every experimental unit has an equal chance of receiving each treatment, it is called complete randomization.

(ii) Replication: In the replication principle, any treatment is repeated a number of times to obtain a valid and more reliable estimate than which is possible with one observation only. Replication provides an efficient way of increasing the precision of an experiment. The precision increases with the increase in the number of observations. Replication provides more observations when the same treatment is used, so it increases precision. For example, if the variance of x is σ^2 than the variance of the sample mean $\bar{x}$ based on n, observation is σ^2/n. So as *n* increases, Var $\bar{x}$ decreases.

(iii) Local control (error cntrol): The replication is used with local control to reduce the experimental error. For example, if the experimental units are divided into different groups such that they are homogeneous within the blocks, then the variation among the blocks is eliminated and ideally, the error component will contain the variation due to the treatments only. This will, in turn, increase the efficiency.

Randomized blocks:

A randomized block design is a restricted randomized design, in which experimental units are first organized into homogeneous blocks and then the treatments are assigned at random to these units within these blocks. The main advantage of this design is, if done properly, it provides more precise results. The main disadvantage is more assumptions are required (no interactions between treatments and blocks and constant variance from block to block) and there are fewer degrees of freedom than for a completely randomized design (R.B.D).

Applications of R.B.D.:

Despite it is agricultural origin, the randomized block design is widely used in many types of studies. For example to determine the differences in productivity of c makes of machines (treatments). We may isolate the possible effects due to differences in efficiency among operators (blocks) by randomly rotating machine assignments in such a way that each operator works on all the machines, the basic idea is to compare treatment levels (the different machines) within block of relatively homogeneous experimental material (the same operator), then repeat the comparison on another block (another operator), and so on for additional repetition of the comparison.

Layout of R.B.D.:

In agricultural experiments, the layout of R.B.D, can be given as follows Let us consider five treatments A, B, C, D and E replicated 4 times. We divide the whole experimental area into four relatively homogenous strata or blocks and each block into five units or plots, treatments are then allocated at random to the plots of a block, fresh randomization done for each block. A particular layout may be as follows:

BLOCK I	**A**	**E**	**B**	**D**	**C**
BLOCK II	**E**	**D**	**C**	**B**	**A**
BLOCK III	**C**	**B**	**A**	**D**	**A**
BLOCK IV	**A**	**D**	**E**	**C**	**B**

For randomization we may use Tippet's randomization tables. Let us select one digit numbers in the order of their occurrence leaving 0 and numbers greater than 5. Suppose we get a random numbers as 1,3,5,4 and 2, so in the first block we allocate treatment A to the first plot, C to the second plot, E to the third plot, D to the fourth plot and B to the fifth plot. Similarly, we use fresh random numbers for each of the other three plots and allocate the treatments accordingly.

Advantages of R.B.D.:

- ✓ Accuracy: This design has been shown to be more efficient or accurate than C.R.D. for most types of experimental work. The elimination of between sum

of squares from residual sum of squares usually results in a decrease of error mean sum of squares.

- ✓ Flexibility: In R.B.D. no restrictions are placed on the number of treatments or the number of replicates.
- ✓ Ease of analysis: Statistical analysis is simple, rapid and straight forward.

Disadvantages of R.B.D.:

- R.B.D. may give misleading results if blocks are not homogeneous.
- R.B.D. is not suitable for large number of treatments because in that case the block size will increase and it may not be possible to keep large blocks homogeneous.
- If the data on more than two plots is missing the statistical analysis becomes tedious and complicated.

Latin Square:

- ➢ The experimenter is concerned with a single factor having p levels. However, variability from two other sources can be controlled in the experiment.
- ➢ If we can control the effect of these other two variables by grouping experimental units into blocks having the same number of treatment levels as the factor of interest, then a *latin square design* may be appropriate.
- ➢ Consider a square with p rows and p columns corresponding to the p levels of each blocking variable. If we assign the p treatments to the rows and columns so that each treatment appears exactly once in each row and in each column, then we have a $p \times p$ latin square design.
- ➢ It is called a "latin" square because we assign "latin" letters A, B, C,..... to the treatments. Examples of a 4×4 and a 6×6 latin square designs are

		Column			
		1	2	3	4
Rows	1	A	C	B	D
	2	D	A	C	B
	3	B	D	A	C
	4	C	B	D	A

		Column					
		1	2	3	4	5	6
Rows	1	A	B	C	D	E	F
	2	B	C	D	E	F	A
	3	C	D	E	F	A	B
	4	D	E	F	A	B	C
	5	E	F	A	B	C	D
	6	F	A	B	C	D	E

- By blocking in two directions, the MSE will (in general) be reduced. This makes detection of significant results for the factor of interest more likely.
- The experimental units should be arranged so that differences among row and columns represent anticipated/potential sources of variability. – In industrial experiments, one blocking variable is often based on units of time. The other blocking variable may represent an effect such as machines or operators.

Six-Hour Work Shift \ Machines	1	2	3	4
1	A	C	B	D
2	D	A	C	B
3	B	D	A	C
4	C	B	D	A

Day \ Operator	1	2	3	4	5
M	A	B	C	D	E
Tu	B	C	D	E	A
W	C	D	E	A	B
Th	D	E	A	B	C
F	E	A	B	C	D

- In agricultural experiments, the experimental units are subplots of land. We would then have the subplots laid out so that soil fertility, moisture, and other sources of variation in two directions are controlled.
- In greenhouse experiments, the subplots are often laid out in a continuous line. In this case, the rows may be blocks of p adjacent subplots and the columns specify the order within each row block.

Rep. 1							Rep. 2							Rep. 3							Rep. 4							Rep. 5							Rep. 6							Rep. 7						
A	D	G	B	E	C	F	G	F	B	C	A	E	D	B	C	D	G	F	A	E	E	G	A	D	C	F	B	C	B	F	E	D	G	A	F	E	C	A	B	D	G	D	A	E	F	G	B	C

After converting Rep. 1 to Rep. 7 into row blocks, we get

Reps \ Column	1	2	3	4	5	6	7
1	A	D	G	B	E	C	F
2	G	F	B	C	A	E	D
3	B	C	D	G	F	A	E
4	E	G	A	D	C	F	B
5	C	B	F	E	D	G	A
6	F	E	C	A	B	D	G
7	D	A	E	F	G	B	C

Split Plot Designs:

In conducting experiments, sometimes some factors have to be applied in larger experimental units while some other factors can be applied in comparatively smaller experimental units. Further some experimental materials may be rare while the other experimental materials may be available in large quantity or when the levels of one (or more) treatment factors are easy to change, while the alteration of levels of other treatment factors are costly, or time-consuming. One more point may be that although two or more different factors are to be tested in the experiment, one factor may require to be tested with higher precision than the others. In all such situations, a design called the split plot design is adopted.

A split plot design is a design with at least one blocking factor where the experimental units within each block are assigned to the treatment factor levels as usual, and in addition, the blocks are assigned at random to the levels of a further treatment factor. The designs have a nested blocking structure. In a block design, the experimental units are nested within the blocks, and a separate random assignment of units to treatments is made within each block. In a split plot design, the experimental units are called split-plots (or sub-plots), and are nested within whole plots (or main plots).

In split plot design, plot size and precision of measurement of effects are not the same for both factors, the assignment of particular factors to either the main plot or the sub-plot is extremely important. To make such a choice, the following guidelines are suggested:

Degree of Precision: For a greater degree of precision for factor B than for factor A, assign factor B to the sub-plot and factor A to the main plot e.g. a plant breeder who plans to evaluate ten promising rice varieties with three levels of fertilization, would probably wish to have greater precision for varietal comparison than for fertilizer response. Thus, he would designate variety as the sub-plot factor and fertilizer as the main plot factor. Or, an agronomist would assign variety to main plot and fertilizer to sub-plot if he wants greater precision for fertilizer response than variety effect.

Relative Size of the Main effects: If the main effect of one factor (A) is expected to be much larger and easier to detect than that of the other factor (B), factor A can be assigned to the main plot and factor B to the sub-plot. This increases the chance of detecting the difference among levels of factor B which has a smaller effect.

Management Practices: The common type of situation when the split plot design is automatically suggestive is the difficulties in the execution of other designs, i.e. practical execution of plans. The cultural practices required by a factor may dictate the use of large plots. For practical expediency, such a factor may be assigned to the main plot e.g. in an experiment to evaluate water management and variety, it may be desirable to assign water management to the main plot to minimize water movement between adjacent plots, facilitate the simulation of the water level required, and reduce border effects. Or, if ploughing is oneof the factors of interest, then one cannot have different depths of ploughing in different plotsscattered randomly apart.

Randomization and Layout:

There are two separate randomization processes in a split plot design – one for the main plot and another for the sub-plot. In each replication, main plot treatments are first randomly assigned to the main plots followed by a random assignment of the sub-plot treatments within each main plot. This procedure is followed for all replications. A possible layout of a split plot experiment with four main plot treatments (a=4), three sub-plot treatments (b=3), and four replications (r=4) is given below –

Layout of Breeding Experiment:

b_1	b_3	b_2	b_2
b_3	b_2	b_1	b_3
b_2	b_1	b_3	b_1

a_4 a_2 a_1 a_3

Rep. II

b_3	b_1	b_2	b_1
b_1	b_2	b_1	b_3
b_2	b_3	b_3	b_2

a_1 a_4 a_2 a_3

Rep. III

b_3	b_1	b_2	b_3
b_2	b_3	b_3	b_2
b_1	b_2	b_1	b_1

a_3 a_2 a_4 a_1

Rep. IV

b_2	b_3	b_3	b_1
b_1	b_2	b_1	b_2
b_3	b_1	b_2	b_3

a_1 a_4 a_3 a_2

The basic objective of plant breeding is the

ultimate crop improvement. It results in development of high yielding varieties hybrids etc., over the existing cultivars and so on. The performances of the new varieties are confirmed from the results obtained from the field experiments. To be explained scientifically the field experiments are laid out following certain rules and the data thus collected are analyzed statistically. The steps involved in this process are explained here under.

Any designing of experiments involves three major steps.

1. Selection of experimental units

The objects on which the treatments are applied is known as experimental units. Eg. Plots in the field, plant, etc.,

2. Fixing of treatments

The objects of comparison are known as treatments. Eg. Varieties, spacing etc.,

3. Arrangement of treatments in the experimental Units

It comprises of three basic principles of design

Replication: repetition of treatments

Randomization: unbiased allocation of treatments to the experimental units

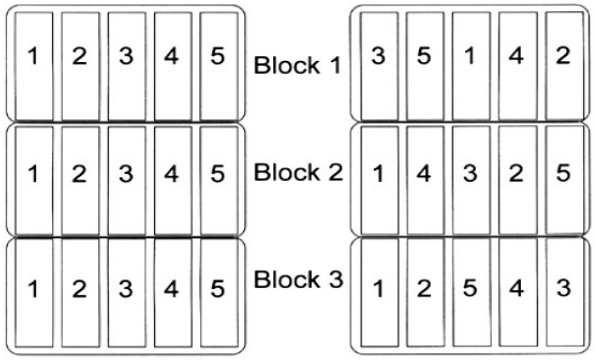


Fig.: Test plots on the left are not randomized. Plots on the right are randomized. The numbers (1-5) represent the five treatments in this test.

Local control: minimizing the effect of heterogeneity of the experimental units

The objective of replication, randomization and local control is to minimize the Experimental Error (EE). EE is nothing but differences in the responses from the

experimental unit to experimental unit under similar environments. Apart from these, EE can be reduced further by proper selection of the experimental units and choosing of most appropriate experimental design for a given number of treatment.

Types of basic experimental designs:

1. Completely Randomized Design (CRD)
2. Randomized Block Design (RBD)
3. Latin Square Design (LSD)

Among these, RBD is the widely used design.

Laying Out of RBD:

A. The experimental material (field) is divided first into blocks consisting of homogenous (uniform) experimental units. Each block is divided into number of treatments equal to the total number of treatments.

B. Randomization should be taken within each block and the treatments are applied following the random number table.

C. Collection and analysis of data: After the collection of data from the individual experimental unit (treatments) ANOVA (Analysis of Variance) table is formed.

The significance of the ANOVA table is that it indicates the sources of variation exhibited by the treatments, the magnitude of variation derived from different sources and their worthiness (significant/ non significant).

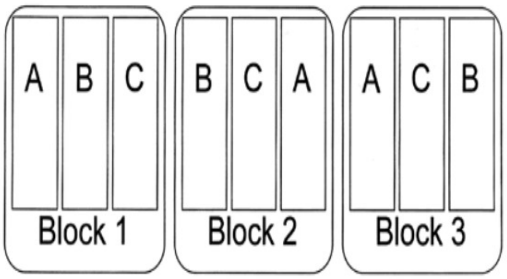


Fig.: An easy way to arrange blocks is to put them side by side across the field. Letters represent different treatments.

ıce between the treatment means, which places the treatments statistically as well

as significantly apart. Otherwise if the difference of two treatments mean is less than CD it can be concluded both the treatments are on par.

RT: Row trial

Row trial is generally conducted in F3 and F4, when the seeds are not sufficient for replication with individual plant progeny rows. Each row consists of about 20 or more plants. Individual plants with desirable characteristics are selected from superior progeny rows. Pest, Disease and lodging susceptible progenies with undesirable characteristics are eliminated.

RRT – Replicated Row Trial

It is generally conducted from F3 generation onwards. Depending on availability of seeds, 3-4 more rows are grown for each progeny to facilitate comparison among progenies adopting suitable replications. Families, which have become reasonably homozygous, may be harvested in bulk. From those families showing segregation, single plants are selected for characters under study. The breeder has to visually assess the yielding potential of progenies and reject the inferior ones in the field and the yield potential has to be assessed in the laboratory for confirmation.

PYT – Preliminary Yield Trial or Initial Yield Evaluation Trial (IYET)

It is conducted from F5 generation onwards. Preliminary yield trial with three or more replications is conduct to evaluate the comparative performance of the culture and to identity the superior cultures among them. The cultures are evaluated for plant height, lodging, pest and disease resistance, flowering time, duration and yield, etc., Quality tests may also be carried out. Standard commercial varieties must be included as checks for comparison. Ten to fifteen outstanding cultures, if superior to checks, would be advanced to the advanced yield trails.

AYT – Advanced Yield Trial

Advanced Yield Trial is conducted from F8 generation on wards. The superior cultures identified from Preliminary Yield Trial are tested in Replicated Yield Trial. In this trial,

the cultures are evaluated for yield, pest, disease and lodging resistance, duration, quality, etc.

MLT – Multi Location Trial

Multi location trial is conducted from F13 onwards for 3 years by the Research Station Scientists. Multi Location Trial are useful for suitability studies i.e. whether a particular culture is able to perform well in all the locations or not and whether the particular culture out yields all the other cultures developed by research stations and the check variety evaluated simultaneously. Based on the evaluation, superior and stable performing culture will be promoted to ART.

ART – Adaptive Research Trial

It is conducted after MLT for 3 years by the Department of Agriculture. Nearly 3-4 cultures are tested and based on the performance of 3 Years in the farmer's field, the best culture over the check may be proposed to SVRC (State Variety Release Committee) for releasing.

If the SVRC finds that the cultivar is suitable for any particular area or through out the state, then the variety is released and is notified by the State Department of Agriculture.

16. Bioreactors: Concept; Types of bioreactors- batch, continuous, multistage and immobilized cell bioreactors; Application in plant tissue culture.

Bioreactors:

A bioreactor (bioreactor) is a closed vessel with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the waste biomass of cultured microorganisms along-with their products.

A bioreactor is used for commercial production in fermentation industries and is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value. Bioreactors are extensively used for food processing, fermentation, waste treatment, etc

Design of bioreactor:

All bioreactors deal with heterogeneous systems dealing with two or more phases, e.g., liquid, gas, solid. Therefore, optimal conditions for fermentation necessitate efficient transfer of mass, heat and momentum from one phase to the other. Chemical engineering principles are employed for design andoperation of bioreactors.

A bioreactor should provide for the following:

(i) Agitation (for mixing of cells and medium),

(ii) Aeration (aerobic bioreactor); for O_2 supply,

(iii) Regulation of factors like temperature, pH, pressure, aeration, nutrient feeding, liquid level etc.,

(iv) Sterilization and maintenance of sterility, and

(v) Withdrawal of cells/medium (for continuous bioreactor).

Modern bioreactors are usually integrated with computers for efficient process monitoring, data acquisition, etc.

Generally, 20-25% of bioreactor volume is left unfilled with medium as “head space” to allow for splashing, foaming and aeration. The bioreactor design varies greatly depending on the type and the fermentation for which it is used. Bioreactors are so designed that they provide the best possible growth and biosynthesis for industrially important cultures and allow ease of manipulation for all operations.

Size of Bioreactors/Bioreactors:

The size of bioreactors ranges from 1-2 litre laboratory bioreactors to 5,00,000 litre or, occasionally, even more, bioreactors of upto 1.2 million litres have been used. The size of the bioreactor used depends on the process and how it is operated. A summary of bioreactor or size of bioreactor (litres)

Construction of Bioreactors:

Industrial bioreactors can be divided into two major classes, anaerobic and aerobic. Anaerobic bioreactors require little special equipment except for removal of heat generated during the fermentation process, whereas aerobic bioreactors require much more elaborate equipment to ensure that mixing and adequate aeration are achieved.

Since most industrial fermentation processes are aerobic, the construction of a typical aerobicbioreactor is the following –

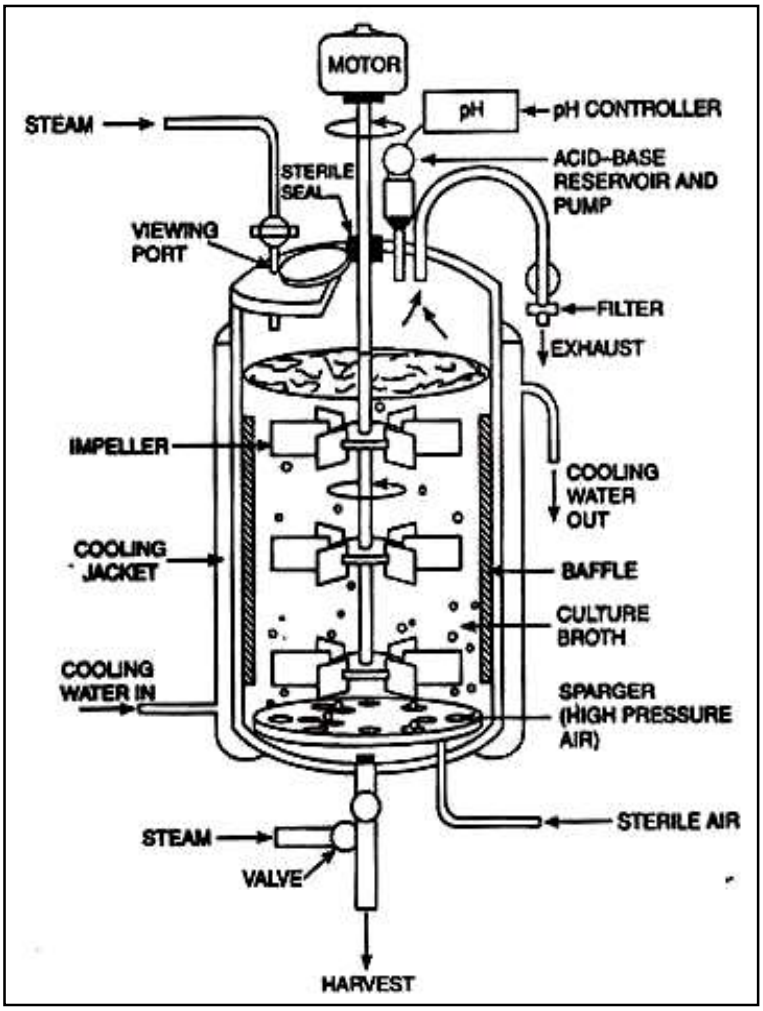


Fig.: An Industrial aerobic bioreactor (Internal View).

1.Cooling Jacket:

Large-scale industrial bioreactors are almost always constructed of stainless steel. A

bioreactor is a large cylinder closed at the top and the bottom and various pipes and valves are fitted into it. The bioreactor is fitted externally with a cooling jacket through which steam (for sterilization) or cooling water (for cooling) is run.

Cooling jacket is necessary because sterilization of the nutrient medium and removal of the heat generated are obligatory for successful completion of the fermentation in the bioreactor. For very largebioreactors, insufficient heat transfer takes place through the jacket and therefore, internal coils are provided through which either steam or cooling water is run.

2. Aeration System:

Aeration system is one of the most critical part of a bioreactor. In a bioreactor with a high microbial population density, there is a tremendous oxygen demand by the culture, but oxygen being poorly soluble in water hardly transfers rapidly throughout the growth medium.

It is necessary, therefore, that elaborate precautions are taken using a good aeration system to ensure proper aeration an oxygen availability throughout the culture. However, two separate aeration devices are used to ensure proper aeration in bioreactor. These devices are sparger and impeller. The sparger is typically just a series of holes in a metal ring or a nozzle through which filter- sterilized air (or oxygen-enriched air) passes into the bioreactor under high pressure. The air enters the bioreactor as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid culture medium. The impeller (also called agitator) is an agitating device necessary for stirring of the fermenter.The stirring accomplishes two things –

(i) It mixes the gas bubbles through the liquid culture medium and

(ii) It mixes the microbial cells through the liquid culture medium. In this way, the stirring ensuresuniform access of microbial cells to the nutrients.

The size and position of the impeller in the bioreactor depends upon the size of the bioreactor. In tall bioreactors, more than one impeller is needed if adequate aeration and agitation is to be obtained. Ideally, the impeller should be 1/3 of the bioreactors diameter fitted above the base of the bioreactor. The number of impeller may vary from size to size to the bioreactor.

3. Baffles:

The baffles are normally incorporated into bioreactors of all sizes to prevent a vortex and to improve aeration efficiency. They are metal strips roughly one-tenth of the bioreactors diameter and attached radially to the walls.

4. Controlling Devices for Environmental Factors:

In any microbial fermentation, it is necessary not only to measure growth and product formation but also to control the process by altering environmental parameters as the process proceeds. For this purpose, various devices are used in a bioreactor. Environmental factors that are frequently controlled includes temperature, oxygen concentration, pH, cells mass, levels of key nutrients, and product concentration.

Use of Computer in Bioreactors:

Computer technology has produced a remarkable impact in fermentation work in recent years and the computers are used to model fermentation processes in industrial bioreactors. Integration of computers into fermentation systems is based on the computers capacity for process monitoring, data acquisition, data storage, and error-detection.

Some typical, on-line data analysis functions include the acquisition measurements, verification of data, filtering, unit conversion, calculations of indirect measurements, differential integration calculations of estimated variables, data reduction, tabulation of results, graphical presentation of results, process stimulation and storage of data.

Types of Bioreactors:

They are broadly of two types — batch reactors and continuous reactors. Other types are stirred tank reactors and plug flow type reactors.

1. Batch Reactors:

In batch reactors, the immobilized enzymes and substrates are placed, and the reaction is allowed to take place under constant stirring. As the reaction is completed, the product is separated from the enzyme (usually by denaturation).

Soluble enzymes are commonly used in batch reactors. It is rather difficult to separate

the soluble enzymes from the products; hence there is a limitation of their reuse. However, special techniques have been developed for recovery of soluble enzymes, although this may result in loss of enzyme activity.

2. Continuous Reactors:

In continuous enzyme reactors, the substrate is added continuously while the product is removed simultaneously. Immobilized enzymes can also be used for continuous operation. Continuous reactors have certain advantages over batch reactors. These include control over the product formation, convenient operation of the system and easy automation of the entire process. There are mainly two types of continuous reactors-continuous stirred tank reactor (CSTR) and plug reactor (PR). CSTR is ideal for good product formation.

3. Stirred tank reactors:

The simplest form of batch reactor is the stirred tank reactor. It is composed of a reactor fitted with a stirrer that allows good mixing, and appropriate temperature and pH control. However, there may occur loss of some enzyme activity. A modification of stirred tank reactor is basket reactor. In this system, the enzyme is retained over the impeller blades. Both stirred tank reactor and basket reactor have a well-mixed flow pattern.

4. Plug flow type reactors:

These reactors are alternatives to flow pattern type of reactors. The flow rate of fluids controlled by a plug system. The plug flow type reactors may be in the form of packed bed or fluidized bed. These reactors are particularly useful when there occurs inadequate product formation in flow type reactors. Further, plug flow reactors are also useful for obtaining kinetic data on the reaction systems.

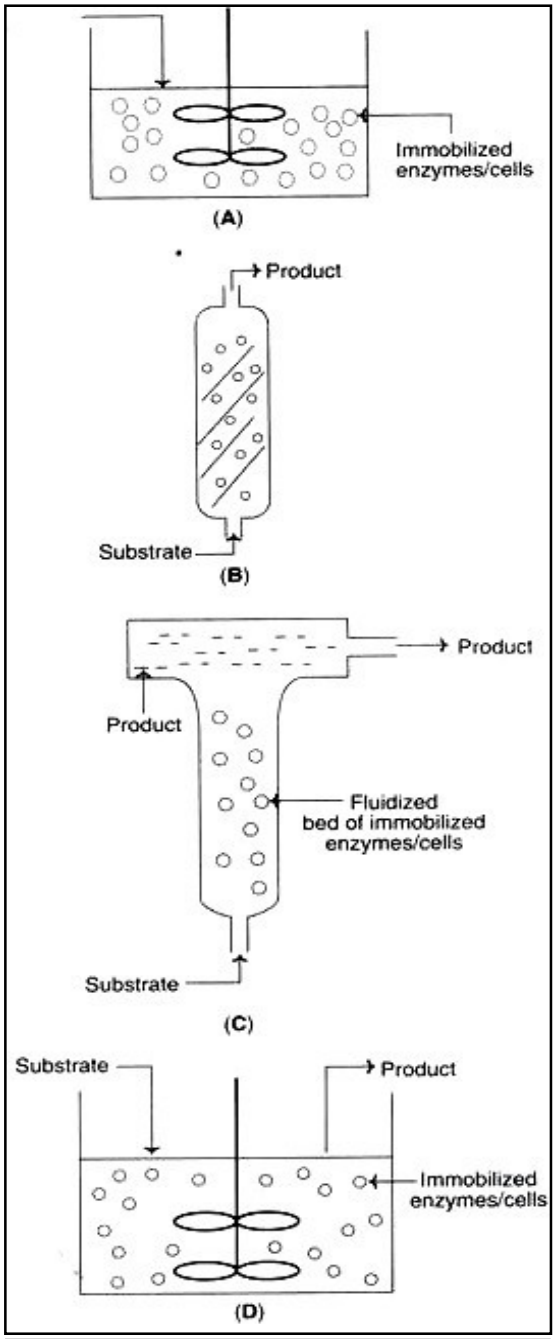


Fig.: Immobilized enzyme (cell) reactors (A) Batch stirred tank reactor, (B) Packed bed reactor, (C) Fluidized bed reactor, (D) Continuous stirred tank reactor.

Membrane Reactors:

Several membranes with a variety of chemical compositions can be used. The commonly used membrane materials include polysulfone, polyamide and cellulose acetate. The biocatalysts (enzymes or cells) are normally retained on the membranes of the reactor. The substrate is introduced into reactor while the product passes out. Good mixing in the reactor can be achieved by using stirrer. In a continuous membrane reactor, the biocatalysts are held over membrane layers on to which substrate molecules are passed. In a recycle model membrane reactor, the contents (i.e. the solution containing enzymes, cofactors, and substrates along with freshly released product are recycled by using apump. The product passes out which can be recovered.

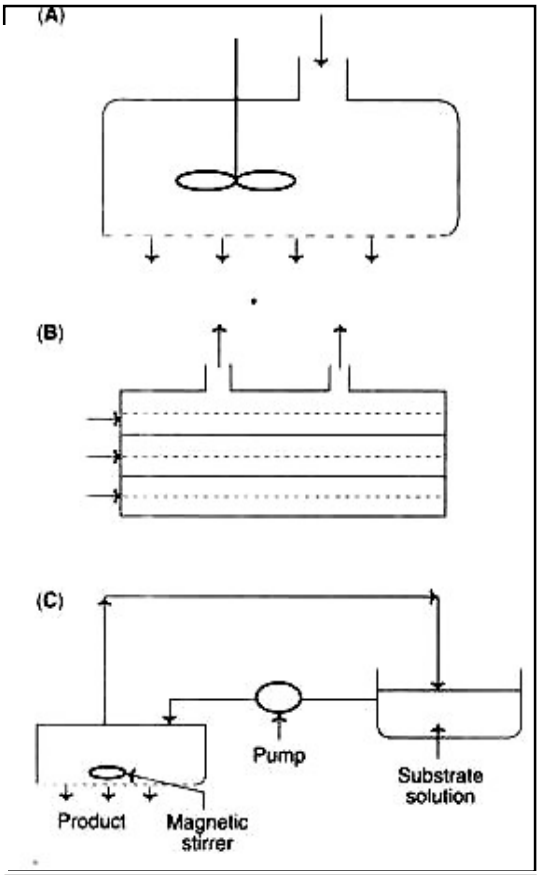
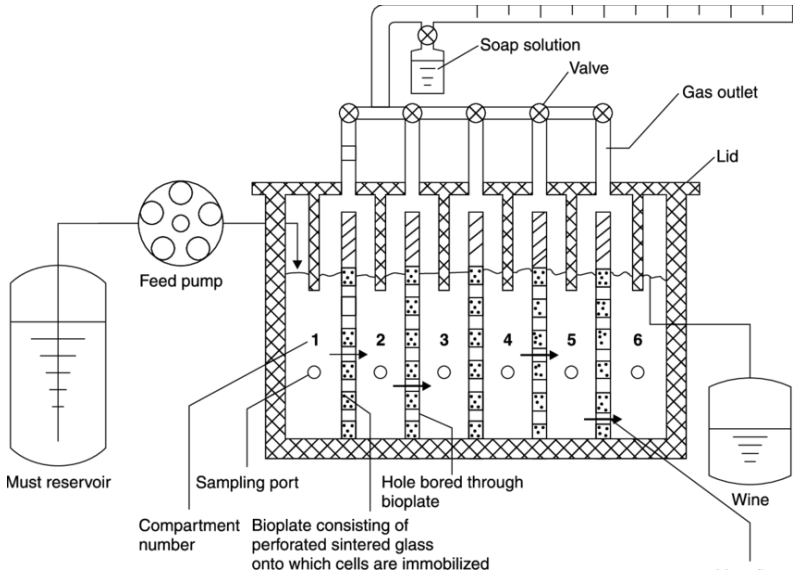


Fig.: Membrane reactors (A) Batch membrane reactor, (B) Continuous membrane reactor, (C) Recycle membrane reactor.

Multistage Bioreactor:

A good alternative to reproduce the partial decoupling between yeast growth and alcohol production during winemaking process is to conduct continuous fermentation in multistage bioreactors. Multi-stage bioreactor devices have been developed previously for use in alcohol production, in combination with cell immobilization and also in the beverage industry in order to increase productivity and optimize the production of aromatic molecules, mainly higher alcohols and esters. However, the potential interest of the multi-stage continuous fermentation system as a tool for investigating the metabolism and physiology of *S. Cerevisiae* during alcoholic fermentation has never been explored.

In multi-stage continuous fermentation (MSCF) system, there are different phases of batch wine fermentation, including the stationary phase (non-proliferating cells). The original device consists of two or more tanks connected in series, the first ones containing micro-organisms in growth phase while the last stages contain non proliferating cells. The dilution rates applied to each tank and the nitrogen content in the feeding medium – which is usually the limiting factor for yeast growth during wine making – need to adjusted and exhaust this nutrient within the first stages of fermentation. It is operated with resting cells (without growth), continuously supplied from the earlier tanks. MSCF system is used for physiological studies to characterize the metabolic specificities of commercial wine yeast.

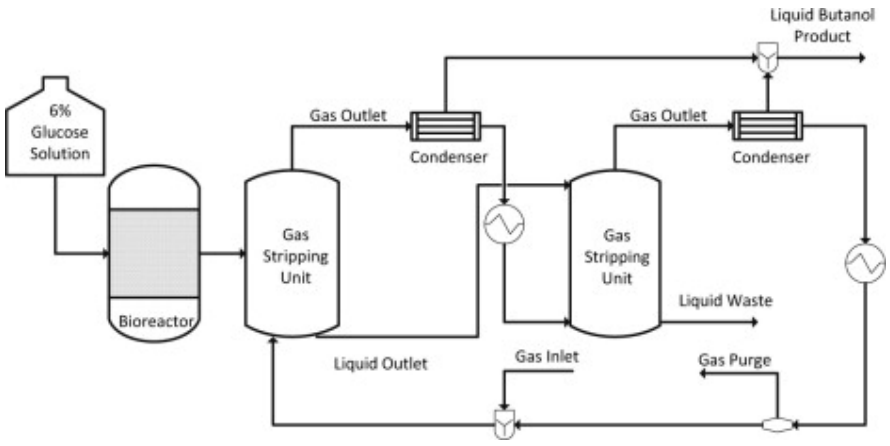


Immobilized Cell Reactors:

In these reactors, enzymes/cells are either attached by adsorption, chemical bonding (cross- linked or covalently bound), or entrapment on a suitable carrier, or are encapsulated and placed/ packed in different types of vessel configurations to serve as a flow reactor.

Reactors with physical adsorption of enzymes or cells encounter practical difficulties because the adsorbed enzyme or cell is weakly bound and is lost rather easily during operation. Gel-entrapped enzyme reactor systems are associated with severe problems of diffusion resistances more exercised by the substrate than the product. Covalently or cross-linked immobilized reactor systems require mild processing conditions.

Large changes in pH and temperature are not permitted. A microencapsulated reactor system is subjected to the requirement of substrate diffusivity across the semi-permeable membrane which contains the enzyme or cells. Despite these disadvantages, the greatest advantage offered by this system is high-productivity.



Application of bioreactors in plant tissue culture:

Bioreactor design specific for plant cell is important for the production of secondary metabolites using cell culture techniques. It is used in the micropropagation of several crops, including ornamental and medicinal plants, vegetables and fruits. By cultivation in bioreactors, different plant parts can be obtained, such as buds, somatic embryos,

bulbs, shoots, calluses, protocorm and others. They are also used for making pharmaceutical products such as flavonoids, phenols and other secondary metabolites. Tissue culture allows the production and propagation of genetically homogeneous, disease-free plant material. Interventions of biotechnological approaches for in vitro regeneration, mass micropropagation techniques and gene transfer studies in tree species have been encouraging.

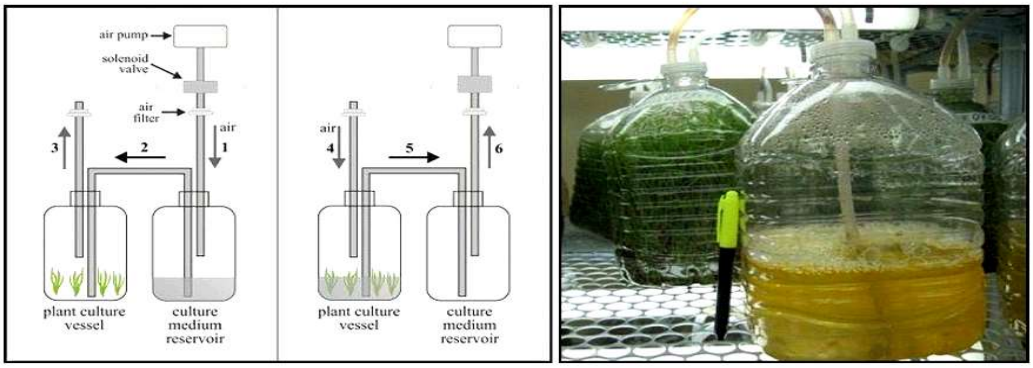


Fig.: Temporary immersion bioreactor (TIB): The transfer of the medium to the plant culture vessel occurs by a positive pressure of the air pump and the explants are temporarily immersed, after this, the air pump starts and the medium returns to the culture medium reservoir (A) scheme, (B) picture.

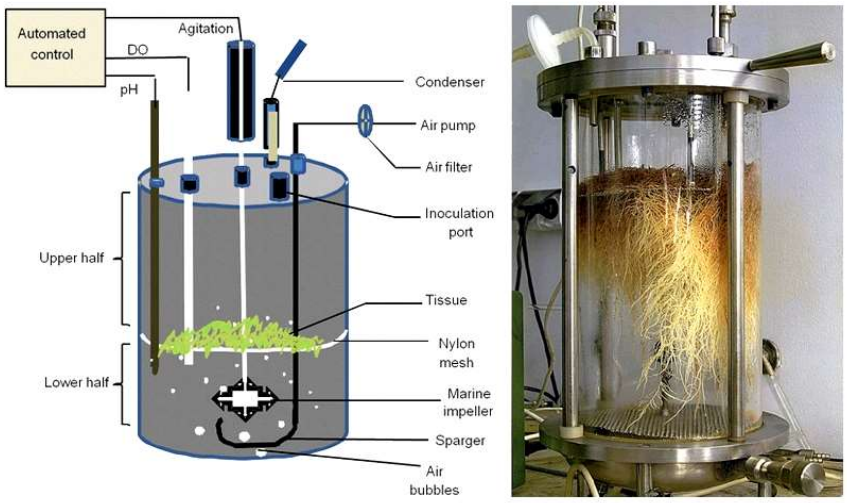


Fig.: Nutrient Mist Bioreactor: A) Hairy Root culture scheme, B) *Platycodon grandiflorum* hairy roots culture.

There are successful examples for cell and tissue culture of medicinal herbs to produce useful secondary metabolites:

1. *Digitalis purpurea* cell line was cultured in airlift bioreactors, and the yield of methyl isopropyl hydroxyl digitoxin reached 430 mg/L.
2. *Phalaenopsis aphrodita* protocorm like body culture in temporary immersion bioreactor for micro-propagation. 3.
3. *Panax ginseng* adventitious root culture in bollon type bubbling bioreactor at scale of 20 tons. All these achievement demonstrated the industrial potential of the application of bioreactor in producing secondary metabolites with high medicinal value.
4. In the propagation of pineapple (*Ananas comosus*), the multiplication rate in bioreactors was four times higher than that obtained in conventional systems. In banana (*Musa acuminata*) this multiplication rate was five times higher and in sugarcane (*Saccharum edule*), it was six.

17. Organogenesis: Developmental sequences, Mechanism of action of plant hormones, Control of in vitro organogenesis by cyclin-dependent kinase activity.

Concept:

Potentiality of a plant cell to regenerate the entire organisms in termed as totipetency coined by Gottlieb Haberlandt of Berlin in 1902. This potentiality has been exploited through the culture of protoplasts, cells, tissues and organs *in vitro*. In cultured material it has been possible to study such processes as differentiation of a parenchyma cell into tracheid (Cyto differentiation), organ formation (Organogenesis) and somatic embryo

(Somatic embryogenesis). Morphogenesis or the origin of form, can be examined through manipulation of physic-chemical (physical and chemical) environment of the cell by physiological, biochemical or even changes at molecular level. Besides the study of fundamental process of differentiation, the capacity of a cell to form organ or embryos can be exploited to regenerate plantlets for clonal propagation. The basic concept mentioned avobe led to the establishment of diverse multiplication technique. The genetic stability of yielded copies (Vegetative produced plantlets) is likely to vary according to the procedure chosen.

Definition

What is Organogenesis?

Organogenesis means the development of adventitious organs or primordial from undifferentiated cell mass in tissue culture by the process of differentiation.

Types of Organogenesis:

In plant tissue culture, Organogenesis is a process of differentiation by which plant organs like roots, shoot buds, bud-etc are promed from the unusual point of origin of an organized explants where an organized meristem is lacking.

Plant development through organogenesis can be achieved by two modes:

(i) Emergence of adventitious organ direating from explains.

(ii) Organogenesis through callus formation with *de novo* origin.

(i) Emergence of adventitious organs directly from explants:

Every cell of plant is derived from the zygote through mitotic division containing the full set of genome. The formation of adventitious buds depends on the reactivation of genes concerned with embryonic phase of development. The addition of growth regulators like auxin and Cytokinin in the medium is required to initiate shoot formation from

different kinds of tissue explants. Adventitious *in vitro* regeneration may give much higher rate of shoot production or meriste moicls (Fig.)

In a suitable medium supplemented with growth regulators the somatic tissues of angiosperms are capable of regenerating adventitious buds/shoots. These buds are formed directly formed directly from a plant organ or any piece of tissue without forming any callus. This type of organogenesis is mostly found in herbaceous plants. Promotion of bud formation by cytokinin occurs in several plant species, though the requirement of exogenous cytokinin and auxin in the process varies with the tissue system of different species. In some plant species, the adventitious buds are produced during vegetative production.

(ii) Organogenesis through callus formation with de novo origin:

In vitro organogenesis from the callus tissue derived from a small piece of plant tissue viz. isolated cells, isolated protoplasts, microspores etc. can be induced by transferring them to suitable medium or a sequence of media that promote proliferation of shoot/root both. The suitable medium is standardized by trial and error method.

When callus is cultured onto a medium favouring organo-genesis, shoot or root regeneration occurs. During shoot regeneration cluster of meristematic cells, called meristemoids, appear on the surface of the explants/callus. Meristemoids are considered to arise in the areas that accumulate starch, which is believed to serve as an energy source for shootbud differentiation. Among the different phytohormones GA_3, is thought to inhibit shoot regeneration by interfering with starch accumulation. Meristemoids may develop vascular elements inside them, while their outside may be made of cambium like cells e.g. in carrot suspension culture. Initially the meristemoids may either produce a root or shoot. In general roots originate from the inside of the meristemoids (endogenous origin), while the shoots develop from the outside (exogenous origin), but in some cases shoots originate endogenously. But in some species e.g. *Cenvolvulus* sp, shoot buds originate independent of vascular tissues, and buds often develop from such nodules that devoid of vascular tissues.

In several cases e.g. tobacco (*N. tabacum*) *Brassica napus* etc. shoot buds originate superficially from epidermal Cells of hypocotyls/Stem explants. When only epidermal cells are peeled and cultured, they usually do not survive. But when superficial peels of stem and leaf are cultured, epidermal cells divide and directly develop into shoot, root or even a flower, depending mainly on the region of the tobacco stem donating the explants and the chemical environment of *in vitro* environment. For example, explants from the basal part yield shoot buds, those from middle portion give rise to both shoot and floral buds, while peels from the upper part regenerate floral buds. Bud peels from floral branches form flower buds only when both IAA and Kinetin are used at 10^{-6}M and 2-3% sucrose is provided. When kinetin concentration was increased to 10^{-5}M only shoot buds were obtained. Similarly, by an increasing IAA to 10^{-5}M and reducing kinetin to 10^{-7}M, only root regeneration could be induced.

The various events occurring during shoot regeneration may be divided into the following two pathways (1) Shoot induction and (2) Shoot differentiation and development. Induction to develop meristemoid consists of molecular events following gene expression which irreversible commit cells of the explants to proceed on a developmental path. The development and differentiation of Callus consists of repeated cell divisions leading to the specific pattern and organ formation. In case of *Brassica* juncea, only roots regenerate when mature cotyledons are cultured on GR-free MS medium. But when the cytoledons are cultured on MS medium containing 5×10^{-6}M BAP for 11 days, shoot buds regenerate from them irrespective of their subsequent culture on GR free or BAP containing medium as follows (Figure):

GR free MS medium

Mature cytoledons ----------→ Root regeneration.

Mature cytoledons ----------→ Transferred ----------→ Multiple shoot

(MS + 5×10^{-6} BAP 11 days to GR free regeneration.

MS medium

MS +

Mature cytoledons ----------→ Multiple shoot Regeneration.

5×10^{-6} MBAP

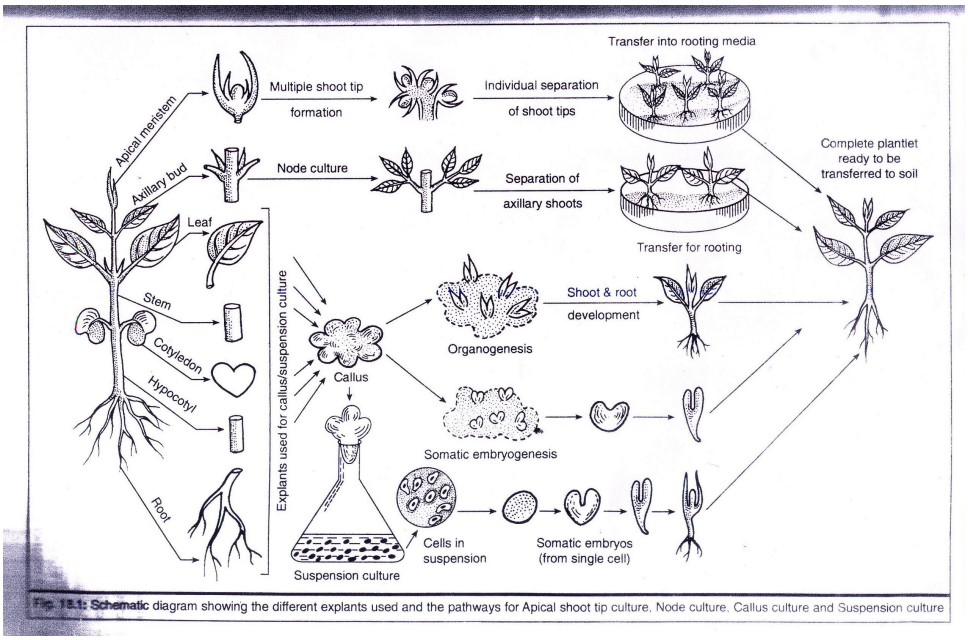


Fig.: Schematic representation of regeneration events in mature Cotyledons of B. juncea. Cultured of excised Cotyledons on GR-free MS medium for more than 7 days abolishes their ability to produce shoots in response to BAP.

The process of organogenesis in response to hormonal treatments is a multistep process. The first phase consists of acquiring competence by the cells to respond to the hormonal treatments. In the second phase, the competent cells are induced to form a specific region. Once induced to form a specific organ the cells become invariably committed to form the organ even if the inductive hormonal treatment is withdrawn.

During the past two decades, *Arabidopsis thaliana* emerged as a model system to press of organogenesis in tissue culture. Root segments of this plant species have been extensively used to understand mechanism of ontogeny and molecular basis of information and a lot of new information has been generated.

A two step process of shoot differentiation in root explants cultured of *A thaliana* has been developed. In the first step 5mm root segments from 7 day old aseptic seedlings are incubated in an auxin rich callus induction medium (CIM; B_5+2.2 μM 2,4-D + 0.2 μM 2 iP). On this medium root cells proliferate and form outgrowths that have referred to as callus. During this phase, the cells are said to acquire competence to form shoots after 2-3 days. After 4-5 days on CIM, the root explants are transferred to shoot induction medium (SIM; B_5+5μM 2 iP+0.9 μM IAA) or to root induction medium (RIM; B_5+0.9 μM IAA). On SIM the so called callus forms shoots.

It has been demonstrated experimentally that Organogenic differentiation is controlled by several genes.

Table: Some genes associated with shoot differentiation in tissue cultures of *A. thaliana*. (*after* Phillips 2004)

Gene	**Putative Function**	**Reference**
CYCD3	Involved in acquisition of competence of organogenesis.	Sugiyama 1999 Fletcher 2002
SRD3	Competence for shoot organogenesis	Sugiyama 1999, 2000
SRD1. *SRD2*	Competence for redifferentiation of shoots	Sugiyama 1999, 2000
ESRI	Enhances shoot regeneration, vegetative-to-Organogenic transition	Zuo et el 2002
CREI	Cytokinin receptor	Zuo et al 2002
CKII	Cytokinin perception	Zuo et al 2002
CLV *WUS*	Preserve stem cell identity in shoot apical meristem	Fletcher 2002
KNI *STM*	Initiate ectopic shoot meristems, shoot apical meriatem function	Fletcher 2002
SHO MGO	Modifiers of the shoot apical meristem involved in leaf	Fletcher 2002

	founder cell recruitment lateral organ primordial	
CUCI	Shoot meristem differentiation	Daimon et al 2003

During organogenesis some genes are over expressed to promote shoot differentiation from *Arabidopsis* explants).

Factors affecting Organogenesis:

Regeneration via denovo organogenesis is a complex multi staged phenomenon. There are many factors, which influence the process. The major factors affecting the process of regeneration through organogenesis are presented below:

- **Source of explants:**

The factors which influence the response of the inoculums in the culture are:

a) The organ that is to be served as tissue source.
b) The physiological and ontogenetic age of the organ.
c) The season in which explants is obtained.
d) The size of the explants.
e) The overall quality of the plant from which explants are taken. Therefore proper selection of explants is made on the basis of experiments with different explants.

- **Role of genotype:**

Significant genotypic influence on shoot regeneration response has been observed.

Among the three monogenomic species of *Brassica, B. oleracea* is most regenerative and *B. campestris* the least, *B. nigra* being intermediate. *B. napus* (amphidiploids of *B. oleracea* and *B. campestris* is highly regerative but less than *B. oleracea* because of the presence of *B. Campetrsis* genome. Several workers have reported that regeneration in Wheat is genetically controlled. (Galiba et al, 1986). According to Galiba et al. the genes controlling regeneration are present on 7B, 7D, and 1D chromosomes.

❖ **Electrical and Ultrasound stimulation of Shoot differentiation:**

Application of very weak (0.1 - 50μA) alternating electric current of low frequency (50 Hz) for long periods (22 – 30 days) in such a way that the callus was made the negative pole and the medium the positive pole, promoted callus growth in tobacco and oil palm respectively by 70 and 50% respectively. Electric stimulation occurred only in the presence of an auxin. In tobacco, microampere current to the callus on shoot differentiation medium caused upto 300 fold increase in the rate of shoot bud differentiation.

A brief sonication treatment of the explants is also reported to stimulate shoot bud differentiation.

❖ **Plant Hormones:**

Of the many factors that influence Organogenesis *in vitro*, the most important single factor seems to be the phytohormones. In their classical experiments with cultured pith tissue of tobacco Skoog and Miller (1951) demonstrated that different types of organogenesis which can be achieved by varying the concentrations of auxin and cytokinin in the culture medium when the concentration of cytokinin are high relative to auxin. Shoot are induced; when the concentrations of cytokinin are low relative to auxins, roots are induced; and at intermediate concentrations the tissue grow as unoganised callus. This basic concept has been used to regenerate a wide variety of dictyledonous plants. In general monocotyledonous plants do not show a pronounced response to cytokinins and need high concentration of auxins such as 2, 4-D to ibtain changes in the development of cultured tissue. Other plant hormones, particularly abscisic acid and gibberellins have some dramatic effect on *in vitro* organogenesis. Very little information is known about how phyto hormones awake a particular pattern of organogenesis. Various hypotheses have been put forward to explain mode of action of phytohormones on organogenesis. The outline of two hypotheses is outlined below. In the first hypothesis, phytohormones are regarded as primary morphogens. According to this hypothesis, the responding cell or groups of cells are competent to react to the hormones but are not commited to a particular development fate. The alternative

hypothesis is that hormone responsive cells are already determined and that the hormones stimulate the expression of the committed state.

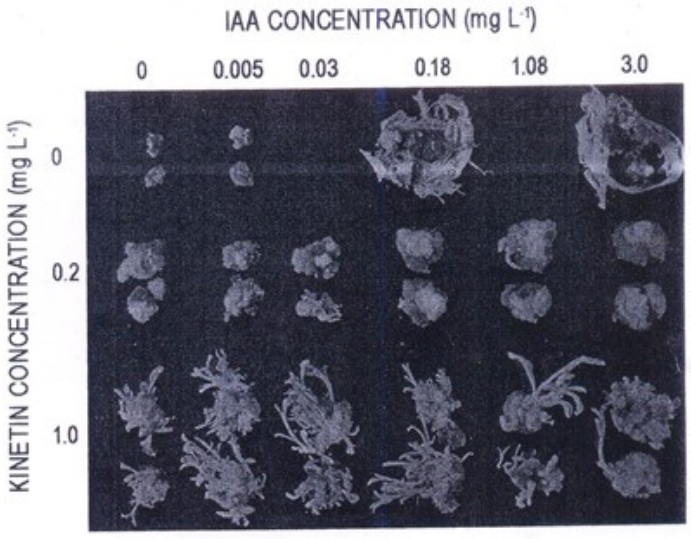


Fig. 6.2 Chemical control of organogenesis in tobacco (Wisconsin No. 38). Effect of increasing concentration of IAA at different levels of kinetin, in the presence of casein hydrolysate (3 mg L^{-1}), on callus growth and differentiation of shoot buds and roots on White's medium. All samples are from 62-days-old cultures. Note root formation in the presence of 0.18–3.0 mg L^{-1} IAA and shoot bud formation in the presence of 1.0 mg L^{-1} kinetin, particularly in combination with IAA in the range of 0.005–0.18 mg L^{-1}. (*after* Skoog and Miller 1957)

Importance of Organogenesis:

- ✓ The regeneration of plant from cell and callus culture via organogenesis bears a wide field of application in plant biology which are as follows:
- ✓ With the discovery of cellular totipotency, a large number of plant species including medicinal plants, timber yielding plants and horticultural plants have been successfully regenerated from callus via organogenesis.
- ✓ The production of large numbers of haploids from microspores and the possibility of raising triploids from endosperm culture plays potential role of plant regeneration via organogenesis in genetics and plant breeding.
- ✓ Improvement of crop through somatic cell hybridization by the fusion of isolated protoplasts, the transfer of foreign genetic material in protoplasts is possible only if somatic hybrid and modified cells are able to give rise a whole plant

through organogenesis of proto clones and genetically transformed callus tissues.

- ✓ Somaclonal variation is a useful source of variability only if plants can be regenerated from the callus culture via organogenesis.

18. Somatic Embryogenesis: Gene expression and signal transduction during embryogeneis-Role of SERK and LEC genes, Brassinosteroid (BR) signaling, Artificial seeds.

Concept:

Somatic embryogenesis is a process by which a single cell or a small group of cells follow a developmental pathway which leads to develop a nonzygotic embryo which leads to develop a complete plant. These non-zygotic embryos may originate directly from androgenetic embryos (from the male gametophyte) or from the any organs of the somatic cells. Generally somatic embryos originated from the somatic tissues in *in vitro* condition.

Types:

The initiation and development of embryos from somatic tissues in plant system was first reported by J. Reinert (1958–59) followed by F.C. Steward, Mo.O. Mapes and K. Mears (1958) from the freely suspended cells of carrot and emphasized the importance of coconut milk for *in vitro* somatic embryogenesis. Since then somatic embryogenesis has been reported in more than 500 species of monocots and dicots.

Kohlenbech (1978) classified embryogenesis in following manner:

1. Zygotic Embryogenesis: These are formed by fertilized egg or the Zygote.

2. Somatic Embryogenesis: These are formed by cells other than egg.
    a) Somatic embryogenesis: These are formed formed by the Somatic cells (except zygote).
    b) Parthenogenetic Embryogenesis: These are formed by unfertilized egg.
    c) Androgenetic embryogenesis: These are formed by the male gametophyte i.e. microspore or pollen grains.

Sharp et al (1980) described mainly routes of somatic embryogenesis.

A. Direct Embryogenesis:

The embryos initiate directly from the explants without callus formation and here some of the cells, which are called 'Pre embryonic determined cells (PEDC). They initiate embryonic development; only those cells need to be released. Such cells are found mostly in embryonic tissues, certain tissues of young grown plants, hypocotyls, nucellus, embryo sac etc.

B. Indirect Embryogenesis:

Here, the embryos are developed through cell proliferation i.e. callus formation. The cells from which embryos arise are called as 'Induced embryogenic determined cells' (IEDC). Here growth regulators with specific cultural conditions are required for initiation of callus and then redetermination of those cells into embryo development. Somatic embryos arise from single cells located within the cluster of meristematic cells either in callus mass or in suspension. Such cells develop into proambryos with polarity following a pattern that tends to mimic the general pattern associated with the development of *in vitro* embryos in the ovule. Proembryo initials may be single cells or multicellular groups. When conditions are suitable these embryos germinate to produce plantlets.

Factors affecting somatic Embryogenesis:

The following factors are responsible for the induction of somatic embryogenesis.

- Type of explants.
- Role of genotype.
- Role of growth regulators and nutrients
- Selective subculture.
- Electrical stimulation.

Role of gene expression and signal molecular during *somatic embryogenesis*:

1. Schemidt (1995) identified several genes from carrot (*Daucus carota*) cell suspension cultures. One of these genes termed "Somatic embryogenesis Receptor Kinase (DcSERK), is a considered as a marker of single competent cells because of tight correlation between the ability of single cells to develop into an embryo and DCSERK expression. Expression of this gene could be detected upto the 100 celled early globular stage of embryogenesis. A similar gene (AtSERK1) has been isolated from *Arabidopsis thalinona* over expression of this gene in the seedling of *A. thaliana* resulted in 3 to 4-fold increase in the efficiency of embryogenesis. In multiple signaling pathways during transition of somatic cell to embryonic cell SERK act as co-receptor via their physical interaction with specific ligard binding Receptor like Proteix llinases (RLKs).
2. A second approach to identify genes that are activated during somatic embryogenesis is the use of loss-of-function mutations as in *Arabidopsis*. Leafy cotyledon (LEC) 1 and 2 are two genes identified through this approach. LEC 1 and LEC 2 cause defects in embryo maturation and partially transform cotyledon into leaves. Ecotopic expression of both LEC1 and LEC2 induces the formation of embryos in vegetative tissue. LEC2 induces somatic embryogenesis in vegetative tissues by enhancing auxin through the direct action of YUCL, an auxin biosynthetic gene. Another pathway depicted for LEC2 action through AGAMOUS – like 15 (AGL15) which unduces enzyme that inactivates GA and thus increases the frequency of somatic embryo formation.

3. The third approach to identify the changes in gene expression that occur during somatic embryogenesis and to detach several molecular markers that play key role in signaling for embryo specific genes which were cloned and analyzed.
    - In *Cichorium* callose deposition in the cell wall and presence of vacuoler Ca2+ are the first signals that allow the recognition of embryogenic cells.
    - Cell wall associated proteins are known to act as short distance signal molecules in development. Some of these, such as members of the group of oligosaccharon participate in regulation of embryogenesis e.g. arabinogalacton protein (AGP).
    - In proliferating cell cultures of carrot two callus specific proteins (C1 and C2) are synthesized.
    - On transfer to an embryogenic specific medium, the protein profile of callus changes and two embryo specific proteins (E1 and E2 appeal). So far three types of extracellular proteins (EPI, EP2 and EP3) have been isolated.
    - Based on 2D gel electrophoresis, Imin et al (2005) identified 54 protein spots where significant changes had occurred during somatic embryogenesis of *Medicago truncatula*.

Applications:

- ✓ Large scale propagation compared to zygotic embryos.
- ✓ Use of bioreactor for commercial propagation i.e. industries.
- ✓ Useful for Embryo cloning.
- ✓ Useful for somatic embryos in gene transfer.
- ✓ Synthetic seed production.
- ✓ Useful for mutagenic studies and mutant production.
- ✓ Useful for pathogen – free plant production.
- ✓ A good source of protoplast culture.
- ✓ Conservation of genetic resources.

19. Somatic Hybridization: Protoplast isolation technique, protoplast fusion, selection of hybrid cells- Homokaryons, Heterokaryons, Symmetric and asymmetric hybrids, fate of plasmagenes, Cybrids.

Protoplast culture has gained the status of a powerful tool for crop improvement, cell modification and direct bridge between tissue culture and genetic engineering. This novel technique has become an ideal tool at the hands of breeding researcher for the production of new type of somatic hybrids. Besides, plant protoplast culture has also been implicated in basic research, particularly in the field of biochemistry for investigation of metabolic pathways, virology for the mode of reproduction, replication and plant physiogists for the investigation of physiological constraints.

Protoplast is a cell without cell wall. According to Torrey Landgreen (1977), protoplast of plant can be interpreted as cell with their cell wall stripped off and isolated from the proximity of their neighbouring cell. This naked cell can be regenerated into whole plant on comply with the concept of totipotency.

Sources of plant material:

Nature of genotype can play a crucial role on the competence of protoplast for colony formation and regeneration. Besides the genotypes, the source of explants and its physiological conditions are the most important factors during isolation and culture of protoplast. In addition, plants grown in the field or greenhouse conditions are readily usable for protoplast isolation. The other feasibility is the excision of explants from *in vitro* grown plants (axenic cluture).

Isolation of plant protoplast:

Generally, isolation of protoplast is categorized into (a) mechanical and (b) enzymatic.

(a) Mechanical:

This method is crude and oldest method for protoplast isolation. It is only an historical perspective. It involves chopping and plasmolysis of tissues. Time consuming and low yield of protoplast are the main constraints in this method.

(b) Enzymatic:

The unprecedented success in protoplast culture was accomplished by employing several enzymes released commercially. The enzymes for protoplast isolation are available in different commercial names. These enzymes are pectinase, cellulose, macerozyme and hemi-cellulase. Pectinase dissolves middle lamella embedded with pectin component. The cellulose and hemi-cellulase degrade the cellulose component of the cell wall, and consequently releases protoplast. The most widely used commercial enzymes are *onozuka* R-10, and macerozyme R-10 *pectolyase* Y-23.

Isolation techniques:

The techniques involved in the isolation of leaf mesophyll protoplast are as follows –

1. Fully expanded leaves obtained from 3 to 10 week old plants are subjected to sterilization by dipping in 70% alcohol for 30–60 s followed by treating them for 20–30 min in 2% sodium hypochloride or calcium hypochloride. The leaves are then rinsed in sterile water in the final step to remove all traces of sterilants.
2. The lower epidermins of leaves are carefully peeled off and then cut into small pieces. The lower epidermis of the peeled segments is placed in a filter sterilized enzyme mixture containing 0.5% macerozyme, 2% cellulose in 13% sorbitol or mannitol. The pH is set to 5.4. the enzyme mixture is incubated for 15-18 h at 26°C.
3. The enzyme mixture is then filtered through nylon mesh (50-100 μ) to remove leaf debris and transferred to screw capped tubes and centrifuged at (100×g) for 1 min and the process is repeated three times.

4. Protoplast are placed in 20% sucrose for final cleaning before washed with sorbitol solution (0.1M) at (200×g) for 1 min. the floating protoplasts are then harvested by gently pipetting out with Pasteur pipette.

Alternatively, a two step method is also followed for the isolation of protoplast as follows.

(a) The peeled segments are placed in enzyme mixture containing macerozyme (0.5%), potassium dextran sulfate (0.3%) in 13% mannitol. After adjusting pH to 5.8, incubation is carried out in water bath at 25°C. enzyme mixture is then replaced with fresh enzyme after 15 min of incubation. These are then filtered through a nylon mesh, centrifuged at 100×g for 1 min. pelleted cells are then washed three times with mannitol (13%) to procure isolated cells.

(b) The cells obtained are mixed with second set of enzyme mixture containing 2% cellulose in 13% mannitol at pH 5.4. The incubation is carried out for 90 min at 30°C. centrifuge the suspension at 100 g for 1 min. pelleted protoplasts are subjected to final cleaning by placing in 2% sucrose and harvested gently by Pasture pipette.

Protoplast can be isolated from cells and cell suspension, pollen grains and pollen mother cells. The filtered cell suspension is directly treated with 2-4% onozuka cellulose in 0.7M mannitol for 5-6h at 30-32°C in water bath. Isolation of protoplast from mature pollen grain is comparatively a difficult task due to the nature of pollen wall, which comprises mainly a durable sporopollenin. This polymer can be dissolved by treatment with KOH and certain organic bases. The pollen grain is squeezed out from the anther and subjected to enzyme treatment. Additional enzymes are required for protoplast isolation. The enzyme mixture contains cellulose 2%, macerozyme 1%, and helicase 0.5%, to release protoplast.

Protoplast culture:

- Agar Plating Method
- Microchamber

- Nurse Culture
- Immobilization of Protoplast
- Nutrient requirement
- Growth Regulators
- Cultural Conditions
- Cell wall regeneration
- Regeneration

Protoplast fusion and somatic hybridization:

Somatic hybridization or parasexual hybridization is a process in which hybrids are obtained through the fusion of protoplasts belonging to two distinct plant species. Somatic hybrids are specifically implicated in the improvement of vegetative propagated crop plants such as potato cassava and banana (Table 2). Greater success in the production of somatic hybrids involves effective fusion of protoplast.

Protoplast fusion:

Fusion of protoplasts can be accomplished by various distinct methods like chemical and electrofusion (Fig. 1). Earliest method known as *mechanical fusion*, where protoplasts are forced to fuse under applied pressure within the pipette. Protoplast in suspension can undergo spontaneous fusion in cultural conditions.

Chemical Fusion

Chemicals such as sodium nitrate, polyethylene glycol and calcium ion at high pH are used for protoplasts fusion with different degrees of success. Sodium nitrate (5%) in 10% sucrose solution is mixed with protoplast suspension, incubated in test tube and maintained at 35°C in water bath for 10 min. this would allow an efficient fusion of protoplast. Calcium ion could play a significant role in inducing protoplast fusion. Addition of calcium ion at high pH (10.5), alone or in combination with other chemical allows more efficient fusion. The process involves centrifugation of protoplast for 3 min

at 100 g in presence of solution containing 0.5M $Cacl_2$ in 0.4M mannitol at pH0.5. Following incubation in water bath for 40 min at 37°C, fusion of protoplast is accomplished. Since calcium ion acts as action, it can alter Zeta potential of charged ratio on the protoplast membrane. Treating protoplast with calcium ion significantly affects *p*-potential of protoplast surface, i.e., from −10 mV to −50 mV. Besides, cell to cell sontact is aided by calcium ion.

Table 1: Recent achiements in regeneration of plants from cultured protoplast

Legumes	**Plant source**
Glycine max	Immature cotyledon
Pisum sativum	Immature cotyledon
Cereals	
Oryza sativa	Embryonic cell suspension (ECS)
Zea mays	ECS
Triticum aestivum	ECS
Vegetables	
Capsicum annum	Leaf
Brassica oleracea	Hypocotyls
Cucumis sativum	Cotyledon, leaf
Oil crops	
Brassica napus	Leaf
Helianthus sp.	Hypocotyls
Woody plants	
Picea glauca	ECS
Coffea camphora	Somatic embryo
Populous sp.	Leaf
Ornamental	**Explants source**
Rose	ECS, root
Chrysanthemum	Leaf

Pelargonium sp.	Cell suspension
Senecio	Leaf

Table 2: Some examples of inter specific and generic fusion

Solanum brevides × *S. tuberosum*
Oat × Maize
Brassica sinensis × *B. oleracea*
Torrential fourneri × *T. bailloni*
Brassica oleracea × *B. campestris*
Nicotiana tobaccum × *N. glatinosa*
Datura innoxia × *Atropa belladonna*
D. innosia × *D. candida*
Arabidopsis thaliana × *Brassica campestris*
Petunia hybrida × *Vicia faba*
Medicago sativa × *M. falcate*
Glycine × *N. glauca*
Hordeum vulgare × *N. tobaccum*
Daucus carrota × *Petroselium hortens*

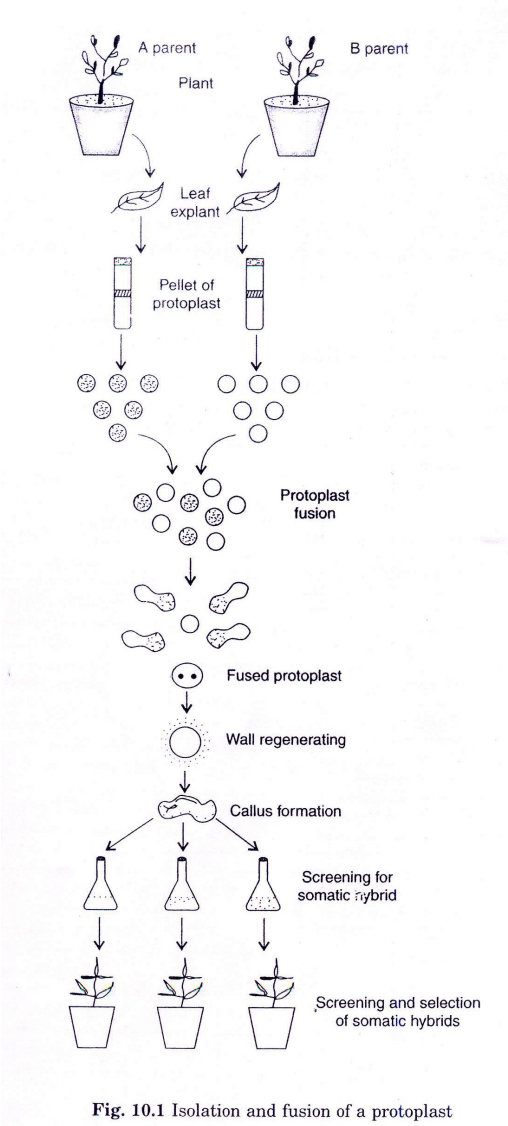


Fig. 10.1 Isolation and fusion of a protoplast

Fig.: Isolation and fusion of a protoplast.

Polyethylene Glycol Mediated Fusion

Polyethylene glycol (PEG) can initiate tight agglutination of protoplast. The protoplast in nutrient medium is mixed with 1 ml of PEG solution and is shaked for 5 s, resulting in agglutination followed by fusion of protoplasts. PEG at the concentration between 36 and 56% is employed for protoplast fusion. Combined role of basic solution (pH 10.5)

containing Ca, 0.05M CaCl2 and PEG treatment improves the efficiency of protoplast fusion. More frequently protoplasts are treated with PEG solution after settlement on the glass cover slips. It has been suggested that combined use of PEG and Ca acts as a bridge between the two protoplasts and it is because of protoplasts' slight negative polarity.

Electrofusion of Protoplast

Electrofusion is extremely a rapid method for protoplast fusion. When protoplasts are exposed to electric field, membrane fusion is achieved by close membrane contact, treatment membrane breakdown and recoganization of membrane contact. Pioneer work on electrofusion has been carried out by Zimmerman. Electrofusion is a biophysical process that involves the application of alternative current (AC) and direct current (DC). In the first step, protoplasts are exposed to a high frequency of AC field strength (0.5 to 1.5 MHz) that generates dipoles through a condition known as *dielectrophoresis*. Due to dielectrophoresis, protoplast moves in the increasing field strength and the mobilization of charge takes place within the protoplasts. As a consequence, protoplast aggregate or bound close together. In the following step, a short pulse of direct current (DC) of sufficient voltage is able membrane breakdown. Once DC is applied, cell membrane breaks down at the point of contact between the cells. This happens because the cell interior acts as a better conductor than surrounding medium. As a consequence, most of the current moves through the cells rather than through the medium. Moving of DC pulse at the point of cell contact leads to cell fusion rather than cell lysis. Duration and nature of DC pulse is crucial in achieving protoplast fusion. The square wave DC pulse, with a rise and decay time of less 2 μs is sufficient to disrupt the membrane junction. Generally a single square wave pulse of 600 – 700 V/cm) will cause fusion. Long pulse duration of 100 μs and high voltage will cause cell lysis (Figure.).

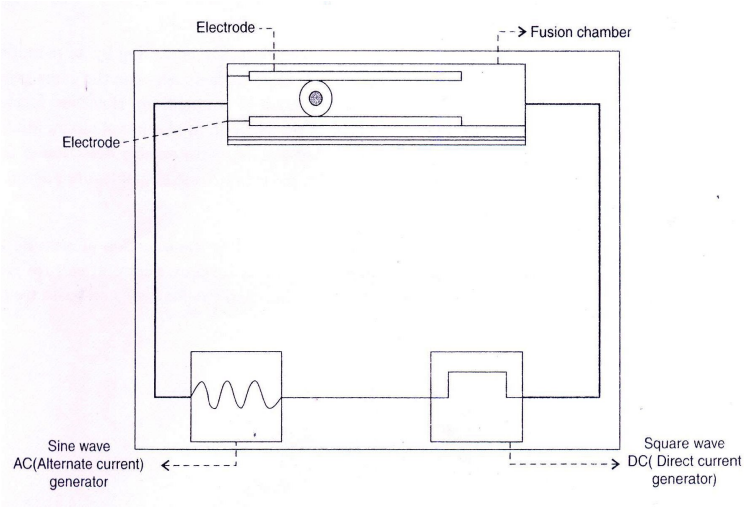


Fig.: Electrofusion of protoplast.

Screening of somatic hybrids:

The recovery of somatic hybrids in the whole process is extremely a difficult task and a huge exercise. Therefore, several screening strategies are to be enforced for the selection of ideal somatic hybrids. More than 40-50% of the protoplasts undergo fusion once the process begins under suitable conditions. The proportion of heterokaryons and survival of the hybrid cells when present, are at lowest frequency within the population. Moreover, survival rate of plant cell at lowest plating density is extremely low. Hence, all these contribute to slim chances of hybrid recovery. Therefore, an efficient selection system is required to track somatic hybrids.

- **Microscopic Visual Selection**

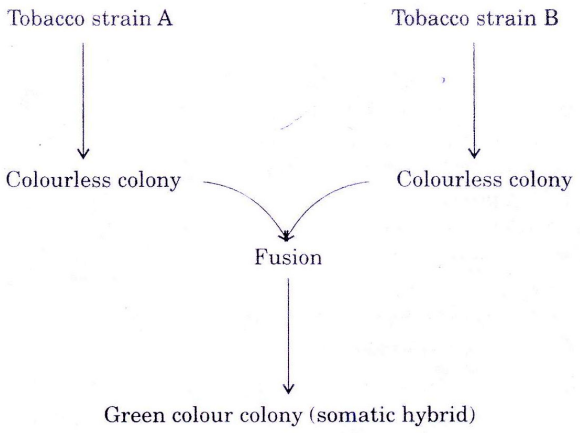
It is based on the fusion between coloured and colourless protoplast. Microscopic observation of heterokaryons formed due to complete integration of structural characters of both parental protoplasts and subsequent culture under non-selection conditions and the development of heterokaryons facilitates the selection of potential hybrid cell line.

- **Auxin Autonomy**

In this selection process, protoplasts are subjected to screening by its potential to grow into cell on the medium devoid of auxin. Fusion of protoplasts between the same genotype and unfused protoplast fails to grow in absence of auxin in the medium. However, fusion between the two target genotype potentiates to grow on the medium in absence of auxin. Mixture of two genetic materials allows hybrid cell lines to become auxin autonomy. Selection of the hybrids based on these approaches has been successful in certain members of leguminaceae.

- **Chlorophyll Complementation**

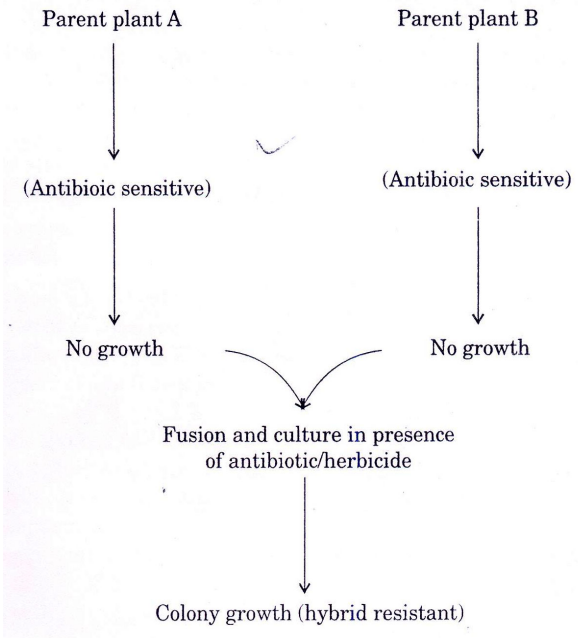
This approach has been successfully implicated in the selection of somatic hybrids in light sensitive tobacco varieties. Development of green colour colony in culture medium ensures hybrids. This was accomplished by fusion between two homozygous recessive albino mutants of tobacco.



- **Biochemical Selection**

This is based on conferring resistance due to dominant character against certain chemicals like antibiotics, herbicides, etc. These are being considered as resistant markers markers in the selection of somatic hybrids. For example, protoplast obtained from each parent, and grown separately in the medium contains antibiotics or herbicides, each parental line exhibiting sensitivity. However, protoplast fusion between two parental types when cultured in the medium containing these chemicals

exhibit resistance. The sensitivity trait of each parent will be dominated by resistant trait and will grow on the medium containing antibiotics or herbicides.



- **Biochemical Verification of Somatic Hybrid**

Isoenzymes are multiple molecular forms of the same enzyme and execute the same function. Depending on the genotype, isoenzyme acts as specific blue print and exhibit specific banding pattern with respect to their complementation of each parental type. In biochemical analysis, electrophoretic banding of isoenzyme can be analyzed for the verification of hybridity. Different nature of protoplasts (fused, unfused) are subjected for electrophoretic separation of isoenzyme bands on acrylamide gel. Somatic hybrids display characteristic banding pattern of both the parents. Comparative enzyme profiles of each parental line and hybrid can be seen. The isoenzymes, which have been extensively used in biochemical analyses are esterases, peroxidases, amylases and alcohol dehydrogenases.

- **Verification by Molecular Screening**

Several molecular techniques like RAPD, RFLP and availability of microsatellites are employed in the screening as well as verification of specific somatic hybrids. Restriction

digestion of DNA obtained from unfused and fused protoplast exhibit specific banding profiles and ensures confirmation of hybrids. Restriction digestion of organelle DNA can boost up effective screening process of hybrid lines and verification of somatic hybrid plant in germplasm. Verification of somatic hybrid, *Nicotiana glauca*, was successfully carried out by assessing restriction fragments of nuclear DNA, which encodes ribosomal RNA. Recently, availability of specific primers for somatic hybrid has been utilized for hybrid identification through PCR technology.

- **Chromosomal Analysis**

Chromosome count can be adapted for the identification of somatic hybrid cell lines. Somatic hybrid contains sum of chromosomes in the protoplast of two parental types. Besides, variation in chromosome number is common in hybrids. Genetic variation due to structural alteration in chromosome might help in the identification of hybrids. Polyploidy conditions have been witnessed in the protoplast culture, which involves the production of inter specific and inter generic somatic hybrids. On several occasions, variation in the chromosome number is mainly due to multiple fusion of protoplast. In addition, unequal rate of DNA replication in the hybridoma cells results in asymmetric hybrids and consequently exhibit chromosomal variations.

Cybrids:

These are cytoplasmic hybrids. In cybrids, nucleus of one parent and cytoplasm of both the parents constitute cytoplasmic hybrids. More precisely, cybrids can be accomplished by fusing protoplast derived from one of the parent plants with enucleated protoplast of other. Cybrids can also be obtained through various other means like chemical treatment of protoplast to induce metabolic inactivation or application of X-ray or gamma irradiation to one of the parental types of protoplast resulting in inactivation or non-dividing status of protoplast. Percoll gradient technique is widely used for the production of enucleated protoplasts by high speed centrifugation upto 35,000 × g for 50–90 min using percoll (10–40%) density gradient. The isolated enucleated protoplast is then used to fuse with other protoplast to accomplish cybrids.

In another approach, when protoplasts are fused, their nucleus may undergo fusion to produce true hybrid or remain separate without fusion. If elimination of one of the nuclei occurs with the retention of other in a mixed cytoplasm, the resulting product is a cybrid.

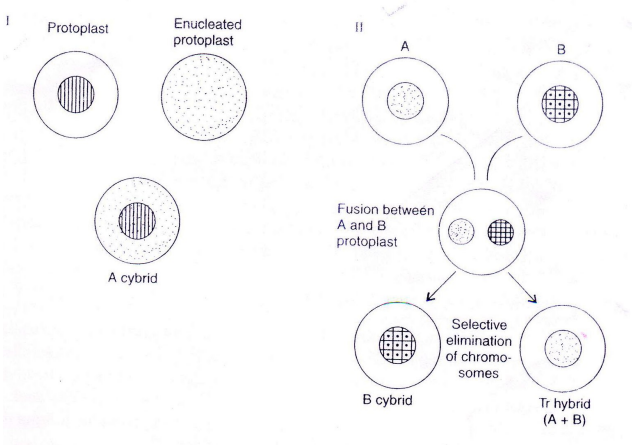


Fig.: Process of Cybrid Production.

Significance of protoplast culture:

1. Cell Modification by DNA Uptake
2. Demonstration of Genetic Complementation
3. Protoplast in Studying Nitrogen Fixation
4. Somatic Hybrid Production
5. Protoplast Culture in Genetic Engineering

Concluding Remarks:

Plant protoplast culture has gained the status of a powerful tool for crop improvement and cell modification. Isolation of protoplast has been successfully accomplished by employing enzymes like cellulose, pectinase and macerozymes. Protoplast can be isolated from any tissue of the plant. However, leaf organ is the choice of the explants for protoplast isolation in most of the species reported so far. Under *in vitro* conditions,

protoplast can be cultured by various methods such as microchamber, nurse culture, immobilization and agar plating techniques. Viability of protoplasts can be assessed by fluorecine diacetate (FDA). Protoplast in culture undergoes divisions, regenerates its own cell wall and transforms into the whole plant.

Fusion of protoplast for the production of somatic hybrids can be achieved by certain chemofusogens such as sodium nitrate, calcium at high pH and polyethylene glycol. However, efficient fusion has been accomplished by subjecting protoplast to electric field. Generally, applying alternative current followed by direct current of appropriate voltage could facilitate fusion of protoplast at a much faster rate. Fusion of protoplast generates different fusion products.hence, screening and verification of suitable hybridity is highly indispensable for the production of successful somatic hybrids. Several screening processes such as microscopic selection, auxin autonomy and chlorophyll complementation are found to be useful tools. However, certain novel techniques like RFLP and RAPD has been considered as the most powerful tool in the screening of somatic hybrids.

Utility of protoplast culture has been positively implicated in several diversified fields for the improvement of crops. Cell modification by DNA uptake, studying nitrogen fixation in protoplast and somatic hybrid production are some of the significant applications of protoplast culture. Transfer of male sterile character is possible by the production of cybrids.

20. In vitro Genetic Variation: Somaclonal and gametoclonal variation, Isolation and characterization of somaclones, Molecular basis of somaclonal variation, Advantages of somaclonal variation over induced mutations, Applications in crop improvement, In vitro mutagenesis and mutant selection.

Concept:

Somaclonal and Gametoclonal variation; Factors contributing to occurrence of somaclonal variation; identification selection and screening of variants. Advantages and application of somaclonal variants in crop improvement.

The growth of plant cells in vitro and their regeneration into whole plants is an asexual process, involving only mitotic division of the cell. Therefore it was expected that the process will produce genetically uniform plants or clonal multiplication is possible through callus regeneration. This expectation has formed the basis to justify genetic fidelity in the micropropagated plant. The occurrence of uncontrolled variation during organogenesis and embryogenesis also can be judged using proper assessment technique.

Micropropagation has been established early more than 40 years back. Interestingly for the commercialization of micropropagated crop when the chromosome numbers were assessed it has been seen that some of the micropropagated and regenerated plants show chromosomal variation and phenotypic variation. As changes in agronomic traits were observed in horticultural and crop plants, interest in exploiting this variation through plant tissue culture from the improvement of the crop plants increased. In 1981, Larkin and Scowcroft, named the phenomenon of variation found in plants regenerated from cell cultures as “Somaclonal variation”. The term is now universally adopted for variation found or created through the use of somatic cells and similar terms like “protoclonal” and “gametoclonal” are used for describing variation from protoplasts and anther culture respectively.

Factors contributing to occurrence of somaclonal variation:

Plant cells are totipotent. It is possible to obtain regeneration from single cells or protoplasts. But in this process, a cell divides and redivides several hundred times to produce callus and subsequently organs. During this process of division several hundred times to produce callus and subsequently organs. During the process of division, several internal and external factors influence the cell. This leads to creation of chromosomal variation in cell cultures, called somaclonal variation. This is the reason that, direct shoot formation from explants through existing meristems and meristem culture are used for clonal propagation and considered that no genetic variation occurs in such system. In comparison to this, callus and cell cultures, organogenesis from cultures and indirect somatic embryogenesis are sources of somaclonal variations. Somaclonal variation is common in long term cultures.

The following are the causes of somaclonal variations.

- **Role of Pre existing variability in the source tissue:**

Heterozygosity, particularly in cross-pollinated crops, is common in field grown population of plants. This heterozygosity is expressed in terms of physiological (alkaloid) and morphological characters (leaf shape, size, plant height etc. gross changes in the genome including endopolyploidy, polyteng, and amplification or diminuation of DNA sequences can occur during somatic differentiation in normal plant growth and development. Variation in alkaloid levels has been observed in field grown plant population of Catharanthus roseus. Therefore, when cultures are raised from different explants they have inherent variation and is expressed as somaclonal variation in culture.

In case of tissue which is obtained from single source, variation is produced during *in vitro* culture involving repeated cell divisions.

- **Role of genotype:**

Variation observed in one cultivar may not be present in other cultivar. Therefore, it is unpredictable to obtain similar variation in related cultivars and the same technique in all the cultivar for their improvement. Secondly, when variation is observed in small

experimental material, it is again not certain that the same variation will be reproduced at large scale variation. Due to these reasons, it is difficult to exploit the variation for crop varieties.

- **Role of Plant growth regulator:**

Selection of specific hormones at higher concentration can induce genetic variation. Auxin such as 2, 4-D is most effective PGR for inducing somaclonal variation. In several plants, specific concentration of hormones decides the nature of variation e.g. in barley plant, variation occurs only at 18μm of 2, 4-D for leaf shape and albino characters. The effect of 2, 4-D in including chromosomal anomally was found higher than NAA. Addition of coconut milk along with 2, 4-D and kinetin further increases conditions in callus Asparagus racemose.

Plant tissues are maintained in closed glass vessels for asepsis requirement. Plant tissues during this period produce plant growth regulators like ethylene in high concentration in closed vessels. Ethylene and other plant growth regulators can cause phenotypic charges that are not sexually transmittable. Therefore, such changes are epigenetic in nature and form important basis for somaclonal variation in the vegetatively propagated (micropropagated) herbaceous material e.g. Omamental and flowering herbs.

- **A long Term culture:**

The long term maintenance may cause somaclonal variations. It has been shown that alternation in Karyotypic structure occurs with increasing time in culture. But regeneration of plants does not display full range of abnormalities. Probably this is due to some kind of selection pressure prior to regeneration.

Tissue culture environment

↓

Cell physiological disturbances

(eg. nucleotide pool imbalance)

↓

DNA modifications

(eg. Hyp and Hypermethylation)

Specific base modifications || Chromatin structure changes

↓ ↓

Single gene Mutations. Base subs-titution of modification	Transposable Element 1. Insertion 2. Excision - Deletion 3. chromosome Breakage	Quantitative trait variation	Late Replication – induced chromosome breakage. 1. Rearrangements dependent on heterochromatin and ploidy. 2. Chromosome type, break, fusion, bridge cycle (during meiosis)

Hypothesis relating DNA modification to various mutational events leading to somaclonal variation.

- **Changes in chromosome Number and Structure:**

Ploidy changes including polyploidy, aneuploidy and mixoploidy are the most commonly observed changes associated with the plants regenerated from cultured cells. Structural changes in chromosomes due to deletion (loss of genes), inversion (alteration of gene order), duplication (duplication of genes) and translocation (movement of chromosome segments to a new location) could also bring about somaclonal variation without altering chromosome number (Fig. Stree).

- **Nucleotide Pool imbalance:**

Availability of dexyribonucleotide reserve within cells exhibit significant influence on the fidelity of prokaryotic and eukaryotic DNA metabolism, including precursor

biosynthesis replication and repair Imbalance in the nucleotide reserve may have serious implications on nuclear DNA as well as organellar DNA mutation. In addition wide array of anomalies like chromosomal aberrations, aneuploidy, sister chromatid exchange, increased sensitivity to external environment result in high degree of variation.

Plant tissue and cell culture provides idea conditions for the induction of imbalanced nucleotide reserve pool particularly during serially transfer from depleted to fresh medium. As a result metabolic processes fluctuate with subculture intervals and may be responsible for somaclonal variation.

- **Cryptic Transposable element:**

Transposable elements are movable genetic elements widely present in prokaryote to eukaryotic system. Transposable elements can influence the expression of neighbouring genetic element due to excision and reinsertion process. The concept of the controlling element was developed by Mcclintock in maize plants. Involvement of transposable element was developed by Larkin and Scowroft. Subsequently reports on maize consolidated his hypothesis.

Activation of maize transposable elements *in vitro* has been reported more than once. The maize plants regenerated from tissue cultures were found to contain an active Ac element. Where as none has been detected in the initial explants. Evolve et al (1984) had earlier observed activation of Spn (En) element in half of regenerated plants in maize. More than 20% of the alfalfa plants regenerated from tissue cultures of a white flowered somaclones exhibited the wild type purple flowered phenotype. Genetic analysis indicated that while the wild type and mutated alleles are stable and sexually transmitted the culture process appeared to trigger reversion, suggesting the involvement of transposable element.

- **Amplification of DNA:**

In higher organisms, certain specific genes can undergo amplification during differentiation or in response to environmental pressure. Similar to animal system plant system has also gene amplification process. Gene amplification in response to herbicide

has been reported in alfalfa and *Petunia* cell culture. In alfalfa cell suspension culture there was 4-11 fold amplification of glutamine synthase (GS) gene and consequential 3 to 7 fold increase of GS enzyme. Similarly selection of tobacco cell lines tolerant to glyphosphate herbicide herbicide was accompanied by amplification of gene for 5-enolpyround shikimate 3 phosphate synthanse (EPSPS). The elevated level of RPSPS mRAN was maintained even in the absence of the herbicide, suggesting that the change was a stable genetic modification.

- **Biochemical selection and isolation:**

Induction and recovery of somaclones are possible based on the display of unique selective characters of plant cells. Resistance to certain toxic chemicals is the unique characteristic feature of certain plant during regeneration process *in vitro*, suggesting simple or multiple alterations within selected variant cells. For example, certain variant cell lines are resistant to isonicotinic acid hydrazide (INH) in irradiated cell cultures of haploid tobacco plant. Regeneration tobacco shows variation in their leaf shape, root formation including growth habit. Another somaclone, Alfalfa was recorded based on the selection for growth on ethionoine containing medium. Careful examination revealed that both resistant and non resistant lines were developed for ethionine toxicity.

- ***In vitro* selection of Saline tolerant cell lines:**

In vitro selection of saline resistant cell lines are possible by exposing callus or free cells to different strength of salt. In the entire process, friable callus system was cut into peces of uniform size and transferred to liquid medium containing different concern trations of sodium chloride (NaCl). Callus cells are able to grow and proliferate under low concentration of salt in the culture media. However as concentration increases it may hinder proliferation rate and eventually lead to death of cells under high salt concentration. Careful observation suggested that few cells are able to survive high salinity in culture conditions and proliferate sporadically. These saline resistant cell lines are isolated, cultured and regenerated for salinity resistant somaclones.

***In vitro* selection of Disease Resistance cell lines:**

In vitro selection of somaclones resistant to toxins of pathogenic microbes has been evidenced. Toxin resistant somaclones cell lines can be induced *in vitro* by growing cells in presence of toxin environment. For example, plant pathogenic fungal resistance cell in a can be established by adding different concentrations of fungal filtrate into the media and followed by culturing free cells or callus. The fungal filtrate presumed to contain toxin range can influence growth rate of cells. The toxin resistant somaclone cell lines are selected, maintained and plantlets are regenerated in *in vitro*. Field tests are carried out to establish disease resistant trait in the plants.

Screening of Somaclonal variants:

- **Cytological Screening:**

This deals with microscope assessment of chromosomal instability in *in vitro* grown plantlets, Callus and cultured cells. Some of the preliminary cytological techniques like pretreatment, fixation, staining followed by squashing provides information about chromosomal numbers as well as structural (viz anauploidy / poly ploidy alteration….. besides these informations linear differentiation by employing more refined technique such as G-banding technique provides information with respect to structural alteration. For example, role of Giamsa chanding with respect structural alteration has been detected *Triticum aestivum, Vicia faba* and *Zea mays*.

- **Bichemical Screening:**

Many isoenzymes are used as biochemical markers in the screening of somaclonal variant plants. Isoenzymes such as peroxidases, esterases and alchydrogeneses can be employed in the screening somaclones. Any minor genetic variation can be visualized by analyzing isoenzyme protein profiles.

- **Molecular Screening:**

Genetic variation at the molecular level can be assessed using DNA markers at single nucleotide level or at the mutational level. From the perusal of literature it becomes clearly evident that RAPD, ISSR, SNP and some other DNA marker can elucidate variation at molecular level including genetic fidelity in the micropropagated crops.

- **Gametoclonal variation:**

As the name indicates, gametoclonal variation is derived from gametic cells. The process as of mitosis is responsible for distributing genetic material in somatic cells and tissues. In meiotic process however, gamates recover half of the gametic complements with allelas after following Mendel's law of segregation and independent assortment. Gametoclones can be observed *in vitro* grown haploid due to expression of recessive and dominant variation in haploids. This is totally different from somaclonal variation. Another difference is recovery of recombinational events in gameto clones. It is due to the result of meiotic crossing over rather than non-mitotic crossing over as in somaclones.

Applications of somaclonal variation:

- ✓ Several significant applications of somaclonal variations have been envisaged in view of its role in crop improvement programme. These are highlighted as follows:
- ✓ Somaclonal variant plants derived from *in vitro* selection process are well known in showing stress tolerance character. Somaclones can be grown in wide range of adverse environmental conditions in the soil as well as in the surrounding environment. Adverse conditions such as soil pH, temperature and water logging conditions will have meager influence on the growth of somaclones. Genetically variable somaclonal plants are also adapted well to the high salinity soil. Several salt tolerable plants have been produced through cell culture system.

- ✓ One of the most significant features of the somaclones is the presence of disease resistant characters against plant pathogenic fungi, bacteria and viruses. *In vitro* plants have shown to exhibit wide range of tolerance against microbial toxins.
- ✓ Somaclones are feasible in the cleaning up soil contaminated with toxic metals. Therefore greater degree of access could be seen in the decontamination of soil by somaclones in a process popularly known as *phytoremediation*.
- ✓ Recovery of somaclones shows resistance to antimetabolites such as amino acids analogous, antibiotic drugs, and pathotoxins.
- ✓ Herbicide tolerant potentials of somaclones have been well documented. These genetically variant plants can detoxify many of the commonly used herbicides which are of immense value in cleaning up of soil contanminated by recalcitrant herbicide chemical.
- ✓ Somaclonal variation offers improvement in the yield of crop plants and significantly contributing in crop productivity. In addition, somaclones improved productivity in plantation crops where generation cycle is long. Therefore, somaclonal plants have several defined edge over conventional plants in acquiring useful traits.

Advantages of somaclonal variation over induced mutations:

- ❖ A rapid source of variation available which can not be created following conventional mutation breeding.
- ❖ Some changes occur at a high frequency. It is believed that the changes, which are not possible by conventional breeding could be produced by somaclonal variation.
- ❖ Many changes in agronomic traits have been observed in somaclonal variants.
- ❖ Some changes can be novel may not be achieved by conventional breeding.
- ❖ *In vitro* selection helps in isolation of lines tolerant to biotic and abiotic stresses.
- ❖ *In vitro* selection shorten the time in somaclone isolation and desirable trait.

- Large population of cells can be used for *in vitro* selection.

Concluding Remarks:

Plant cell and tissue culture often provides suitable conditions for the generation of genetic variations in plants. Callus mediated regeneration of plants generally exhibit genetic variations commonly referred as somaclonal variations, earlier discovered by Larkin and Scowcraft. Genetically variant plants or somaclones can be recovered from the plants which are preexisted in the tissues and organs. But more importantly genetic variations can be induced and recovered in the *in vitro* process.

21. Micropropagation: Overview, Stages of micropropagation, Advantages and limitations, Horticultural Uses, Production of virus-free plants, Molecular and immunological techniques of plant virus detection, Genetic assessment by RAPD, RFLP, ISSR and SSR markers.

Micropropagation:

Micropropagation is the practice of rapidly multiplying stock plant material to produce many progenyplants, using modern plant tissue culture methods.

Micropropagation is used to multiply plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

Cornell University botanist Frederick Campion Steward discovered and pioneered micropropagation and plant tissue culture in the late 1950s and early 1960s.

Stages:

Stage 0: Mother plant selection, maintenance and preparation for culture initiation

Stage I: Initiation and establishing an aseptic culture

Stage II: Multiplication of suitable propagules

Stage III: Preparation for growth in the natural environment

Stage IV: Transfer to the natural environment

Applications of Micro propagation:

Micro propagation has become a suitable alternative to conventional methods of vegetativepropagation of plants. There are several advantages of micro propagation.

1. ***High Rate of Plant Propagation:***

Through micro propagation, a large number of plants can be grown from a piece of plant tissue withina short period. Another advantage is that micro propagation can be carried out throughout the year, irrespective of the seasonal variations. Further, for many plants that are highly resistant to conventional propagation, micro propagation is the suitable alternative. The small sized propagules obtained in micro propagation can be easily stored for many years (germplasm storage), and transported across international boundaries.

2. ***Production of Disease-free Plants:***

It is possible to produce disease-free plants through micro propagation. Meristem tip cultures are generally employed to develop pathogen-free plants .In fact, micro propagation is successfully used for the production of virus-free plants of sweet potato (*Ipomea batatus*), cassava (*Manihot esculenta*) and yam (*Discorea rotundata*).

3. ***Production of Seeds in Some Crops:***

Micro propagation, through axillary bud proliferation method, is suitable for seed production in some plants. This is required in certain plants where the limitation for' seed production is high degree of genetic conservation e.g. cauliflower, onion.

4. ***Cost-effective Process:***

Micro propagation requires minimum growing space. Thus, millions of plant species can be maintained inside culture vials in a small room in a nursery. The production cost is

relatively low particularly in developing countries (like India) where the manpower and labour charges are low.

5. ***Automated Micropropagation:***

It has now become possible to automate micro propagation at various stages. In fact, bio- reactors have been set up for large scale multiplication of shoots and bulbs. Some workers employ robots (in place of labourers) for micro- propagation, and this further reduces production cost of plants.

Disadvantages of Micro propagation:

1. **Contamination of Cultures:**

During the course of micro propagation, several slow-growing microorganisms (e.g. *Eswinia* sp, *Bacillus* sp) contaminate and grow in cultures. The microbial infection can be controlled by addition of antibiotics or fungicides. However, this will adversely influence propagation of plants.

2. ***Brewing of Medium:***

Micro propagation of certain plants (e.g. woody perennials) is often associated with accumulation of growth inhibitory substances in the medium. Chemically, these substances are phenolic compounds, which can turn the medium into dark colour. Phenolic compounds are toxic and can inhibit the growth of tissues. Brewing of the medium can be prevented by the addition of ascorbic acid or citric acid or polyvinyl pyrrolidone to the medium.

3. ***Genetic Variability:***

When micro propagation is carried out through shoot tip cultures, genetic variability is very low. However, use of adventitious shoots is often associated with pronounced genetic variability.

4. ***Vitrification:***

During the course of repeated in vitro shoot multiplication, the cultures exhibit water soaked or almost translucent leaves. Such shoots cannot grow and even may die. This phenomenon is referred to as vitrification. Vitrification may be prevented by increasing the agar concentration (from 0.6 to 1%) in the medium. However, increased agar

concentration reduces the growth rate of tissues.

5. ***Cost Factor:***

For some micro propagation techniques, expensive equipment, sophisticated facilities and trained manpower are needed. This limits its use.

Production of virus free plants:

Viruses are very small (submicroscopic) infectious particles (virions) composed of a protein coat and a nucleic acid core. They carry genetic information encoded in their nucleic acid, which typically specifies two or more proteins. Translation of the genome (to produce proteins) or transcription and replication (to produce more nucleic acid) takes place within the host cell and uses some of the host's biochemical "machinery".

Viruses cause many important plant diseases and are responsible for huge losses in crop production and quality in all parts of the world. Infected plants may show a range of symptoms ranging from leaf yellowing (either of the whole leaf or in a pattern of stripes or blotches), leaf distortion (e.g. curling) and/or other growth distortions (e.g. stunting of the whole plant, abnormalities in flower or fruit formation). Viral diseases occur in virtually all seed propagated crops.

There is no commercially available treatment to cure virus-infected plants.

To produce disease free plants a healthy nucleus stock is needed. However, when the whole population is infected then the only way to obtain a pathogen free plant is through tissue culture.

Virus replication is unable to keep pace with cell proliferation.

Apical meristems in the infected plants are generally either free or carry a very low concentration of the viruses. However, the titer of the viruses increases in the older tissues corresponding to the increase in the distance from the meristem tips.

Reasons attributed to the escape of the meristems by virus invasions are –

- Viruses **move rapidly in a plant body** through the vascular system which in meristems isabsent.
- A high **metabolic activity** in the in the actively dividing meristematic cells does not allowvirus replication.

- A **high endogenous auxin** level in shoot apices may inhibit virus multiplication.

- A meristem-tip culture has also enabled plants to be freed from other pathogens includingviroids, mycoplasmas, bacteria and fungi.

Methods of Virus Elimination:

A general term 'virus free' is used by commercial horticulturists for plants free of any type of virus. Virus free material can be re-infected if proper precautionary measures are not adopted.

1. Heat treatment
2. Meristem-tip culture
3. Chemical treatment
4. Other in vitro methods

1. Heat treatment

Before the advent of the meristem cultures the in vivo eradication of viruses from plants can be achieved by **heat treatment (thermotherapy)** of whole plants.

At temperature higher than optimum for many viruses in plant tissues are partially or completely inactivated with little or no injury to the host tissues. Heat treatment is given through hot water or hotair.

The hot water treatment effectively eliminates viruses in dormant buds, where as hot air treatment is recommended for elimination of viruses from actively growing shoots. The survival rate of the host tissue is better in hot air treatment.

Hot Air Treatment

I. Temperature: **35-40°C [few minutes to several months]**
II. Adequate supply of **humidity and light**
III. Plants must have **ample carbohydrate** reserves to withstand the heat treatment
IV. The **temperature** of the air should be **gradually raised** during the first few days until thedesired temperature is raised.
V. **Small cuttings are taken** from the shoot tips **immediately** after heat treatment and graftedonto healthy rootstocks.

VI. Generally the **percentage** of plants that survive after heat treatment is **small.**

2. Meristem-tip culture

For purposes of virus elimination the chance are better if culture are initiated with shoot tips ofsmaller size comprising mostly meristematic cell.

Apical meristem: The portion of the shoot lying distal to the youngest leaf primordium measuring upto about 100 mm in diameter and 250 mm in length.

Shoot apex: The apical meristem together with one to three young leaf primordia measuring 100-500mm.

In most published work explants of larger size (100-1000 mm long) have been cultured to raisevirus free plants.

Under a stereoscopic microscope (8-40 magnification) with a suitable light source explants are placed on a Petri plate lined with a sterile moist filter paper to avoid desiccation.

Meristem tips taken from terminal buds observed to give better result than those from axillary buds. The percentage of virus free plants can also depend on season, especially with crops which displayperiodic growth.

Although the apical meristems are often virus free, there may be exceptions. Some viruses are known to actually invade the meristimatic region of the growing tips in certain plants. i.e. TMV.

In such cases it has also been possible to obtain virus free plants by combining meristem tip culture with thermotherapy. The duration of heat treatment has to be decided carefully since excessive exposure can damage the plant tissues. To avoid deterioration of meristem tip cultures by continuous exposure to high temperature, the treatment with diurnal or daily cycle of high and low temperature can be tried. i.e. CMV in tissue cultures of *Nicotina rustica* and *Stellaria indica* can be inactivated by following the diurnal cycles of 40°C (16hrs) and then 22°C (8 hrs) per day.

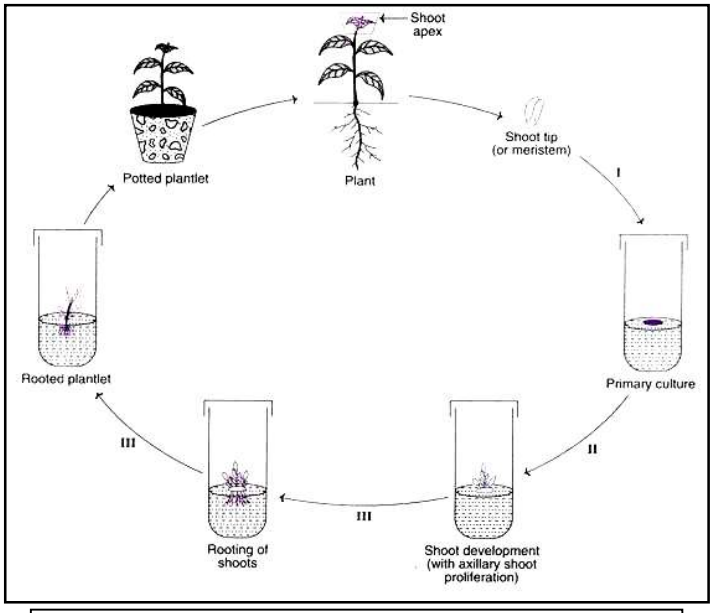


Fig.: A diagrammatic representation of shoot tip (or meristem) culture in micropropagation.

Meristem-tip cultures are influenced by the following factors:

i. **Physiological condition of the explant** — actively growing buds are more effective.
ii. **Thermotherapy prior to meristem-tip culture** — for certain plants (possessing viruses in the meristematic regions), heat treatment is first given and then the meristem-tips are isolated and cultured.
iii. **Culture medium —**MS medium with low concentrations of auxins and cytokinins is ideal.

A selected list of the plants from which viruses have been eliminated by meristem cultures is given in Table.

Table: A selected list of the plants with virus elimination by meristem cultures

Plant species	*Virus eliminated*
Solanum tuberosum (potato)	Leaf roll, potato viruses — A, X, Y, S
Nicotiana tabacum (tobacco)	Tobacco mosaic virus
Saccharum officinarum (sugar cane)	Mosaic virus
Allium sativum (garlic)	Mosaic virus
Anenas sativus (pineapple)	Mosaic virus
Brassica oleracea (cauliflower)	Cauliflower/mosaic virus turnip mosaic virus
Ipomoea batata (sweet potato)	Fealthery mottle virus
Ribes grassularia	Vein banding virus
Humulus lupulus	Hop latent virus
Armoracia rusticena	Turnip mosaic virus
Musa sp (Banana)	Cucumber mosaic virus
Hycinthus sp	Hycinth mosaic virus
Dahlia sp	Dahlia mosaic virus
Chrysanthemum sp	Virus B
Petunia sp	Tobacco mosaic virus
Iris sp	Iris mosaic virus
Cymbidium sp	Cymbidium mosaic virus
Fragaria sp	Pallidosis virus, yellow virus complex
Freesia sp	Freesia mosaic virus

3. Chemical treatment

In the absence of effective therapeutic chemicals capable of eradicating virus from infected plants, there are reports of some attempts to suppress viruses in plant tissue and protoplast by the addition of some chemicals in the media. In some instances virus multiplication was suppressed by **the addition of cytokinins and other growth substances** while in others it was actually stimulated. Antimetabolites **malachite green, thiouraciol and acetylsalicylic acid** had little or a limited effect on virus elimination in meristem-tip regenerated plants, but the incorporation of nucleoside analog **rivavirin** (PVX,CMV, alfalfa mosic virus), **vidarbin, cyclohexamide** and **actinomycin-D** inhbit virus replication in plant protoplasts**.**

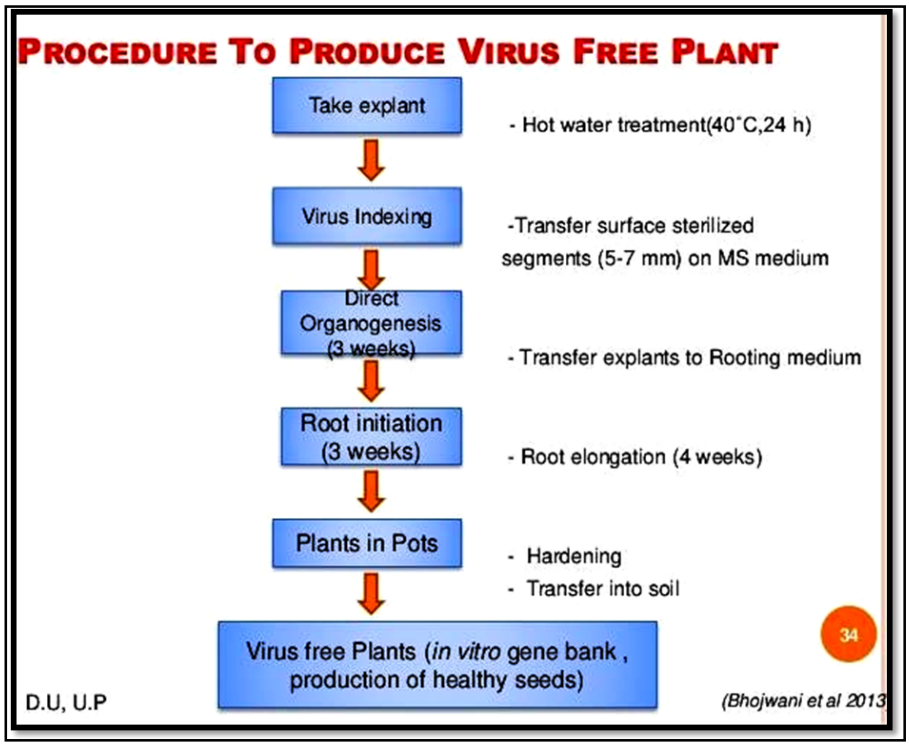
4. Cryotherapy

Cryotherapy is the prolonged exposure to a low temperature followed by shoot tip culture. It is a good method of virus elimination. Shoot tip cultures from *Chrysanthemum* plants treated with 5°C for 4 months yielded 67% plants free from *Chrysanthemum*

stunt virus (CSV) and 22% plants free from *Chrysanthemum* chlorotic motile virus (CCMV). Cold treatment is preferable to heat treatment as it is less injurious to the plants and often more effective in virus elimination.

5. *Electrotherapy*

Electrotherapy assays were carried out either on infected in vivo or in vitro plants. Pulses of 15 V were applied for 5 min to 2- 3 cm long explants containing apical meristem. The meristems were then excised and placed on an MS culture medium. The efficiency of electrotherapy in producing virus- free regenerants from BSV-infected banana plants (cv. *W. Bungulan*) is 40-80 %.



Virus free assessment methods through Molecular and immunological techniques:

Tissue culture offers great promise for the production of quality planting material on account of disease free plants produced through meristem culture. But all the plants obtained through meristem culture with or without the therapeutic treatments are not virus free. Testing plants for the presence or absence of viruses is known as virus indexing. Every meristem tip or callus derived plant must be tested before using it as a mother plant to produce virus free stock. The three methods which are followed for virus indexing are sap transmission test, serology and EM examination.

Sap transmission test

It is done by taking the saps (filtered leaf extract) from test plants and which may be used to inoculate highly sensitive and healthy indicator plants. An Indicator plant for a virus is that plant species or variety which is highly susceptible to the virus and readily develops the symptoms. The inoculated indicator plants are maintained in a green house or aphid- proof cages separated from each other and from other plants. Sap transmission test is the most sensitive test among the three methods and can be easily performed on a commercial scale.

Serological test

It is a highly sensitive and precise technique for virus indexing employing antibodies specific to the concerned viruses. This test is performed by adding a drop of centrifuged sap from a test plant to a drop of antiserum taken from the blood of a rabbit. If the virus is present, the precipitation will take place due to the presence of specific antibodies in the blood. The ELISA (enzyme linked immunosorbent assay) is one of the serological methods used to identify viruses based on antibody reaction. ELISA is the most convenient, rapid and efficient test especially when a large number of samples are to be handled.

EM (Electron microscopy) examinations

These are particularly useful for identifying latent viruses (viruses those exhibit no visible symptoms). This method is not usually implemented as specialized equipment and trained personnel are required to carry out EM studies. Immunosorbent Electrom Microscopy (ISEM) described combines both serology and EM studies for detection of viruse.

Genetic assessment by RAPD, RFL, ISSR and SSR markers:

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism. Currently, the restriction fragment length polymorphism (RFLP) assay has been the choice for many species to measure genetic diversity and construct a genetic linkage map. However, an RFLP assay which detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridisation, is, in general, time consuming and laborious. Over the last decade, polymerase chain reaction (PCR) technology has become a widespread research technique and has led to the development of several novel genetic assays based on selective amplification of DNA. This popularity of PCR is primarily due to its apparent simplicity and high probability of success.

Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes.

Randomly amplified polymorphic DNA markers (RAPD):

RAPD markers detect nucleotide sequence polymorphisms between individuals by employing a single, short (decamer), random oligonucleotide primers. DNA polymorphisms are detected due to the changes of nucleotide at or between oligonucleotide primer binding sites in the genome. RAPD are dominant markers that can detect several loci in a single assay ranging from 0.5 to 5 kb. DNA fragments are fractionated by agarose gel electrophoresis and visualized with ethidium bromide (EtBr) that scores differences in the amplification pattern by their presence (+) and absence (-) at particular loci. Simplicity in methodology, no prior sequence information for primer designing, cost efficiency as well as no requirement radioactive probes has made RAPD technique a promising tool for identification of markers linked to agronomically important traits. However, inherent problems of reproducibility,

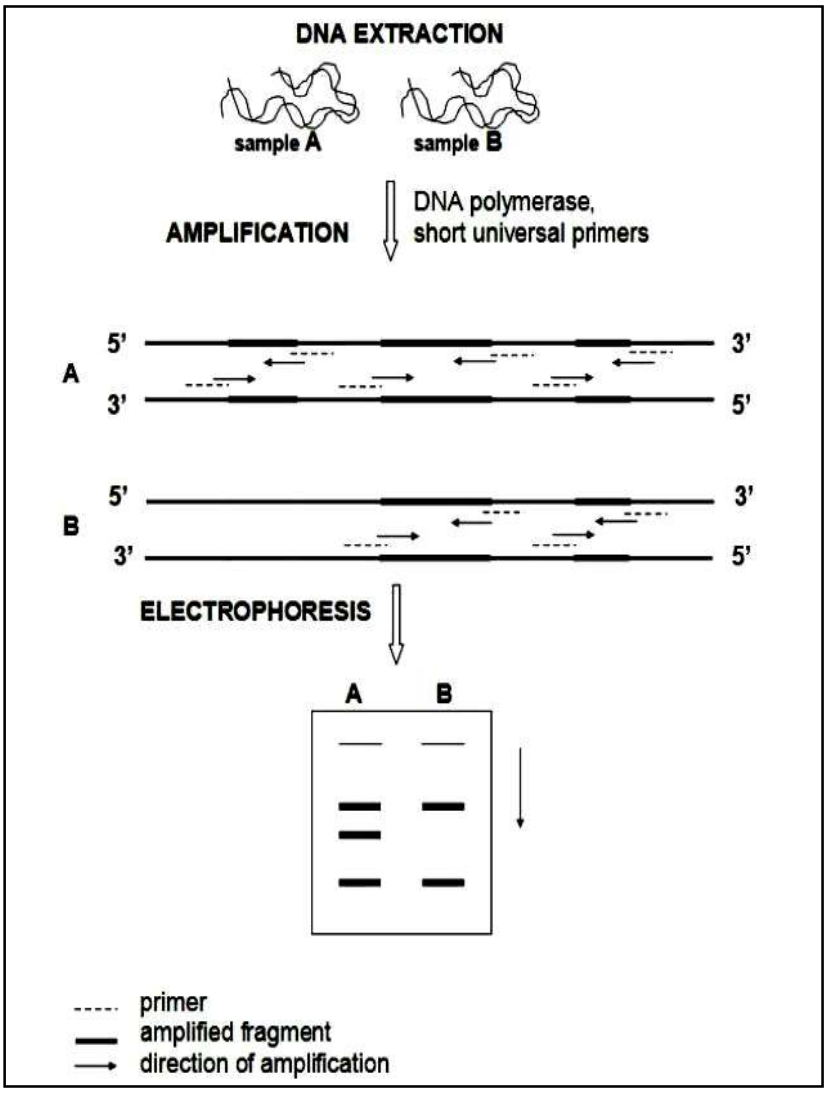
sensitivity to experimental conditions, presence of artefactual bands (false positives) and inability to distinguish heterozygous from homozygous individuals makes them less preferential tool for genome wide studies.

RAPD primer	Sequence (5′-3′)	No. of amplification products
2	AGACGCGTAG	7
3	TGGACCCACA	3
4	GTGGCTTCTC	5
5	GCGCAGTATC	8
6	CCACCGTACT	6
7	TCCGGCTGTT	6
8	TGTCCCGTTG	7
11	CACACGAGAC	7
12	CGTACACCAG	5

RAPD involves following steps:-

1. The DNA of a selected species isisolated.
2. An excess of selected deca oligonucleotide added.
3. This mixture is kept in PCRequipment and is subjected torepeated cycles of DNAdenaturation-renaturation- DNAreplication.
4. During this process, the decaoligonucleotidenwill pair with the homologous sequence present atdifferent locations in the DNA.
5. DNA replication extends the decaoligonucleotide and copy the sequence continuous with the sequence with which the selected oligonucleotide has paired.
6. The repeated cycles of denaturation -renaturation-DNA replication will amplify this sequence of DNA.
7. Amplification will takes place only of those regions of the genome that has the sequence complementary to the decaoligonucleotide at their both ends.

8. After several cycles of amplification the DNA is subjected to gel electrophoresis.
9. The amplified DNA will form a distinct band. It is detected by ethidium bromide staining and visible fluorescence's under U.V. light.



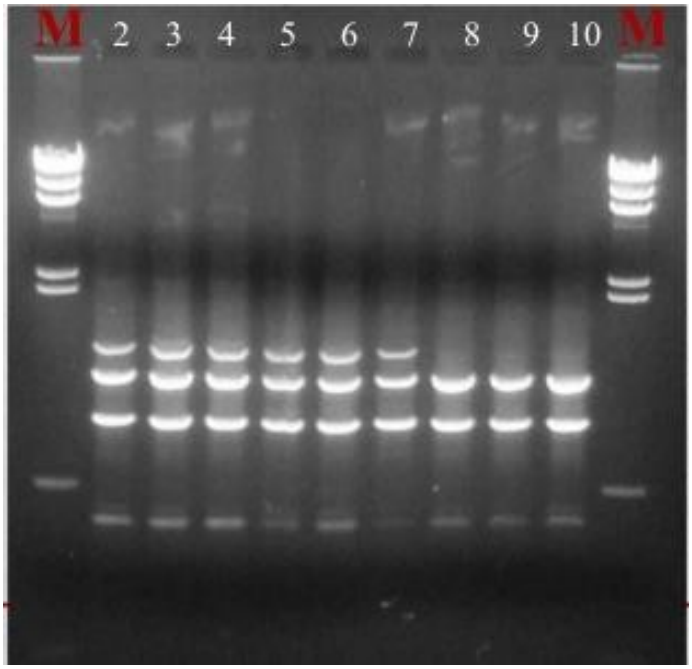


Fig.: Separated RAPD

Advantages:

RAPD primers are readily available being universal. They provide moderately high genotyping throughput. This technique is simple PCR assay (no blotting and no radioactivity). It does not require special equipment. Only PCR is needed. The start-up cost is low. RAPD marker assays can be performed using very small DNA samples (5 to 25 ng per sample). RAPD primers are universal and can be commercially purchased. RAPD markers can be easily shared between laboratories. Locus-specific, co-dominant PCR-based markers can be developed from RAPD markers. It provides more polymorphism than RFLPs.

Disadvantages:

The detection of polymorphism is limited. The maximum polymorphic information content for any bi-allelic marker is 0.5. This technique only detects dominant markers. The reproducibility of RAPD assays across laboratories is often low. The homology of

fragments across genotypes cannot be ascertained without mapping. It is not applicable in marker assisted breeding programme.

Uses:

This technique can be used in various ways such as for varietal identification, DNA fingerprinting, gene tagging and construction of linkage maps. It can also be used to study phylogenetic relationship among species and sub-species and assessment of variability in breeding populations.

Restriction Fragment Length Polymorphism (RFLP):

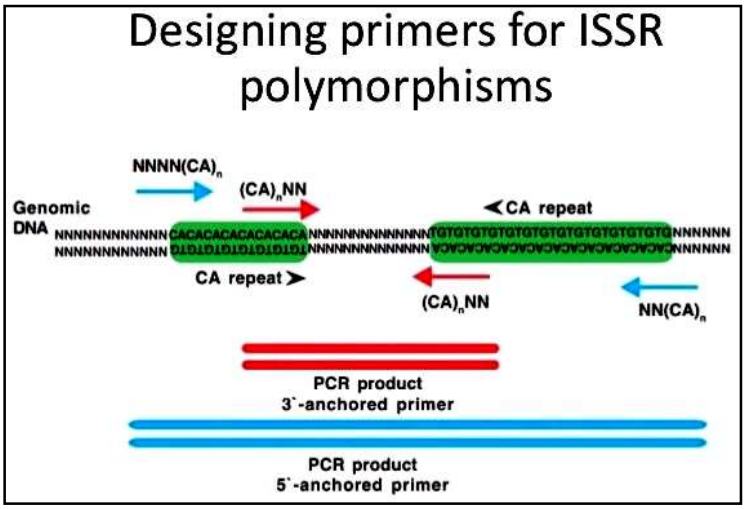
RFLP is the first molecular marker technique which was developed by Botstein et al., (1980) and was based on restriction enzyme digestion of genomic DNA. This is a landmark invention in molecular biology which revolutionized the field of molecular genetics. Training Manual on Genetic Fidelity Testing of Tissue Culture-Raised Plants (2016-17) 14 Polymorphisms are detected based on the restriction enzyme digestion and in combination with probe hybridization (a labelled DNA fragment which is used for hybridization with the restriction digested genomic DNA). The Southern blots of restricted genomic DNA from different strains of the same species are denatured and hybridized to a probe. This results in the visualisation of variation in the size and/or number of detected restriction fragments generated from the different strains. The detected length variation is because of the differences in restriction sites caused by insertions or deletions. RFLP is largely codominant. It could be multi or single locus depending upon the source and sequence of the probe used.

Inter Simple Sequence Repeat (ISSR):

ISSR involves the amplification of DNA fragments flanked by inversely oriented SSR-microsatellites. The DNA polymorphism depends on the abundance and hypervariability of microsatellites in the genome. This technique uses microsatellites consisting of di-, tri-, tetra- or penta-nucleotides as primers complementary to microsatellite regions in the genome and amplify inter simple sequence repeats of different sizes. Depending on the usage ISSR primers can be either unanchored or

anchored at 3' or 5' end with 1–4 degenerate bases extended into the flanking sequences. Primer extension with 1–4 degenerate nucleotide at 3' or 5' end avoids internal priming and smear formation. The size of the amplified products varies between 200 and 2000 bp and can be separated using both agarose and polyacrylamide gelelectrophoresis. The technique issimple, quick and need no sequence information for primer synthesis. ISSRs use longer semi-arbitrary SSR primers (15–30 mers) as compared to RAPD primers (10 mers), which allow the subsequent use of high annealing temperature leading to higher stringency and greater band reproducibility. However, dominant inheritance and homoplasy are the main limitations of ISSRs.

During the last twenty years, tissue culture and molecular biology have experienced a dynamic synergism. While tissue culture has provided model systems for molecular biology studies, molecular biology techniques have been applied to address limitations of tissue culture systems. Molecular marker systems have investigated genetic stability or somaclonal variation of plants after long term preservation or cryopreservation under in vitro conditions, and also of plants coming from long term culture conditions or following a large number of subcultures. Quality control of tissue cultured plants, molecular breeding in in vitro conditions and determining the genes involved in tissue culture responsiveness are other areas of usage of molecular markers.



Simple Sequence Repeat (SSR):

The SSR molecular markers are used for different applications including assessment of *in vitro* plants fidelity testing. are RAPD, They are cost effective and require low amounts of DNA. These types of markers were suitable for establishing genetic stability of several micropropagated plants in crops such as wheat. In this study several wheat accessions were characterized, which in particular are not morphologically identifiable. In potato, SSR markers proved that *in vitro* culture is a safe method for conservation of potato microtubers to produce true-to-type plants. SSR markers were applied in tree analysis as well, as for in vitro plants characterization of *Populus tremuloides* and *Pinus pinea*. In case of white or pedunculated oak (*Quercus robur*) seedlings, epicormic, crown and micropropagated shoots from mature trees were analysed by SSR markers and no intraclonal or interclonal polymorphism was detected. *In vitro* plants of *Robinia pseudacaccia* multiplied by axillary buds showed no variations in RAPD banding but SSR markers showed high level of mutations in somatic tissues.

The simple sequence repeats are present over a hundred repeats of a 1-4 nucleotide sequences in the genome of all eukaryotes. In higher organisms, the simple repetitive DNA sequences are of three types viz., satellite, minisatellite and microsatellites DNAs according to their size. The DNA consisting of long repeats of about 100-1000s are called satellite DNA while tandem repeats having shorter repeat units of 10-100s are described as minisatellite DNA and very short repeat units (1-4) are termed as microsatellite. Primers can be designed for the nucleotide sequences flanking these short repeats and polymorphism can be generated because of the fragment variation in the length of repeat regions present in genome. During DNA synthesis, if slipped-strand mispairing occurs within a microsatellite array, a gain or loss may have happened depending on whether the newly synthesized DNA chains loops out or the template chain loops out, respectively. So, SSR allelic differences are the results of variable number of repeat units within the microsatellite structure. A popular example of a microsatellite is (CA) n, n=10 to 100 and these markers often present in higher level of inter and intra-specific polymorphism particularly when tandem repeats number is ten or greater. Di-nucleotide repeat arrays occur much frequently than tri and tetra-

nucleotides so that it is easier to run combinatorial screen for them but gives fewer stutter bands. PCR for SSR runs in the presence of forward and reverse primers that anneal at 5' and 3' ends of DNA templates. The amplification products are either separated by polyacrylamide gels and polymorphism detected with $AgNO_3$ staining or agarose gel electrophoresis through autoradiography. Because of production of high polymorphism even among the genetically much closed lines, SSRs have become markers of choice in most areas of molecular genetics. They are codominant markers with high genomic abundance and reproducibility, require small amount of DNA, can be easily automated for high through put screening and excellent for studies of population genetics and mapping. The main limitation of SSR marker is the difficulty of cloning and sequencing the flanking regions. The tedious process of developing protocols for cloning and sequencing of SSRs has to be performed for each plant species under study.

RAPD and ISSR markers have been extensively applied to serve the purposes in the following areas:

- ***Determination of genetic stability or detection of somaclonal variation***

Somaclonal variation is the variation observed among plants regenerated from in vitro culture. These variations are heritable, i.e., transmitted through meiosis, and are usually irreversible. Many factors such as plant growth regulator balance (auxin and cytokinin concentration), culture duration (subculture number), macro and micro elements used in in vitro culture, and physiological stress induce somaclonal variation under in vitro conditionsThe source of explants and their pattern of regeneration are known to play major roles in determining the extent of genetic or somatic variation. Plants regenerated from adventitious buds around axillary buds, or from other well developed meristematic tissue, show the lowest tendency for genetic variation, whereas more changes are detected in plants derived from callus compared with those coming from embryogenic tissue.

- ***Genetic stability of plants after long term in vitro conservation and cryopreservation***

Germplasm preservation is essential to maintain biodiversity and avoid genetic erosion. In vitro culture may provide an alternative to standard methods for the conservation of many woody plants. Genetic conservation is based on the assumption that the material is conserved under conditions ensuring genetic stability. However, many factors associated with in vitro culture conservation procedures may result in somatic variation. It is essential to observe genetic stability during in vitro conservations. Currently, RAPD and ISSR markers are being increasingly used for monitoring geneticstability of germplasm during their long-term conservation.

- ***Genetic stability of plants after long subculture***

In some plants the variable number of proliferation cycles increases the occurrence of the somaclonal variation, mainly, due to chromosomal abnormalities.

- ***Molecular markers for quality control of in vitro plants***

The micropropagation industry is expanding and somaclonal variation and bacterial and viral contamination is becoming a problem for commercial propagators of a genotype. It is necessary that the plant material produced through tissue culture be certified before being distributed to growers. Quality control of most micropropagated plants is being ensured using molecular markers (RAPD and ISSR) and DNA fingerprinting techniques. Polymorphism obtained in the progenies of tissue culture plants in comparison to the control mother explant source could be correlated with the apparent morphological changes. Although this is very time consuming, once done this will give an ideal marker system. These markers will further enhance the ability to understand the effect of different variables on the production of somaclonal variation in plants regenerated from tissue culture.

- ***Developing molecular markers linked to a trait in question***

The identification of gene or genomic regions that influence agriculturally related traits is very important in DNA marker-trait association study. This provides the basis for marker assisted selection (MAS) in plant breeding. Random molecular markers (RAPD

and ISSR) are traditionally applied to establish a genetic linkage with a phenotypic trait. Having a tight linkage with the phenotypic trait RAPD and ISSR markers are the method of choice in many cases.

22. Let's sum up

- Selection is basic to any crop improvement. Isolation of desirable plant types from the population is known as selection. It is one of the two fundamental steps of any breeding programme viz., 1. Creation of variation and 2. Selection.
- Natural variability in self-pollinated population is exhausted during selection, for further improvements new genetic variability has to be created by crossing two different pure lines. Hybridization means the mating or crossing of two plants or lines of dissimilar genotypes.
- A clone may be defined as a group of plants derived from a single plant by vegetative propagation.
- Variation encompasses the differences observed within a population. Classifying variation into discrete and continuous categories is essential for understanding genetic diversity and evolution.
- A polygene is a member of a group of non-epistatic genes that interact additively to influence a phenotypic trait, thus contributing to multiple-gene inheritance (polygenic inheritance, multigenic inheritance, quantitative inheritance), a type of non-Mendelian inheritance, as opposed to single-gene inheritance, which is the core notion of Mendelian inheritance.

- Polygenic locus is any individual locus which is included in the system of genes responsible for the genetic component of variation in a quantitative (polygenic) character.
- Self-incompatibility or intraspecific incompatibility is a well-designed genetic mechanism by which certain plants recognize and reject their own pollen thus forcing outbreeding.
- The best studied mechanisms of Self Incompatibility act by inhibiting the germination of pollen on stigmas, or the elongation of the pollen tube in the styles. These mechanisms are based on protein-protein interactions, and the best-understood mechanisms are controlled by a single locus termed S, which has many different alleles in the species population.
- Male sterility is defined as an absence or non-function of pollen grain in plant or incapability of plants to produce or release functional pollen grains as a result of formation or development of functional stamens, microspores or gametes.
- Genetic male sterility is governed by nuclear genes. It is wide occurrence in plants. Male sterility genes are generally recessive (*ms ms*) but dominant gene governing male sterility are also occur in safflower, and arise through spontaneous mutation or may be induced mutagen treatments.
- Heterosis may be defined as the superiority of an F1 hybrid over both of its parents in terms of yield or some other character.
- Performance or expression of any character or trait is influenced by many genetic factors — some are positive (stimulating) and others are negative (decreasing). Expressivity of the genes or the degree of manifestation of a character is the result of genetic balance in the action of differently directed factors.
- The essential concept that aided molecular understanding of heterosis by making molecular markers accessible, allowing for a more precise method to mapping genes and detecting them in complicated phenotypes.
- The quantitative trait loci (QTL) for specific variables associated in the formation of heterosis in parental inbred lines were found using Marker Assisted Selection

- Mating between individuals related by descent or ancestry is called inbreeding. When the individuals are closely related, e.g., in brother-sister mating or sib mating, the degree of inbreeding is high.
- Molecular markers have changed the entire scenario of life sciences with their broad application to understand the cellular and molecular responses, providing breeders a simple but powerful weapon for precision selection of a desired genotype.
- A molecular marker is a DNA sequence with a known position on a linkage map/chromosome that may or may not be linked with phenotypic expression of a gene that can easily distinguish between two closely related individuals.
- RFLPs markers are relatively highly polymorphic, co-dominantly inherited and highly reproducible. The polymorphism in restricted fragments due to DNA rearrangements that occur due to evolutionary processes, point mutations with in the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over.
- SNP markers are highly polymorphic and mostly bialleleic. The genotyping throughput is very high. SNP markers are locus specific.
- Expressed Sequence Tags (ESTs) are small pieces of DNA and their location and sequence on the chromosome are known. The variations which are found at a single nucleotide position are known.
- Mini-satellites are tandem repeats of DNA sequence with 10-100 bp repeat motifs whereas, microsatellites are tandem repeat of DNA sequence with 2-6 bp repeat motifs. These are also referred to as Variable Number Tandem Repeats (VNTRS) and this is one of the basis of polymorphism at a locus.
- A genetic map is a schematic representation of the various genetic markers in the specific order in which way they are located in a chromosome as well as the relative distance between these markers. Three different strategies have been used to construct genetic maps.

- A linkage map depicts the order of genetic markers and the relative distances between them as measured in terms of recombination frequencies between the markers.
- High-density genetic map is a valuable tool for exploring novel genomic information, quantitative trait locus (QTL) mapping and gene discovery of economically agronomic traits in plant species.
- A high-density genetic map is not only a key resource for studies on genome structure and genetic relationships but also provides the basis for quantitative trait locus (QTL) mapping and marker-assisted selection (MAS) based on the numbers of polymorphic markers.
- Doubled haploid lines contain two identical sets of chromosomes in each cell. They are completely homozygous, as only one allele is available for all genes. Doubled haploids can be produced from haploid lines.
- Association mapping identifies quantitative trait loci (QTLs) by examining the marker-trait associations that can be attributed to the strength of linkage disequilibrium between markers and phenotype across a set of diverse.
- Association mapping, also known as "linkage disequilibrium mapping", is a method of mapping quantitative trait loci (QTLs) that takes advantage of linkage disequilibrium to link phenotypes to genotypes. It offers greater precision in QTL location than family-based linkage analysis. It does not require family or pedigree information, can be applied to a range of experimental and non-experimental populations.
- Marker Assisted Selection (MAS) refers to indirect selection for a desired plant phenotype based on the banding pattern of linked molecular (DNA) markers. MAS is based on the concept that it is possible to infer the presence of a gene from the presence of a marker which is tightly linked to the gene of interest.
- MAS has been used for genetic improvement of different field crops such as maize, barley, rice, wheat, sorghum, soybean, chickpea, pea, sunflower, tomato, potato and some fruit crops for various economic characters.

- Genome selection (GS) is a specialized form of MAS, in which information from genotype data on marker alleles covering the entire genome forms the basis of selection.
- GWAS is a method for the study of associations between a genome-wide set of single-nucleotide polymorphisms (SNPs) and desired phenotypic traits. The quantitative evaluation is based on linkage disequilibrium (LD) through genotyping and phenotyping of diverse individuals.
- All methods for the isolation of mutant genotypes in sexually reproduced plants are based on the pedigree method, modified to account for the chimeric structure of the M_1 plants.
- Phenotypic buffering is another property of polyploids that restricts mutability of many characters, especially those essential for the whole life of the plant: e.g. the process of chlorophyll formation. Thus, chlorophyll mutations decrease with the increasing level of ploidy; however, the total rate of mutation increases.
- Backcross method of breeding change the genotype of recurrent parent only for the gene(s) under a transfer to correct the specific defect of the recurrent parent. But some unexpected changes in one or more character may also occur due to gene tightly linked with the gene being transferred.
- Multiline variety is a mixture of several pure lines of similar phenotype(height, seed color flowering time, maturity time and various other agronomic characteristics) but have different genes for the character under consideration the disease resistance means these are isogenic lines.
- Heritability, amount of phenotypic (observable) variation in a population that is attributable to individual genetic differences. Heritability, in a general sense, is the ratio of variation due to differences between genotypes to the total phenotypic variation for a character or trait in a population.
- Improvement in the mean genotypic value of selected plants over the parental population is known as genetic advance. It is the measure of genetic gain under selection.

- One of the main objectives of designing an experiment is how to verify the hypothesis in an efficient and economical way.
- Randomization forms a basis of a valid experiment but replication is also needed for the validity of the experiment.
- A split plot design is a design with at least one blocking factor where the experimental units within each block are assigned to the treatment factor levels as usual, and in addition, the blocks are assigned at random to the levels of a further treatment factor.
- A bioreactor (bioreactor) is a closed vessel with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the waste biomass of cultured microorganisms along-with their products.
- All bioreactors deal with heterogeneous systems dealing with two or more phases, e.g., liquid, gas, solid. Therefore, optimal conditions for fermentation necessitate efficient transfer of mass, heat and momentum from one phase to the other.
- Organogenesis means the development of adventitious organs or primordial from undifferentiated cell mass in tissue culture by the process of differentiation.
- *In vitro* organogenesis from the callus tissue derived from a small piece of plant tissue viz. isolated cells, isolated protoplasts, microspores etc. can be induced by transferring them to suitable medium or a sequence of media that promote proliferation of shoot/root both. The suitable medium is standardized by trial and error method.
- Somatic embryogenesis is a process by which a single cell or a small group of cells follow a developmental pathway which leads to develop a non-zygotic embryo which leads to develop a complete plant.
- The embryos initiate directly from the explants without callus formation and here some of the cells, which are called 'Pre embryonic determined cells (PEDC).

- The embryos are developed through cell proliferation i.e. callus formation. The cells from which embryos arise are called as 'Induced embryogenic determined cells' (IEDC).
- Somaclonal and Gametoclonal variation; Factors contributing to occurrence of somaclonal variation; identification selection and screening of variants. Advantages and application of somaclonal variants in crop improvement.
- Plant cell and tissue culture often provides suitable conditions for the generation of genetic variations in plants. Callus mediated regeneration of plants generally exhibit genetic variations commonly referred as somaclonal variations.
- Micropropagation is the practice of rapidly multiplying stock plant material to produce many progeny plants, using modern plant tissue culture methods. It is used to multiply plants such as those that have been genetically modified or bred through conventional plant breeding methods.
- Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism.
- Utility of protoplast culture has been positively implicated in several diversified fields for the improvement of crops. Cell modification by DNA uptake, studying nitrogen fixation in protoplast and somatic hybrid production are some of the significant applications of protoplast culture. Transfer of male sterile character is possible by the production of cybrids.

23. Suggested Readings

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24. Assignments

1. Describe the application of male sterility.
2. Write down the genetic and molecular basis molecular basis of self incompatibility.
3. What are the barriers of distant hybridization? Discuss its achievements.
4. Short note on a) back cross, b) multiline breeding
5. Give the differences between vertical and horizontal disease resistance
6. Explain Flor Hypothesis with suitable examples.
7. What is Vertifolia effect?
8. What are the possible methods of breeding for disease resistance?
9. Enumerate the broad sense and narrow sense heritability with classical examples.
10. How does split plot design differ from randomized block and Latin square designs?
11. How would you induce haploids?
12. What is meant by cellular totipotency?
13. Differentiate between somatic and zygotic embryogenesis.

14. What is a bioreactor? How is it used to scale up multiplication of commercially importantplants?
15. What conditions do plant cells need to multiply *in vitro*?
16. Define embryoids.
17. Briefly describe the stages of somatic embryogenesis.
18. Mention the role of SERK and LEC genes during somatic embryogenesis.
19. What is its significance in micropropagation?
20. What is meant by Cybrids?

All the materials are self writing and collected from e-book, journals and websites.