Brief content of lectures

Discipline “Molecular biochemical markers related to disease resistance”

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Discipline “Molecular biochemical markers related to disease resistance” is one of the important part of

L. PRINCIPLES OF Plant Pathology

1. What is Plant Pathology?
2. Objectives of Plant Pathology
3. Disease & Disorder
4. Plant Pathogens
5. Classification of diseases

What is Plant Pathology?
Phytopathology (Phyton : plant) Greek - Pathos (suffering) + Logos (study) = The study of the suffering plant
Plant pathology is that branch of agricultural, botanical or biological sciences which deals with the study of cause of the disease resulting losses and Control of plant diseases

Objectives of Plant pathology

Study of origin, causes or reasons. Study of living, non-living and other causes of disease or disorder in plants- Etiology:
2.Study of mechanism of disease development i.e. processes of infection and colonization of the host by the pathogen. This phase involves complex host-pathogen interactions-
Pathogenesis study the interaction between the causal agent and the diseased plants in relation to environmental conditions. Generally at the population level- Epidemiology
4 Development of management systems of the diseases land reduction of losses caused by them- Control/ Management.

The Concept of Disease in Plants plant is healthy, or normal, when it can carry out its physiological functions to the best of its genetic potential.
 Any deviation- Disease
 The kinds of cells and tissues that become affected determine the type of physiological function that will be
 For example, infection of roots may cause roots to rot and make them unable to absorb water and nutrients from the soil;
 infection of xylem vessels, interferes with the translocation of water and minerals to the crown of the plant;
 infection of the foliage, (leaf spots, blights, rusts, mildews, mosaics etc.), interferes with photosynthesis

L. 2 What is a Disease

Stakman & Harrar (1957) defined disease as physiological disorder or structural abnormality that is deleterious to the plant or its part or product, that reduces the economic value of the plant e.g., wilt, potato blight, Loose smut of wheat, karnal bunt of wheat.

Disease – the process in which a pathogen interferes with one or more essential plant cell functions

Marshall Ward (1901): disease represents a condition in which functions of the plant are not properly discharged.

Disease is a harmful deviation from normal functioning of physiological processes. (British Mycological Society, 1950)

Horsfall & Diamond (1957): Disease can be defined as a physiological disorder or structural abnormality that is deleterious or harmful toe the plant or its part or product that reduces its economic value.

Disease can be defined as the result of interaction between host, pathogen and environment.

How Pathogens affect Plants?

There are many ways in which plant disease pathogens can affect plants

–By utilizing host cell contents

–By killing host or by interfering with its metabolic processes through their enzymes, toxins etc.

–By weakening the host due t continuous loss of the nutrients.

–By interfering with the translocation of the food, minerals land water.

–They can suppress the chlorophyll content.

–They can reduce the leaf area.

–They can curb the movement of solutes and water through the stems.

–They sometimes reduce the water-absorbing capacity of the roots.

–They suppress the translocation of photosynthates away from the leaves.

–They sometimes promote wasteful use of the products of photosynthesis as in the formation of galls.

- **Lecture 3. SIGNAL TRANSDUCTION of pathogen**
- 1. Steps of Signal Transduction
- 2. Gene-for-gene hypothesis
- 3. Cyclic adenosine monophosphate (cAMP)
- 4. Mitogen-activated protein kinases (MAPKs
- SIGNAL TRANSDUCTION
- Biologically, signal transduction refers to any process by which a cell converts one kind of signal or stimulus into another.
- Signal transduction at the cellular level refers to the movement of signals.
- Signal Transduction Pathways
- Refers to a series of sequential events, such as protein phosphorylations, consequent upon binding of ligand by a transmembrane receptor, that transfer a signal through a series of intermediate molecules until final regulatory molecules, such as transcription factors, are modified in response to the signal.
- Steps of Signal Transduction:
- Signal perception
- Signal transduction
- Signal response
- Termination of signaling events
- Generalized view of signal transduction
- Signal Perception
- It is a surface level phenomena in which elicitor from the pathogen are recognized by host receptor.
- •Explained by Flor gene-for-gene hypothesis and consist of a receptor-ligand interaction.
- The perception of signals
- The perception of signals from plant surfaces by pathogenic fungi is the result of signaling pathways mediated by

1. cyclic adenosine monophosphate (cAMP)
2. mitogen-activated protein kinase (MAPK), which have been implicated in regulating the development of infection-related phenomena in many different fungi.

- Gene-for-gene hypothesis
- For each gene that determines resistance in the host, there is a corresponding gene in the pathogen that determines (a) virulence.
- The perception of pathogen signals
- The perception of signals from plant surfaces by pathogenic fungi is the result of signaling pathways mediated by:

1. cyclic adenosine monophosphate (cAMP)
2. and mitogen-activated protein kinase (MAPK),

which have been implicated in regulating the development of infection-related phenomena in many different fungi.

- In some fungi, cAMP (cyclic adenosine monophosphate (cAMP) signaling is required for the initiation of appressorium development, at which time intracellular cAMP concentrations rise during differentiation of conidia and emergence of the appressorium germtube. Subsequently, cAMP levels fall as the germ tube extends –and, if more cAMP is added at this point, further development of the germ tube is inhibited.
- Appressorium formation
- Cyclic adenosine monophosphate (cAMP)
- –Transmission of the cAMP signal proceeds via the cAMP-dependent activity of protein kinase A (= PKA) and subsequent phosphorylation of target proteins.
- –The major activity of PKA in developing germ tubes is the mobilization of carbohydrates and lipids to the appressoria.
- Mitogen-activated protein kinases (MAPKs)
- Signaling pathways for infection-related development are also achieved through mitogen-activated protein kinases (MAPKs) and their upstream regulatory kinases.
- •All of these together comprise a functional unit that transmits input signals from the periphery of the cell to the cell nucleus to elicit the expression of appropriate genes.
- A MAP kinase, K1 or P1, regulates appressorium formation in response to a signal from the plant surface but it is also required for invasive growth or viability in its host plant.
- RECEPTOR-LIGAND INTERACTION
- Elicitors released during pathogen attack recognized by receptor of the plasma membrane.
- G-Proteins and Cyclic Nucleotides
- Guanine nucleotide binding proteins regulate a variety of physiological processes, including:

1. sensual perception,
2. protein synthesis,
3. hormonal regulation,
4. vesicular and nuclear transport,
5. cell growth and differentiation.

This superfamily includes members of small monomeric Ras-related proteins, the heterotrimeric G-proteins and the factors involved in protein synthesis. They act as molecular mediators,

- G-proteins are composed of three subunits: α, β, γ, where specificity mainly determined by α.The α-subunit consists of two domains: GTPase domain and α-helical domain. Activation results in conformational changes around so called switch I, II and III regions in GTPase- domain.
- G Proteins and cyclic nucleotides
- Signal Transduction
- After perception next step is signal transduction.

- Perception of environmental signals, mediated by specific receptors, likely initiates internal signal pathways.
- There are two major pathways by which signal can be transduced i.e. Via protein kinases & Via G-protein.
- Detection of signal by the host receptor activates the signal transduction pathways.
- Cyclic Nucleotide Metabolism-cAMP
- cAMP- second messengers of intracellular events
  •receptors- stimulates conversion of ATP to cAMP associated with G proteins
  binding with membrane bound receptor leads to activation of á-subunit of G protein
  •Gs-stimulates, Gi-inhibits– Adenylnyl cyclase (AC)
  •AC stimulation- catalyze ATP to cAMP conversion
  •activation cAMP- dependent protein kinase A –PKA
  •PKA phosphorylates protein- intracellular enzyme
  •either increase or decrease the activity
- G Protein –Coupled Receptor Signal Transduction Pathways
- Various second messenger system regulated by g-protein activation:
  •Adenylyl cyclase
  •Phospholipase C
  –Inositol Triphophate (IP3)
  –Diacylglycerol (DAG)
  Ion channel activity
  •The G-protein involves in most signaling processes
  •Heterotrimers: á, â and .
  Subunits
- Protein Phosphorylation
- Mitogen-activated Protein Kinase (MAPK) Cascades
- MAPK cascades fulfill essential functions in transduction of extracellular signals to cellular and nuclear responses.
- MAPK is activated by dual phosphorylation of a threonine catalyzed by MAPK kinase (MAPKK)
- MAPKKs activated by serine/threonine phosphorylation by a MAPKK kinase (MAPKKK).
- All MAPK pathways operate through sequential phosphorylation events to phosphorylate transcription factors and regulate gene expression.

Signal Receptor

- Ca^{2+} binding and Ca^{2+} dependent pathways
- Ca^{2+} -important second messenger. Regulatory functions of Ca^{2+} ion exerted mostly by the small cytoplasmic protein calmodulin.
- On binding Ca^{2+}, calmodulin undergoes a major conformational change that allows it to bind to calmodulin-dependent enzymes.
- Calmodulin-activated protein phosphatase and the calmodulin-dependent protein kinase (CDPK).
- Small change in cytoplasmic Ca^{2+} concentrations 'switch on' the active form of calmodulin.
- Ion Fluxes
- *Secondary Messengers*

Secondary messenger system exists in plants to transmit the primary elicitation signal of pathogen and/or host.

These are:

1. • Ca^{2+} ion influx
2. •Protein phosphorylation
3. •cAMP

4. •Active oxygen species
5. •Salicylic acid
6. •Methyl Jasmonic and Jasmonic Acid
7. •Ethylene
8. •Nitric Oxide

- Active oxygen species
- Several active oxygen species (AOS) involved in signal transduction like:
- •H_2O_2
- •Superoxide anion (O-2)
- •Singlet oxygen($1O_2$) and
- •Hydroxyl radical (OH)
- • H_2O_2 is the most important second messenger
- Salicylic Acid
- Salicylic acid (SA) is a phenolic compound commonly present in plant kingdom. One of the most important signal molecules.
- Salicylic acid acts locally in intracellular signal transduction. SA suppress the H_2O_2 degrading activity of catalase .
- Salicylic acid (SA) is an essential signal molecule for the onset of SAR.
- Methyl Jasmonic and Jasmonic Acid
- Naturally occuring compounds in plants

•JAs affect a variety of physiological processes and mediate plant responses to stresses by pathogen.

•Low concentration induce different enzymes involved in plant defense

•Phenylalanine ammonia-lyase (PAL) and lipoxygenase.

- Ethylene
- Ethylene is a volatile plant hormone

•Synthesized from amino acid methionine.

•Ethylene is produced upon wounding or infection by pathogen as well as by treatment by elicitors of defense responses.

- The increased production of ethylene is one of the earliest chemically detectable events in pathogen-infected plants or treated plants with elicitors.
- ISR is commonly regulated by jasmonic acid (JA)- and ethylene (ET)-dependent signaling pathways.
- Nitric Oxide
- NO signaling involves cyclic GMP- dependent pathways.
- •NO signaling in tobacco requires cGMP synthesis
- •NO activates MAP kinases in tobacco and Arabidopsis.
- •Biosynthesis of NO is catalyzed by nitric oxide synthase (NOS) enzyme.
- •ROS known to work with nitric oxide (NO) in defense responses.
-

SIGNAL RESPONSES -Massive changes in gene expression

- Plants response to pathogen infection is associated with massive changes in gene expression
- In Arabidopsis more than 2000 genes changed expression levels within 9 h of inoculation with the pathogen Pseudomonas syringae
- Signal termination
- The signal should terminate when it is
- induced and responded to.
- Conclusion
- The interaction between plant and pathogen are specific, complex and dynamic.
- Signals for activation of various defenses initiate in response to recognition.

- The outcome of interaction dependent on initial sensing of the other organism via exchange of molecular signal through signaling cascade and modified gene expression. Recognition is the first step by which response is generated which is involved in defense signal transduction.
- Conclusion
- Secondary messenger system required in plants to transmit the primary elicitation signals.
- Many secondary molecules are involved in signal transduction process.
- Signaling outcome leads to massive changes in gene expression.

- **LECTURE 3** GENE FOR GENE CONCEPT
- 1. Gene to gene concept. Different two theories
- Interaction between two "R" and "Avr' genes"
- 3. Biochemical basis gene to gene hypotesis
- Gene for Gene Concept
- "for each gene conditioning rust reaction in the host there is a specific gene conditioning pathogenicity in the parasite"
- For each resistance gene in the host there is a corresponding gene for avirulence in the pathogen conferring resistance and viceversa
- Flor (1946) showed correlation between inheritance of pathogenicity and resistance to linseed rust caused by Melampsora lini which is now commonly known as gene -for - gene hypothesis.
- that "for each gene conditioning rust reaction in the host there is a specific gene conditioning pathogenicity in the parasite.
- Gene for Gene Concept
- - = Incompatible reaction
- += Com- = Incompatible reaction
- - Incompatible reaction
- += Compatible reaction
- 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 .
- 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.
- HOW CAN WE EXPLAIN THIS BIOCHEMICALLY?
- PATHOGEN (Has general pathogenicity genes and specific avirulence (A1) or virulence (a1) gene)
  A1 a1
  Pathogen produces Pathogen produces no
  avrA1 gene product elicit specific (elicitor)
  Host (Has general resistance genes and specific resistance (R1) or lack of resistance (r1) genes) R1
- There are two different schools of thought pertaining to biochemical basis of gene-for - gene interactions.
- According to first specificity in gene- for- gene systems lies in susceptibility(Van der Plank, 1978)
- whereas to other specificity lies in resistance (Ellingboe, 1981).

- 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.
- 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
- 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.
- the biochemical explanation of gene for gene systems is based on the fact that specificity lies in resistance and not in susceptibility as proposed by Vander Plank (1978).
- Gene for- gene hypothesis
- Flor's gene-for- gene hypothesis is purely a hypothesis of identities.
- The resistance gene in the host and the corresponding virulence gene can be identified by this hypothesis.
- But it does not tell us about the gene quality. A second gene V for -gene hypothesis, which is an extension of Flor's hypothesis, tells us about the quality of genes.
- The quality of resistance gene in the host determines the fitness of matching gene in the pathogen to survive, when this gene for virulence is unnecessary.
- Unnecessary gene means- a gene for virulence in the pathogen population against which matching resistance gene in the host is not present.
- Reciprocally, the fitness of the virulence gene in the parasite to survive when it is unnecessary determines the quality of matching resistance gene in the host.
- For instance, there are ten or more genes in the host for resistance to late blight of potato, R1, R2, R3----------R10.
- Of these, the first four R1---R4 have been well studied. These genes have not been found of equal importance and strength.
- From the reports available in the literature, R4 has not been successfully used on its own by breeders although it has occasionally been used in combination with other genes.

- The R1 gene has often been used alone and it has given protection to the varieties against blight.
- The difference between these R genes is that virulences on R4 preexisted in population of Phytophthora infestans whereas virulences on R1 don't (Van der Plank, 1975).
- The ratio for virulence between R1 and R4 genes has been found to differ significantly. Thus there is difference in the quality of resistance genes R1 and R4.
- The quality of resistance gene in the host determines the fitness of matching gene in the pathogen to survive, when this gene for virulence is unnecessary.
- Unnecessary gene means- a gene for virulence in the pathogen population against which matching resistance gene in the host is not present.
- Reciprocally, the fitness of the virulence gene in the parasite to survive when it is unnecessary determines the quality of matching resistance gene in the host.

- **LECTURE** 4. *THE PRACTICAL USE OF GENE TO GENE RELATIONSHIPS*
- The quality of resistance gene in the host
- The quality of resistance gene in the host determines the fitness of matching gene in the pathogen to survive, when this gene for virulence is unnecessary.
- Unnecessary gene means- a gene for virulence in the pathogen population against which matching resistance gene in the host is not present.
- Reciprocally, the fitness of the virulence gene in the parasite to survive when it is unnecessary determines the quality of matching resistance gene in the host.
- For instance, there are ten or more genes in the host for resistance to late blight of potato, R1, R2, R3----------R10.
- Of these, the first four R1---R4 have been well studied. These genes have not been found of equal importance and strength.
- From the literature, R4 has not been successfully used on its own by breeders although it has occasionally been used in combination with other genes.
- The R1 gene has often been used alone and it has given protection to the varieties against blight. The difference between these R genes is that virulences on R4 preexisted in population of Phytophthora infestans whereas virulences on R1 don't (Van der Plank, 1975).
- The ratio for virulence between R1 and R4 genes has been found to differ significantly. Thus there is difference in the quality of resistance genes R1 and R4.
- The source of pathogenic variability in pathogens
- „The mutability of resistance and virulence genes
- „Why host resistance is expressed under one set of conditions and not others
- „Prediction of putative genotypes
- „Race nomenclature
- „Genetic dissection of complex loci
- „Cataloguing and storing of R genes in the form of plant seeds or cuttings and V genes in the form of pathogen strains
- „Management and deployment of resistance genes in space and tome
- „Detection of linkage and allelic relationship
- „Geographic distribution of R and V genes
- „Synthesis of multilines and multigene cultivars.
- Historical Overview
- Resistance in Mendelian fashion (Biffen, 1905)
- Pathogenicity is inherited in Mendelian fashion (Newton,1929)
- Surface Carbohydrate elicitor - receptor model
- (Albersheim and Anderson Prouty, 1975)
- Modified as elicitor- receptor model (Keen and Bruegger, 1977)
- Genetic and physiological evidences elicitor-receptor models (N T Keen ,1982)
- Dimer Model (Ellingboe, 1982)

- Historical Overview
- First Avr gene cloned from Pseudomonas syringae (Staskawicz et al., 1990)
- First R gene (Hm1) was cloned (Johal and Briggs, 1992)
- R proteins are dynamic and subject to intra-molecular interactions (Moffet et al., 2002)
- Several host proteins as pathogen virulence targets were discovered (Mackney et al. , 2003, Axtel et al., 2003, Rooney et al., 2005)
- The soft wired model to explain the interaction of NBS-LRR domains (Bekhaldir et al.,

Lecture 5 Biochemical relationship in resistant and susceptible cultivars

- sugars
- Biochemical reactions
- It is well known that the disease resistance mechanism is a complex phenomenon and in response to invasion by a disease causing organism, plant produces various kinds of biochemical reactions.
- Therefore, analysis of biochemicals in selected resistant and susceptible cultivars to spot blotch disease was carried out at two stages to understand their role in resistance/ susceptibility of wheat genotypes
- The disease reaction has been correlated with **the sugar level** in different crop plants.
- Generally **high levels of total sugars in the host** plant are stated to be responsible for disease resistance.

The amount of total sugar in resistant as well as susceptible wheat genotypes decreased as the age of the crop advanced

Sugars act as precursor for synthesis of: phenolics,

- **phytoalexins,**
- **lignin and**
- **cellulose .**
- **They** play an important role in defence mechanism of plants against invading pathogens.
- In the present study **(spot blotch infected in wheat)** also resistant genotypes recorded ***higher sugars.***
- **High sugar content in resistant wheat genotypes is responsible for lower development of the spot blotch disease.**
- **Spot blotch disease infected in wheat**
- *phytoalexins*
- *phytoalexins*
- *Lignin Synthesis*

Role of lignification in plant defense

- **Lignification** is a mechanism for disease resistant in plants.
- During defense responses, lignin or lignin-like phenolic compound accumulation was shown to occur in a variety of plant-microbe interactions.
- Plants assemble CWAs, also called papillae, at the sites of attempted penetration of biotrophic fungi such as powdery mildew.
- Lignin, a major component of cell walls of vascular plants, was shown to accumulate in CWAs and surrounding halo areas and is, thus, considered a first line defense against successful penetration of invasive pathogens.
- Lignification renders the cell wall more resistant to mechanical pressure applied during penetration by fungal appressoria as well as more water resistant and thus less accessible to cell wall-degrading enzymes.
- Lignification is essential for the structural integrity of plant cell walls and is crucial for plant development but the monomeric composition of lignin can vary depending on the developmental process:

- thus, defense lignin accumulated by an elicitor treatment was shown to be significantly different from lignin in vascular tissues, suggesting that lignin biosynthesis is differentially regulated.
- **Cellulose**
- **Structural Defenses**
- **The Plant Cell**
- All plant tissues contain pre-formed structural barriers that help limit pathogen attachment, invasion and infection.
- The **cell wall** is a major line of defense against fungal and bacterial pathogens.
- It provides an excellent structural barrier that also incorporates a wide variety of chemical defenses that can be rapidly activated when the cell detects the presence of potential pathogens.
- All plant cells have a **primary cell wall**, which provides structural support and is essential for turgor pressure, and many also form a **secondary cell wall** that develops inside of the primary cell wall after the cell stops growing.
- **The primary cell wall** consists mostly of **cellulose**, a complex polysaccharide consisting of thousands of glucose monomers linked together to form long polymer chains.

Cellulose

- **Structural Defenses**
- These chains are bundled into fibers called **microfibrils**, which give strength and flexibility to the wall. The cell wall may also contain two groups of branched polysaccharides:
- cross-linking glycans and pectins.
- **Cross-linking glycans** include **hemicellulose** fibers that give the wall strength via cross-linkages with cellulose. **Pectins** form hydrated gels that help "cement" neighboring cells together and regulate the water content of the wall. Soft-rot pathogens often target pectins for digestion using specialized enzymes that cause cells to break apart: these organisms are extremely common, and anyone who has seen fruits or vegetables become brown and "mushy" have seen these pathogens in action.
- **Cellulose**
- Many cell walls also contain **lignin**, a heterogeneous polymer composed of phenolic compounds that gives the cell rigidity. Lignin is the primary component of wood, and cell walls that become "lignified" are highly impermeable to pathogens and difficult for small insects to chew. **Cutin, suberin**, and **waxes** are fatty substances that may be deposited in either primary or secondary cell walls (or both) and outer protective tissues of the plant body, including **bark**.
- **Cellulose**
- **Cell walls** contain proteins and enzymes that actively work to reshape the wall during cell growth yet thicken and strengthen the wall during induced defense. When a plant cell detects the presence of a potential pathogen, enzymes catalyze an **oxidative burst** that produces highly reactive oxygen molecules capable of damaging the cells of invading organisms. Reactive oxygen molecules also help strengthen the cell wall by catalyzing cross-linkages between cell wall polymers, and they serve as a signal to neighboring cells that an attack is underway. Plant cells also respond to microbial attack by rapidly synthesizing and depositing **callose** between the cell wall and cell membrane adjacent to the invading pathogen. Callose deposits, called **papillae**, are polysaccharide polymers that impede cellular penetration at the site of infection, and these are often produced as part of the induced basal defense response.
- **Cellulose**
- Some plant cells are highly specialized for plant defense. **Idioblasts** ("crazy cells") help protect plants against herbivory because they contain toxic chemicals or sharp crystals that tear the mouthparts of insects and mammals as they feed. There are many classes of idioblasts including pigmented cells, sclereids, crystalliferous cells, and silica cells.

- **Pigmented cells** often contain bitter-tasting tannins that make plant parts undesirable as a food source. Young red wines often contain high levels of tannins that give wine a sharp, biting taste. **Sclereids** are irregularly-shaped cells with thick secondary walls that are difficult to chew: the rough texture of pear fruit (*Pyrus* spp.) is caused by thousands of sclereid **stone cells** that can abrasively wear down the teeth of feeding animals. Stinging nettles (*Urtica dioica*) produce **stinging cells** shaped like hypodermic needles that break off when disturbed and inject highly irritating toxins into herbivore tissues. Some stinging cells contain **prostaglandins**, hormones that amplify pain receptors in vertebrate animals and increase the sensation of pain.
- **Cellulose**
- **Crystalliferous cells** contain crystals of calcium oxalate that may tear herbivore mouthparts when chewed and can be toxic if ingested. Members of the genera *Philodendron* and *Dieffenbachia* are very common tropical house plants that contain large amounts of these cells. Humans and pets who chew the leaves of these plants may experience a burning sensation in the mouth and throat that is often accompanied by swelling, choking, and an inability to speak. For these reasons, species of Dieffenbachia are commonly called **dumb cane**. Grasses and sedges contain rows of **silica cells** in their epidermal layers which give strength and rigidity to the growing leaf blades and deter feeding by chewing insects.

Biochemical markers

- Biochemical markers are proteins produced by gene expression. These proteins can be isolated and identified by electrophoresis and staining.
- Isozymes are proteins that catalyze the same enzymatic reaction; they are revealed on electrophoregrams through a colored reaction associated with the enzymatic activity.
- They are the product of the various alleles of one or several genes.
- Monomeric (fig 1 a) and dimeric (fig 1b) isozymes are the most often used because the analysis of their segregation is easier.
- Isozymes are generally **codominant.**
- **Examples of isozyme systems routinely used in plant breeding are:**
- isozyme Aps-1 for its linkage to the tomato resistance to nematode (Meloidogyne spp) from Lycopersicon peruvianum (Rick and Fobes, 1974); isozyme Got2 for its linkage to the tomato resistance to Fusarium oxysporum race 3 from L penellii;
- isozyme Est5 for its linkage to the wheat resistance to
- Biochemical markers
- The post infection resistance (active resistance) might be correlated with specific biochemical changes in phenols, sugars, amino acids, phytoalexin accumulation, lignifications and activation of oxidative enzymes in host plant.
- Also, the accumulation of host synthesized new polypeptides is associated with the disease resistance.
- The new protein contents depended on host genotype and virulence genes of the pathogens

LECTURE 6. *Examples of isozyme systems routinely used in plant breeding for disease resistance*

The first molecular markers that initially were used extensively to study systematic of plants,
animals and insects were isozyme (or isoenzyme).
Isozyme analysis was initially utilized to study the taxonomy of plant pathogenic fungi**.**

The method involves extraction of crude proteins and separation by electrophoresis on starch, nondenaturing poly acrylamide gels (PAGE) or isoelectric focusing.

Isozyme zones are visualized after supplying the appropriate substrate necessary for the specific activity of each enzyme.
The resulting Isozyme banding patterns (zymograms) are used to infer genetic relationships based on interpretations of banding polymorphisms assuming that isozyme zones correspond to equivalent loci.
Analysis of isozyme variation found application in distinguishing fungi bearing overlapping morphological or cultural characters, such as species of the genus *Phytophthora.*

- By comparing 18 Isozyme loci, three species of Phytophthora (P. *cambivora, P. cinnamomi, P. cactorum*) could be separated and subsequently the systematic of twelve papillate Phytophthora species were re-evaluated

Because isozymes are post transcriptional markers, their expression is influenced by environmental changes leading to polymorphisms that might not reflect real differences at the molecular level.

This was the basic reason that application of isozyme in studying fungal plant pathogen variation was rather limited although in general they may provide satisfactory levels of polymorphic loci.

- Panama disease of banana (*Musa* spp) caused by the fungus *Fusarium oxysporum* f. sp. *Cubense* (FOC), is a serious constraint both to the commercial production of banana and cultivation for subsistence agriculture.
- Chemical control is not economically effective and is also hazardous to the environment and human health. Breeding for disease resistance is an alternative strategy, which leads to the development of resistance clones.
- Field evaluation is the most reliable method of screening for disease resistance, but it is demanding in terms of cost, manpower and space requirements.
- Another approach of screening hybrids at the sucker's stage (planting material) through biochemical markers is effective in early identification of resistant hybrids.
- The resistance mechanisms involving the role of phenol, PAL,
- oxidative enzymes like peroxidase (PO),
- polyphenol oxidase (PPO),
- superoxide dismutase (SOD),
- catalase and PR-proteins like chitinase,
- β-1-3 glucanase were studied.
- These enzymes showed relatively higher activity in resistant hybrids than susceptible hybrids.
- Isozyme analysis of peroxidase (PO) and
- polyphenol oxidase (PPO) was also carried out in cultivars and hybrids, which revealed the induction of specific isoforms in the resistant hybrids upon challenge inoculation.
- This could be a useful tool for early identification of *F. oxysporum* f. sp. *cubense* resistance banana clones.

LECTURE 7. RFLP markers. Application for Determination of plants resistance.

RFLP markers are the first generation of DNA markers and one of the important tools for plant genome mapping.

They are a type of Southern-Boltting-based markers.

In living organisms, **mutation events (deletion and insertion)** may occur at restriction sites or between adjacent restriction sites in the genome.

Gain or loss of restriction sites resulting from base pair changes and insertions or deletions at restriction sites within the restriction

fragments may cause differences in size of restriction fragments. These variations may cause alter-

nation or elimination of the recognition sites for restriction enzymes.

Most RFLP markers are **co-dominant and locus-specific**. RFLP genotyping is highly reproducible, and the methodology is simple and no special equipment is required.

By using an improved RFLP technique, i.e., **cleaved amplified polymorphism sequence (CAPS), also known as PCRRFLP,** high-throughput markers can be developed from RFLP probe sequences.

Very few CAPS are developed from probe sequences, which are complex to interpret.

Most **CAPS** are developed from SNPs found in other sequences followed by PCR and detection of restriction sites.

CAPS technique consists of digesting a PCR-amplified fragment and detecting the polymorphism by the presence/absence of restriction sites.

Another advantage of RFLP markers is that the sequence used as a probe need not be known. All that a researcher needs is a genomic clone that can be used to detect the polymorphism. Very few RFLPs have been sequenced to determine what sequence variation is responsible for the polymorphism. However, it may be problematic to interpret complex RFLP allelic systems in the absence of sequence information. RFLP analysis requires large amounts of high-quality DNA, has low genotyping throughput, and is very difficult to automate. Radioactive autography involving in genotyping and physical maintenance of RFLP probes limit its use and share between laboratories. RFLP markers were predominantly used in 1980s and 1990s, but since last decade fewer direct uses of RFLP markers in genetic research and plant breeding have been reported. Most plant breeders would think that RFLP is too laborious and demands too much pure DNA to be important for plant breeding. It was and is, however, central for various types of scientific studies.

LECTURE 8. Random Amplified Polymorphic DNA (RAPD).

RAPD is based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence [39]. These primers detect polymorphisms in the absence of specific nucleotide sequence information and the polymorphisms function as genetic markers and can be used to construct genetic maps. Since most of the RAPD markers are dominant, it is not possible to distinguish whether the amplified DNA segment is heterozygous (two different copies) or homozygous (two identical copies) at a particular locus. In rare cases, co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, may be detected.

The basic technique of RAPD involves (i) extraction of highly pure DNA, (ii) addition of single arbitrary primer, (iii) polymerase chain reaction (PCR), (iv) separation of fragments by gel electrophoresis, (v) visualization of RAPD-PCR fragments after ethidium bromide staining under UV light and (vi) determination of fragment size comparing with known molecular marker with the help of gel analysis software. A diagrammatic presentation of these steps is given in Figure 4. It is

important to note that RAPD technique requires maintaining strictly consistent reaction conditions in order to achieve reproducible profiles. In practice, band profiles can be difficult to reproduce between (and even within) laboratories, if personnel, equipment or conditions are changed. Despite these limitations, the enormous attraction of this technique is that there is no requirement for DNA probes or sequence information for primer design. The procedure involves no blotting or hybridizing steps. The technique is quick, simple and efficient and requires only the purchase of a thermocycling machine and agarose gel apparatus and relevant chemicals, which are available as commercial kits (e.g., Ready-To-Go RAPD analysis beads; GE Healthcare, Buckinghamshire, UK). Another advantage is the requirement for only small amounts of DNA (10-100 ng per reaction).

LECTURE 9. Microsatellites

Microsatellites, or simple sequence repeats (SSRs), are polymorphic loci present in DNA that consist of repeating units of one to six base pairs in length]. One common example of a microsatellite is a (CA) n repeat, where n is variable among different alleles. These markers often)n present high levels of inter- and intra-specific polymorphism, particularly when the tandem repeats number is 10 or greater. The repeated sequence is often simple, consisting of two, three or four nucleotides (di-, tri- and tetra- nucleotide repeats) and can be repeated many times. The basic principle of microsatellite is illustrated in Figure 6.

Microsatellites can be amplified for identification by PCR using the unique sequences of flanking regions as primers. The most common way to detect microsatellites is to design PCR primers that are unique to one locus in the genome and that base pair on either side of the repeated portion. Therefore, a single pair of PCR primers will work for every individual in the species and produce different sized products for each of the different length of microsatellites. The PCR products are separated either by slab gel electrophoresis or capillary gel electrophoresis in an automated sequencer. Microsatellites have proved to be versatile molecular markers, particularly for population analysis, but they are not without limitations. With the abundance of PCR technology, primers that flank microsatellite loci are simple and quick to use, but the development of correctly functioning primers is often a tedious and costly process. Microsatellites developed for particular species can often be applied to closely related species, but the percentage of loci that successfully amplify may decrease with increasing genetic distance. Microsatellite technique has recently been used to establish conservation strategy of endangered plants like *Calystegia soldanella* , *Tricyrtis ishiiana* and Galium catalinense subspecies acrispum.

- A microsatellite is a tract of tandemly repeated (i.e. adjacent) DNA motifs that range in length from two to five nucleotides, and are typically repeated 5-50 times.
- For example, the **sequence TATATATATA** is a dinucleotide microsatellite, and **GTC**GTCGTCGTCGTC is a trinucleotide microsatellite (with A being Adenine, G Guanine, C Cytosine, and T Thymine).
- Repeat units of **four and five nucleotides** are referred to as **tetra**- and **penta** nucleotide mo-

tifs, respectively.

- **Microsatellites are distributed throughout the genome.**
- **Many are located in non-coding parts of the human genome and are therefore do not produce proteins, however they can also be located in regulatory regions and within the coding region.**

LECTURE 10. *Retrotransposons - molecular markers.*

Transposons are mobile genetic elements capable of changing their locations in the genome. Transposons elements were discovered in maize almost 60 years ago. There are two classes of transposable elements. Class I known as retro-elements, such as retrotranspo sons.

Retrotransposons may be short interspersed nuclear elements or long interspersed nuclear elements and they are the mRNA-encoded element. In this class, a new copy of transposon is produced after each transposition event; however, the original copy remains intact at the donor site. Class II contains DNA transposons and their locations change by the cut-and-paste method in the genome.

Retrotransposons are an important class of repetitive DNA constituting 40%–60% of the entire plant genome. Retrotransposons belong to class I of transposon elements and they transpose through an RNA intermediate, which is not present in class II transposable elements.

Retrotransposons are grouped into two subclasses on the basis of their structure and transposition cycle. Long terminal repeats (LTRs) retrotransposons (LINE; long interspersed nuclear elements) and non-LTR retrotransposons (SINE; short interspersed nuclear elements).

These two subclasses can be differentiated based on the presence or absence of LTRs at their ends. LTR retrotransposons are widely distributed in the plant genome and in many crop plants, nearly 40%–70% of their DNA contains LTR retrotransposons. On the basis of integration, target site duplications of 4–6 bp are often produced by LTR retrotransposons. LTR retrotransposons contain ORFs, POL and GAG, as they are widely distributed within plant genomes. LTR retrotransposons are further divided into Ty1/copia and Ty3/gypsy retrotransposons on the basis of encoded gene order. Class II of trans posable elements is further divided into terminal inverted repeat (TIR) and non-TIR subclasses [60]. As transposon elements have great abundance and wide dispersion in the genome, they are an ideal source for the development of molecular markers. The follow ing are some important retrotransposon-based molecular markers.

LECTURE 11. *Inter simple sequence repeat (ISSR).*

This technique was developed by Zietkiewicz et al. It is based on amplification of DNA segments located in between two identical but oppositely oriented microsat Elite repeat regions, at a distance which allows amplify cation.

Primers used in this technique are also known as microsatellite and they might be di-, tri- and tetra-

or penta-nucleotide repeats. Normally long primers having a size of 15–30 bases are used in this technique. The pri mers used in Inter simple sequence repeat (ISSR) may be unanchored or more typically they are anchored at the 30 or 50 end having 1 to 4 degenerate bases, which are extended into the flanking sequences. ISSR allows the successful usage of high annealing temperature oC); (about 45–60 the amplified products are 200– 2000 bp long and can be visualized through agarose or PAGE. Segregating by simple Mendelian laws of inheritance, they are characterized as dominant markers ; however, they can also be used in the develop ment of co-dominant markers [49]. ISSRs are simple, easy to understand as compared to RAPD and there is no need of prior knowledge of DNA sequences.

However, they are dominant markers and they have less reproducibility with homology of co-migrating amplifica tion products

LECTURE 12. *Inter simple sequence repeat (ISSR). Cleaved amplified polymorphic sequences (CAPS) - molecular markers.*

This technique was developed by Flavell et al. In it, the presence or absence of retrotransposon sequences is investigated, which can be used as molecular marker.

In this technique, DNA amplification is achieved through a primer having 3' and 5' end regions flanking the retrotransposons insertion site. Detection of the presence of insertion is achieved through the development of primer from LTR. Sequence information about the regions flanking the retrotransposon insertion sites is needed in this technique and it results in the typing of a single locus as compared to other retrotransposon-based markers.

Agarose gel electrophoresis is used for the detection of polymorphism. Tagged microarray marker, which is based upon fluorescent microarray marker scoring, is used for high-throughput retrotransposon-based insertion polymorphism (RBIP) analysis.

Cleaved amplified polymorphic sequences (CAPS). markers

(CAPS) originally named as the PCR–RFLP markers due to combination of RFLP and PCR [71]. In this technique, target DNA is amplified using PCR and then its digestion is performed with restriction enzymes [72,73]. Agarose gel or acrylamide gel is used for the visualization of CAPS products. The primers used in this technique are developed from sequence information present in a databank of genomics or cloned RAPD bands or cDNA sequences.

CAPS markers are versatile and the possibility to fin

DNA polymorphism can be increased by combining CAPS with single-strand conformational polymorphism,

SCAR, AFLP or RAPD. CAPS markers are co-dominant markers and have been used in genotyping, map-based cloning and molecular identification studies

LECTURE 13. *High-Throughput Marker Systems. Single Nucleotide Polymorphisms (SNPs)*

SNP is a single nucleotide base **difference between two DNA sequences or individuals**.

SNPs are typically biallelic and arise either due to: **substitutions/point mutations** (**transversion and transition**), **or as a result of deletion/ insertion of** nucleotides and are detectable when similar genomic regions from different genotypes of different or same species are aligned

Although SNPs are less polymorphic than SSR markers because of their biallelic nature, they easily compensate this drawback by being:

abundant,

ubiquitous, and

amenable to high- and

ultra-high-throughput automation.

However, despite these obvious advantages, there were only a limited number of examples of application of SNP markers in plant breeding by 2009.

LECTURE 14. *Marker-assisted Selection for Disease Resistance: Applications in Breeding.*

- is an indirect selection process where a **trait** of interest is selected based on a **marker** **(morphological, biochemical or DNA/RNA** variation) linked to a trait of interest (e.g. productivity, disease resistance, abiotic stress tolerance, and quality), rather than on the trait itself. This process is used in **plant and animal breeding.**
- For example, using MAS to select individuals with disease resistance involves identifying a marker **allele** that is linked with disease resistance rather than the level of disease resistance.
- The assumption is that the marker associates at high frequency with the **gene or quantitative trait locus** (QTL) of interest, due to genetic linkage (close proximity, on the chromosome, of the marker locus and the disease resistance-determining locus). MAS can be useful to select for traits that are difficult or expensive to measure, exhibit low **heritability** and/or are expressed late in development. At certain points in the breeding process the specimens are examined to ensure that they express the desired trait.
- The following terms are generally less relevant to discussions of MAS in plant and animal breeding, but are highly relevant in molecular biology research:
- **Positive** selectable markers are selectable markers that confer selective advantage to the host organism. An example would be antibiotic resistance, which allows the host organism to survive antibiotic selection.
- **Negative** selectable markers are selectable markers that eliminate or inhibit growth of the host organism upon selection. An example would be thymidine kinase, which makes the host sensitive to ganciclovir selection.
- A distinction can be made between selectable markers (which eliminate certain genotypes from the population) and screenable markers (which cause certain genotypes to be readily

identifiable, at which point the experimenter must "score" or evaluate the population and act to retain the preferred genotypes). Most MAS uses screenable markers rather than selectable markers.

SITUATIONS THAT ARE FAVORABLE FOR MOLECULAR MARKER SELECTION

- There are several indications for the use of molecular markers in the selection of a genetic trait.
- In such situations that:
- the selected character is expressed late in plant development, like fruit and flower features or adult characters with a juvenile period (so that it is not necessary to wait for the organism to become fully developed before arrangements can be made for propagation)
- the expression of the target gene is recessive (so that individuals which are **heterozygous positive** for the recessive allele can be crossed to produce some homozygous offspring with the desired trait)
- there is requirement for the presence of special conditions in order to invoke expression of the target gene(s), as in the case of breeding for disease and pest resistance (where inoculation with the disease or subjection to pests would otherwise be required). This advantage derives from the errors due to unreliable inoculation methods and the fact that field inoculation with the pathogen is not allowed in many areas for safety reasons. Moreover, problems in the recognition of the environmentally unstable genes can be eluded.
- the phenotype is affected by two or more unlinked genes (epistatis). For example, selection for multiple genes which provide resistance against diseases or insect pests for **gene pyramiding.**
- The cost of genotyping (an example of a molecular marker assay) is reducing while the cost of phenotyping is increasing particularly in developed countries thus increasing the attractiveness of MAS as the development of the technology continues.

LECTURE 15. COMBINED MARKER ASSISTED SELECTION (MAS): THE DEVELOPMENT.

a- Reliability: Molecular markers should co-segregate or tightly linked to traits of interest, preferably less than 5 cM genetic distance. The use of flanking markers or
intragenic markers will greatly increase the reliability of the markers to predict phenotype.
b- DNA quantity and quality: Some marker techniques require large amounts and high quality DNA, which may sometimes be difficult to obtain in practice, and this adds to the cost of the procedures.
c- Technical procedure: Molecular markers should have high reproducibility
across laboratories and transferability between researchers.
The Level of simplicity and time required for the technique are

critical considerations. High-throughput simple and quick methods are highly desirable.

d- Level of polymorphism: Ideally, the marker should be highly polymorphic in breeding material and it should be co-dominant for differentiation of homozygous and heterozygous individuals in segregating progenies.

e- Cost: Molecular markers should be user-friendly, cheap and easy to use for efficient screening of large populations. The marker assay must be cost-effective in order for MAS to be feasible.

Screening or MAS alone in order to maximize genetic gain (Lande and Thompson 1990). This approach could be adopted when additional QTLs controlling a trait remain unidentified or when a large number of QTLs need to be manipulated. In some situations a marker assay may not predict phenotype with 100% reliability.

However, plant selection using such markers may still be useful for breeders in order to select a subset of plants using the markers to reduce the number of plants that need to be phenotypically evaluated. This may be particularly advantageous when the cost of marker genotyping is cheaper than phenotypic screening.

This was referred to as ‘tandem selection’ by Han et al. (1997) and ‘stepwise selection’ by Langridge and Chalmers (2005).

Simulation studies indicate that this approach is more efficient than phenotypic screening alone, especially when large population sizes

Are used and trait heritability is low (Hospital and Charcosset.1997). Zhou et al. (2003) observed in wheat that, MAS combined with phenotypic screening was more effective than phenotypic screening alone for major QTL on chromosome 3BS for Fusarium head blight resistance.

In practice, all MAS schemes will be used in the context of the overall breeding programme, and this will involve phenotypic selection at various Stages to confirm the results of MAS as well as to select for trait or genes for which the map location is unknown.