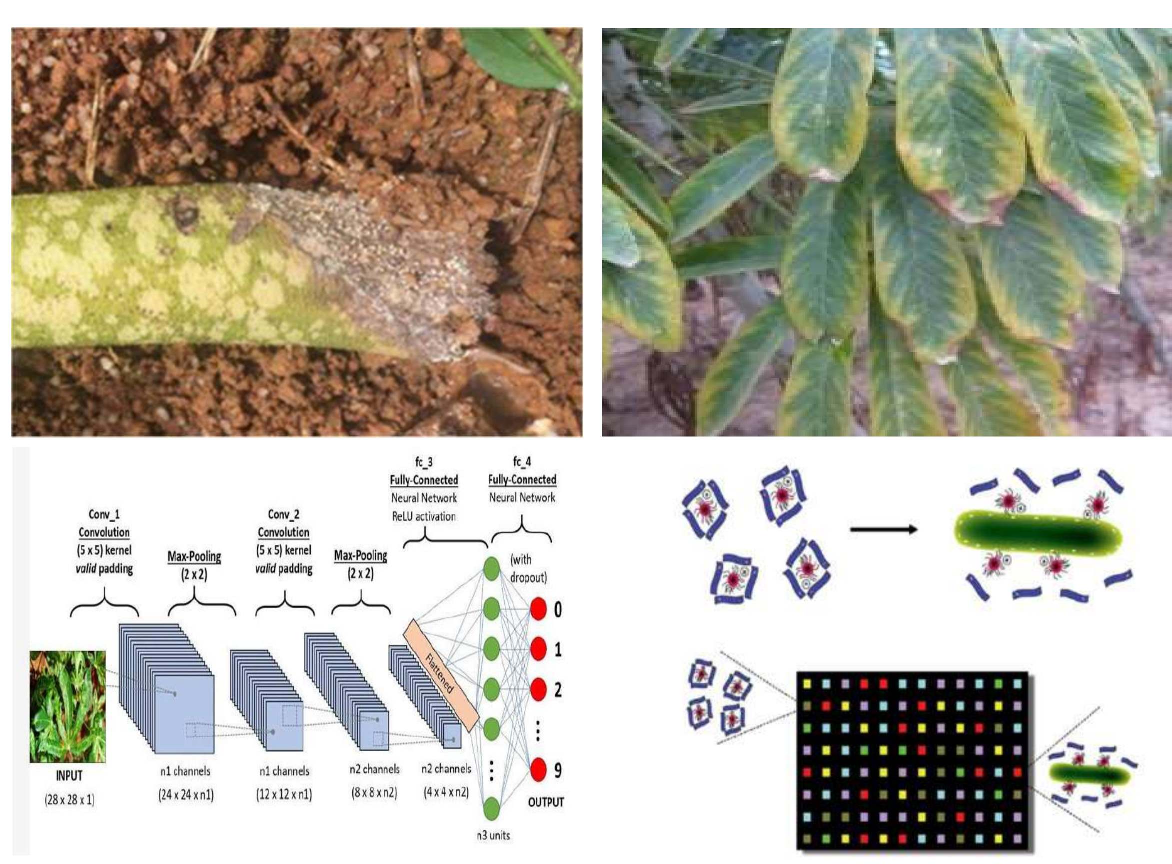
Training on Diagnosis of Plant Diseases Through Novel Approaches

February 19 to 23, 2024



भाकृअनुप - केन्द्रीय कंद फसल अनुसंधान संस्थान
ICAR - Central Tuber Crops Research Institute
श्रीकार्यम, तिरुवनंतपुरम, केरल
Sreekariyam, Thiruvananthapuram, Kerala

ICAR-Central Tuber Crops Research Institute

Sreekariyam, Thiruvananthapuram, 695017, Kerala, India

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February 19 to 23, 2024

Programme schedule

Theory Sessions

S.No	Date and Time (hrs)	Topic	Resource persons
19.02.2024 (9.30-10.00)		**Inaugural program**	
1	19.02.2024 (10.00 to 11.00)	Research activities at ICAR-CTCRI	Dr.G.Byju Director, ICAR-CTCRI
2	19.02.2024 (11.00 to 12.00)	Plant fungal diseases and conventional diagnosis	Dr. Veena SS Principal Scientist, ICAR-CTCRI
3	20.02.2024 (9.30 to 10.30)	Plant viral diseases and conventional diagnosis	Dr. M. L. Jeeva Principal Scientist, ICAR-CTCRI
4	20.02.2024 (10.30 to 11.30)	Serodiagnosis of plant diseases	Dr. K. Umamaheswaran Retd. Professor, KAU
5	20.02.2024 (15.30- 16.30)	Nucleic acid based diagnosis of plant diseases	Dr. Arutselvan Scientist, ICAR-CTCRI
6	21.02.2024 (09.30 to 10.30)	Nano technology in plant disease diagnosis	D. T. Makeshkumar Principal Scientist & Head, ICAR-CTCRI
7	21.02.2024 (14.30 to 15.30)	Nutrient deficiencies to differentiate from plant diseases	Dr. K. Susan John Principal Scientist, ICAR-CTCRI
8	22.02.2024 (09.30 to 10.30)	Applications of diagnosis in plant disease management	Dr. P. Lavakumar Head, Germplasm Health Unit, IITA
9	22.02.2024 (10.30 to 11.30)	Next generation sequencing in plant disease diagnosis	Dr. Stephen Winter Head, Plant Virus division, DSMZ
10	23.02.2024 (09.30 to 10.30)	Molecular and Bioinformatics approaches in plant disease diagnosis	Dr. J.Sreekumar Principal Scientist, ICAR-CTCRI
11	23.02.2024 (10.30 to 11.30)	Role of AI and IOT in plant disease diagnosis	Dr. V.S.Santhosh Mithra Principal Scientist, ICAR-CTCRI
23.02.2024 (16.00 -17.00)		**Valedictory program**	

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Practical Sessions

S.No	Date and *Time	Topic	Resource persons
1	19.02.2024	Isolation of nucleic acids from diseased samples	Dr.B.G.Sangeetha & Ms.Divya K
2	20.02.2024 & 21.02.2024	Direct Antibody Coating -Enzyme linked Immunosorbant assay (DAC-ELISA)	Dr.S.Karthikeyan & Mrs.Merlin Grace Cherian
4	20.02.2024 & 21.02.2024	Polymerase chain reaction and Reverse transcription-PCR	Dr.P.S.Hareesh
5	21.02.2024	Real time PCR	Dr.Sangeetha G & Summaya M
6	22.02.2024	Rolling circle amplification	Dr.T.R.Resmi
7	23.02.2024	Loop mediated isothermal amplification	Dr.Pravi Vidyadharan

* Time will be intimated during theory classes

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Plant Fungal Diseases and Conventional Diagnosis

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Introduction

Crop plants are infected by numerous fungal pathogens from seedling stage to the seed maturing stage under the natural conditions, either singly or in combination with other kinds of microbial plant pathogens. Fungi are the most diverse group of plant pathogens accounting for 70–80% of plant diseases (Persley, 1993). Over 20,000 species of fungi are parasitic in nature and can cause diseases in crops and plants. Fungal diseases in crop plants result in a significant reduction in both quality and yield, often leading to the loss of an entire plant. The fungal diseases can, at times, seriously compromise food security. For example, *Phytophthora infestans* caused the widespread devastation of potato crops in Ireland and northern Europe beginning in 1845, leading to the Irish Potato Famine.

Detection of fungal pathogen

In order to minimize the losses, it is essential to detect and precisely identify the pathogens at an early stage. The ability to detect, identify and quantify plant pathogens accurately is the milestone of plant pathology. The reliable identification of the organisms responsible for a plant disease is an essential prerequisite for the implementation of disease management strategies. Therefore, in the diagnosis of plant fungal infections, it is essential to carry out a precise disease detection process. Because many fungal pathogens produce similar symptoms and therefore it is important to be able to distinguish between different species.

Conventional methods of fungal pathogen detection

Traditional techniques are the "gold standard" in fungal detection which relies on visual symptoms, culture-based isolation of the fungi in pure cultures using appropriate media and identification of the putative pathogen based on morphological and biochemical characteristics. In recent times, with the advancement of biotechnology, molecular and immunological approaches have revolutionized fungal disease detection. But the drawback lies in the fact that these methods require specific and expensive equipments.

Visual examination of diseases

Traditional method for plant fungal pathogen involves interpreting visual symptoms of disease (e.g., spots, blight, galls, tumors, cankers, wilts, rots or damping-off), followed by isolation of pathogen and microscopy techniques. Visual examination has become more accurate, reliable and has been the subject of intensive research and investigation due to the availability of detailed guidelines and standards required for assessment (Nutter and Guan, 2001). Nevertheless, visual examination is always subject to an individual's experience and therefore the results are not always conclusive i.e., these can be affected by temporal variation. Moreover, visual identification procedure needs expertise in plant pathology.

Signs and symptoms of fungal pathogens

The most distinctive and easily identifiable characteristics of fungal infections are the physical presence of signs of the pathogen. A sign of plant disease is physical evidence of the pathogen. A symptom of plant disease is a visible effect of a pathogen on the plant. Signs include hyphae, mycelia, fruiting bodies and spores of the fungal pathogen are significant clues to proper identification and diagnosis of a disease. The fruiting bodies of fungi range from microscopic to macroscopic. They come in many shapes and configurations and have their individual characteristics. The fruiting bodies, along with spores, and mycelium, in most cases can lead to an accurate identification of the disease.

The following symptoms are common in fungal infections whether alone or in combination with other fungal pathogens.

Leaf Spots Leaf spots are very common in both biotic and abiotic plant disorders. Fungal leaf spots often take the form of localized lesions consisting of necrotic and collapsed tissue. Leaf spots can vary in size and are generally round and concentric, but can be ovoid or elongated on both leaves and stems of the host. The typical fungal leaf spot will have a "bulls-eye-like" appearance consisting of roughly concentric rings that may display zones of different colors such as yellow, red or purple, and will often have a tan center.	
Anthracnose An ulcer-like lesion that can be necrotic and sunken. These lesions can appear on the fruit, flowers and stems of the host. On mango, anthracnose symptoms occur on leaves, twigs, petioles, flower clusters (panicles), and fruits. On leaves, lesions start as small, angular, brown to black spots that can enlarge to form extensive dead areas.	
Damping Off Rapid collapse and death of very young seedlings. Either the seed rots before emergence or the seedling rots at the soil line and falls over and dies. Several soil-born fungi cause this disease. The most common genera involved are Fusarium, Rhizoctonia and Pythium.	

Blight Rapid generalized browning and death of leaves, floral organs, stems and branches. Blights can refer to both biotic and abiotic disorders.	
Soft and dry root rots Rot and disintegration of fleshy leaves, roots, tubers and fruits.	
Canker A localized necrotic lesion on woody tissue, often sunken	
Dieback Progressive death of shoots and twigs generally starting at the tip of the infected plant part.	
Wilts Generalized loss of turgidity as in vascular wilts. Both woody and herbaceous plants are subject to wilts Fusarium wilt	

Mildews Mycelium, fruiting bodies visible and the attack cause necrosis. Powdery Mildews, Downey Mildews	
Rusts Infected plants will most of the time have many small lesions on stems or leaves, usually a rust color but can also be black or white.	
Smuts Mycelium or black spores on seeds, in the form of galls or seeds "replaced" by spores.	

Culturing method of identification

Culturing method involves isolation of fungi using appropriate artificial media grown onto various artificial media under a variety of conditions followed by microscopy techniques. Microscopic observation involves diagnosis of pathogen based on its morphological characteristics such as spore morphology, sporulation patterns, production and characteristics of sporulating structures producing asexual and sexual spore forms, which are used for taxonomic classification of fungi (Narayanasamy, 2011). Culture methods have been used for detection of wide range of fungal pathogens. Examples include species of Fusarium, Colletotrichum, Phytophthora etc.

Although this method is cheapest and simplest, the accuracy and reliability of the method depends on the experience and skill of the person making the diagnosis (McCartney et al., 2003). Moreover, this method is time-consuming as it usually takes few days or weeks for confirmation and thus is not suitable for rapid pathogen diagnosis. In spite of the disadvantages, culture methods are still used in combination with other pathogen detection methods like biochemical-based method to yield better results.

Proving pathogenicity- Koch's postulates

Koch's postulates are four criteria designed to establish a causal relationship between a causative microbe and a disease. The postulates were formulated by Robert Koch and Friedrich Loeffler in 1884 and refined and published by Koch in 1890. Koch applied the postulates to establish the etiology of anthrax and tuberculosis, but they have been generalized to other diseases.

Postulate 1: The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.

Postulate 2: The microorganism must be isolated from a diseased organism and grown in pure culture.

Postulate 3: The cultured microorganism should cause disease when introduced into a healthy organism.

Postulate 4: The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Plant Viral Diseases and Conventional Diagnosis

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The livelihoods of millions in developing countries rely on agriculture, particularly staple crops like rice, wheat, maize, banana, cassava, potato, and sweet potato. However, these crops are vulnerable to biotic stresses, notably viral pathogens, leading to substantial economic losses. Viral diseases, transmitted by insect vectors, spread rapidly with no effective chemical controls. In India, diseases like banana bunchy top, cassava mosaic, and various viral diseases in pulses, vegetables and oil crop cause significant annual losses, impact the economic status of dependent communities. The lack of chemical solutions exacerbates the threat posed by viral pathogens, emphasizing the need for ongoing research on diagnosis and effective management strategies to safeguard food security and the livelihoods of agricultural communities.

Viral diseases affecting plants in the developing nations are very important because the damage caused to the staple food crops such as rice, wheat and maize, will lead into food and nutritional insecurity. There are 23 viruses affect rice crop, of which rice tungro disease caused by *Rice tungro bacillifiom virus* and *Rice tungro spherical virus* is an economically significant disease in India. PCR based test has been developed for the detection of RTBV In case of wheat more than 44 viruses have been reported but mostly from temperate countries and none of the reported viruses are very serious in developing world. Maize is the third largest food crop grown widely in developing countries of Africa and China and India. Though more than 57 viruses have been reported to infect the crop, maize streak disease caused by maize streak virus, a geminate particles containing ss DNA virus transmitted by leaf hoppers can reduce the yield drastically. *Maize streak vir*us is limited to Africa, Madagascar and Marutious. Important viral diseases occurring India and the virus species, and its genus are furnished in **Table 1**

Table 1.Nationally important viral diseases of crops and the yield loss they cause

Crop	**Disease**	**Yield loss (%)**	**Virus**	**Virus genus**
Banana	Bunchy top	100	*Banana bunchy top virus*	*Babuvirus*
Cassava	Mosaic	18-25	*Indian cassava mosaic virus*	*Begomovirus*
Chilli	Mottling and deformation	Upto 50	*Chilli Veinal Mottle Virus*	*Potyvirus*
Citrus group of plants	Decline (quick and slow), stem-pitting,	0 to 100	*Citrus Tristeza Virus*	*Closterovirus*

Crop	Disease	Yield loss (%)	Virus	Virus genus
	and seedling yellows			
Cotton	Leaf curl	68-71	*Cotton leaf curl virus*	*Begomovirus*
Groundnut	Bud necrosis	>80	*Groundnut bud necrosis*	*Tospovirus*
Mung Bean Black gram Soya bean	Yellow mosaic	21-70	*Mung bean yellow mosaic virus*	*Begomovirus*
Papaya	Ring spots on leaves and fruits	Upto 70	*Papaya Ringspot Virus*	*Potyvirus*
Pigeon pea	Sterility mosaic	>80	*Pigeon pea sterility mosaic virus*	*Tenuivirus*
Potato	Mosaic	85	*Potato virus Y*	*Potyvirus*
Rice	Rice tungro	10	*Rice tungro badna virus* *Rice tungro spherical virus*	*Badna virus and* *Waika virus*
Sugarcane	Mosaic	20 -30	*Sugarcane mosaic Virus*	*Potyvirus*
Sun flower	Necrosis	12-17	*Sunflower necrosis virus*	*Ilarvirus*
Tobacco and other solanaceous crops like tomato and potato	Mosaic	20-100	*Tobacco mosaic Virus*	*Tobamovirus*
Tomato and many cucurbits	Leaf curl	40-100	*Tomato leaf curl virus*	*Begomovirus*

Diseases caused by *Tospovirus*

Viruses belonging to genus, Tospovirus; family, Bunyaviridae is a cause of concern for field, horticultural and ornamental crops grown in developing countries. The type species of this genus is Tomato spotted wilt virus has a broadest host range. The other viral diseases caused by Tospovirus are Groundnut ringspot tosposvirus, Impatient necrotic spot virus, Watermelon silver mottle virus,

watermelon bud necrosis, Iris yellow spot virus and Ground nut bud necrosis virus. Tospoviruses are transmitted by thrips speices in a circulative and propagative manner.

Diseases caused by *Begomovirus*

The virus belonging to the family *Geminviridae* is one of the economically important groups of plant pathogens emerging worldwide. *Begomovirus* are either monopartite or bipartitie having ss DNA as their genome. The important diseases caused by this genus are cassava mosaic, leaf curl in cotton and tomato, yellow vein mosaic in okra, yellow mosaic in grain legumes, and yellow mottle in cucurbits. This virus is transmitted by whiteflies in a persistent manner.

Diseases caused by *Potyvirus*

Potyviridae is the largest single taxonomic group of plant viruses. Potyvirus genus is as old as flowering plants. Potato virus Y, *Bean yellow mosaic virus*, *Bean common mosaic virus*, *soybean mosaic virus*, *Chilli vein mottle virus*, *Papaya ring spot virus*, *Sweet potato feathery mottle virus*, *Sugarcane mosaic* and *streak mosaic viruses* and *Banana bract mosaic virus* are some of the important viruses affecting crops in India. This virus is transmitted by many species of aphids in non-persistent manner. This virus can also spread mechanically through sap. *Rice necrosis mosaic virus* belongs to *Bymovirus* genus of *Potyviridae* also an emerging plant disease in India. A lot of research papers have been appeared for the detection of this group of viruses. *Potyviurs* group specific antiserum and degenerate primers are available for the detection of wide number of viruses infecting various plant speices. Multiplex PCR approach, real time PCR, microarray and dip stick technologies are available for the detection of *potyviruses*.

Diseases caused by *Ilarvirus*

This genus contains ss RNA genome belongs to family, *bromoviridae*. *Tobacco streak virus* is the type species. Sunflower necrosis is one of the serious diseases leading to great loss to the growers in India. Apple mosaic and prunus necrotic ring spot are some fruit tree infecting ilarviruses. Ground nut stem necrosis caused by *tobacco streak virus* became a major problem in Andhra Pradesh. Group specific polyclonal antiserum and specific primers have been reported to detect the virus in many plant species.

Diseases caused by *Cucumovirus*

Cucumber mosaic virus belongs to *cucumovirus* is one of the dangerous virus specices in the earth. It infects more than 1000 plant species belonging to 85 plant families and 365 genera. This virus is transmitted mechanically through sap and also by several aphid species. Crops like banana, black pepper, oranamental crops, cucurbits, tomato and other vegetable crops, pulses and oil seeds are affected by this virus. Cucumber mosaic virus contains two sub groups and which can be detected using degenerate primers. The other viruses belongs to this genus are *peanut stunt virus* and *tomato aspermy virus*. ELISA, PCR, Real time PCR, Microarray and dip stick techniques have been reported to detect CMV.

Diseases caused by *Badnavirus*

This genus is of recent occurrence in India. The bacilliform DNA virus belongs to genus *badnavirus* and family, *Caulimoviridae*. The important viral diseases caused by badnaviruses in India are banana streak, sugarcane yellow mosaic, citrus yellow mosaic, rice tungro disease, black pepper yellow mottle and yam yellow mosaic. These viruses are transmitted by mealy bug species in a persistent

manner. Among these viral diseases the banana streak caused by many species of BSV are more diverse and the viral genomes are known to have integration into the host chromosome leading difficulties in detection of virus by PCR based approaches. PCR based detection has been widely used for detection of badnaviruses.

Important viruses in vegetatively propagated or mass propagated through Tissue culture technology in India

There are many horticultural crops are vegetatively propagated and those crops also being multiplied by tissue culture technology. Fruits crops like apple, banana, citrus and strawberry are multiplied through grafts/corms and tissue culture. The important viral diseases affecting these crops are *Banana bunchy top virus*, *bract mosaic and Cucumber mosaic virus*, C*itrus trizteza virus*, *Indian citrus ring spot virus* and *Apple mosaic virus* Besides fruit crops, potato, sugarcane, spice crops such as black pepper, cardamom, vanilla are also propagated vegetatively and can spread the viruses if they are indexed. Viruses belonging to different genus are known to infect the sugarcane and spice crops. Ornamental such as orchids, gladiolus and lily etc., are multiplied through tissue culture. These crops are also known to be infected with many debilitating viruses which need to be detected before mass multiplication. Bamboo is another important forest plant multiplied successfully in tissue culture. These vegetatively or TC propagated crops also known to harbour many viruses which can harm the plant and reduce the production and productivity. Tissue culture can eliminate most of the pathogens belong to fungi, bacteria and also parasitic nematodes but viruses cannot be eliminated in shoot tip cultures. Plant Tissue Culture Technology offers great promise for the production of quality planting material on account of disease free and true to type plants produced through micropropagation techniques. The need for a certification programme for the tissue culture plants is imperative since inadvertent micropropagation of virus infected plants will not only result in its poor performance, but also in undesirable spread of viruses wherever such plants are grown. In India, the certification programme for tissue culture raised plants has been introduced for which virus diagnostics is mandatory. The tissue culture production units have to get the approval from the certification agency for quality certification of the TC plants. There are many more crops like tuber crops and fruit crops like papaya have not been included in the certification because they are not commercially exploited by tissue culture industries.

Plant virus diagnostics

Diagnostic techniques for viruses fall into two broad categories: biological properties related to the interaction of the virus with its host and/or vector (e.g., symptomatology and transmission tests) and intrinsic properties of the virus itself (coat protein and nucleic acid), conventional and advanced respectively. Availability of these diagnostic methods provides greater flexibility, increased sensitivity, and specificity for rapid diagnosis of virus diseases in disease surveys, epidemiological studies, plant quarantine and seed certification, and breeding programs.

Conventional diagnosis

Visual diagnosis based on symptoms

Visual diagnosis based on symptoms is a preliminary step, and confirmation often requires laboratory tests such as serological assays or molecular techniques. Additionally, environmental factors, nutrient deficiencies, and other stressors can sometimes mimic viral symptoms, emphasizing the need for a comprehensive approach in plant health assessment.

Symptoms on plants commonly are used to characterize a disease having viral etiology. Visual inspection is relatively easy when symptoms clearly are characteristic of particular disease. However, many factors such as virus strain, host plant cultivar/variety, time of infection, and the environment can influence the symptoms exhibited (Matthews 1980). Plants can also exhibit typical symptoms of viral disease such as bunchy top, mosaic pattern, ring spot, enation, curling of leaves etc., but there are virus-like symptoms as a response to unfavourable weather conditions, soil mineral/nutrient imbalances, infection by nonviral pathogens, damage caused by insect/mite/nematode pests, air pollution, and pesticides (particularly herbicides). Some viruses may induce no apparent symptoms or cause symptomless infection. In addition, different viruses can produce similar symptoms or different strains of a virus cause distinct symptoms in the same host. While symptoms provide vital information on virus diseases, adequate field experience is required when attempting to indentify the diseases based on symptomatology alone. Usually, it is necessary that visual inspection for symptoms in the field is done in conjunction with other confirmatory tests to ensure accurate diagnosis of virus infection. The major symptoms of viral diseases are given below.

Leaf Mottling and Mosaic Patterns: Irregular patterns of light and dark green areas on leaves caused by various viruses, such as *Tobacco mosaic virus* (TMV) or *Cucumber mosaic virus* (CMV).

Leaf Curling and Distortion: Curling, twisting, or distortion of leaves caused by *Tomato yellow leaf curl virus* (TYLCV) and other geminiviruses.

Stunting and Reduced Growth: Abnormal stunting, dwarfing, or reduced growth caused by numerous viruses, including *Potato virus Y* (PVY) and *Cauliflower mosaic virus* (CaMV).

Necrosis and Yellowing: Yellowing (chlorosis) or necrotic areas on leaves caused by *Bean common mosaic virus* (BCMV) and *Cucumber mosaic virus* (CMV).

Ring Spots and Lesions: Circular lesions or ring spots on leaves.

Viruses: Papaya ringspot virus (PRSV) and Ring spot virus in beans. The distinctive ring-like patterns help in the identification of specific viruses.

Wilting and Decline:

Sudden wilting or overall decline in plant health caused by viruses like *Citrus Tristeza virus, Cucumber mosaic virus* and *Tomato spotted wilt virus*

Transmission tests/ Bioassay

Virus detection and identification techniques originated with mechanical, graft, and vector transmission of the viruses to susceptible indicator plants (Jones 1993). Mechanical transmission by sap inoculation to herbaceous indicator plants can be done with minimal facilities and characteristic symptoms produced by these plants allow both the detection and identification of many viruses. The reliability of host-range tests for diagnosis can be increased with hands-on experience and by using a suitable range of plant species. Viruses that are not mechanically transmissible and viruses of tree fruit and small fruit can be diagnosed by vector transmission or grafting onto suitable indicator hosts. While these assays are used in many laboratories both for diagnosis and maintaining virus cultures, they are time and resource consuming, and beset with the same difficulties in discerning viruses based on symptoms expressed in the field.

Electron microscopy

Electron microscopy is a valuable tool in plant virus diagnosis due to its ability to provide high-resolution images of viral particles directly in plant tissues, aiding in the identification, classification, and understanding of viral infections

High Magnification and Resolution: Electron microscopes use a beam of electrons rather than light, allowing for much higher magnification and resolution. This enables researchers to observe structures at the nanoscale, making it possible to visualize individual virus particles.

Virus Morphology: Electron microscopy provides detailed information about the morphology of viral particles. Different viruses have distinct shapes and structures, such as helical, icosahedral, or complex forms. Examining the morphology aids in identifying and classifying viruses.

Direct Observation in Plant Tissues: In plant virus diagnosis, samples are prepared from infected plant tissues. Thin sections or negatively stained samples are examined under the electron microscope, allowing for the direct observation of virus particles within the cellular context.

Identification of Unknown Viruses: Electron microscopy is particularly valuable for detecting unknown or less common viruses. When faced with a new or unidentified pathogen, observing its morphology can provide initial insights into its classification and characteristics.

Complementing other diagnostic methods: While techniques like serological tests (antibody-based assays) and molecular methods (PCR, RT-PCR) are widely used for virus detection, electron microscopy offers a complementary approach. It provides visual evidence of viral presence, aiding in a comprehensive understanding of the infection.

Limitations: Despite its strengths, electron microscopy has some limitations, such as the need for specialized equipment, technical expertise, and the inability to identify specific viral strains. Molecular techniques are often required for precise strain identification.

Serodiagnosis of Plant Diseases

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The basis of serological technique is the affinity of specific antibody to its antigen. As viral pathogen's protein coat can effectively induce to produce antibodies in warm-blooded animals. These protein antibody reaction forms the basis of serological techniques. Kohler and Milstein (1975) first produced hybridomas cells to produce single kind of antibody (monoclonal antibodies), since then many serological techniques based on polyclonal and monoclonal antibodies have evolved to detect, quantify and to find the relationships in viral antigens.

Most of the traditional methods in early days involved direct observation of specific precipitates of virus and antibody either in liquid or in agar gels. But past two decades lot of development has taken place in modern virology in developing new diagnostic techniques based on antigen (virus) and antibody reaction.

Precipitation methods

Plant viruses' are polyvalent i.e. each particle can combine with many antibody molecules. A lattice structured precipitate forms between the virus media in tubes. In case of ring test, the diluted antiserum is placed in small glass tube over which the antigen is slowly overlaid. The diffusion takes place and a ring of precipitation forms at the juncture.

Immunodiffusion reaction in gels

It can be done in small tubes or agar plates. In plates the antiserum is placed in central well and antigen solution to be tested placed in surrounding wells. Both diffuse and a zone of precipitatory complex forms if they are related. These bands can be visually seed. In case immuno electrophoresis, a mixture of antigen is first separated by migration in an electric field in agar gel. Antiserum kept in trough parallel to the path of electrophoretic migration and the immunodiffusion is carried out.

Enzyme linked immunosorbent assays

Enzyme – amplified immunoassays have been developed to enhance the detectability of antigen – antibody reactions. Commonly used immunoassay is enzyme linked immunosorbent assay (ELISA). Clark and Adams (1977) developed this ELISA technique, since then many modifications have taken place to improve the sensitivity and reliability. ELISA is carried out in wells of polystyrene microtitre plates. Double antibody sandwich – ELISA developed by Clark and Adams in 1977 is still the best ELISA technique with regard to specificity. Before ELISA technology, many serological techniques were based on antibody – antigen reaction in liquid media or in agar gels. Tube precipitin test and precipitin ring tests were used in liquid media. Immuno diffusion tests were also widely used to study the relatedness of different viruses based on precipitin rings. The ELISA has the following advantages over other serological tests.

1. Very low concentration of virus (1-10 ng per ml) can be detected.
2. Very small amount of antibodies are required.
3. This ELISA test can be applied for both crude and purified virus preparation.

4. Highly suitable for large scale indexing.
5. Viruses can be quantified by method.
6. Cost wise cheapest for large scale testing.

Types of ELISA

1. Direct ELISA
2. Indirect ELISA

In the first procedure, the polystyrene coated well are first coated with specific IgG, and then the test sample is added to it, over which enzyme labeled primary antibody is added to bind with trapped virus. Then the substrate is added for colour development. Depending on the quantity of virus, the colour intensity will change. The colour developed can be read in colorimeter (ELISA reader) with approximate filters.

In indirect protocol, the enzyme is conjugated with anti globulin antibody (Universal conjugate), which is used to quantity IgG (specific antibody) attached with the test virus/antigen. Indirect ELISA can detect broad range of related viruses. Many variants of indirect ELISA protocols are available. Direct antigen coating – ELISA, F (ab') $_2$ ELISA, Triple antibody sandwich ELISA, protein A-ELISA.

Variants of ELISA

Variants of direct ELISA

a) Double antibody sandwich (DAS) assay:
b) Two step DAS assay:
   The test sample and the antibody enzyme conjugate preincubated is added.
c) DAS- assay based on antigen specific antibodies from two animal species
   In the first step, the specific antibodies or IgGs produced in animal species 1 are coated and in the third step the specific IgGs produced in animal species II and conjugated with enzyme are used.

Variants of indirect –ELISA

a) Direct antigen coating (DAC) assay:
b) Two step DAC assay: The specific antibody and anti IgG enzyme conjugate complex preincubated is added.
c) F(ab')$_2$ and antibody sandwich assay: Before the first step, F(ab')$_2$ fragment of specific IgG is added (additional step)
d) DAS- assay based on antigen specific antibodies from two animal species: Before the first step, the specific antibodies or IgGs produced in animal species I are coated. In the second step the specific antibodies or IgGs produced in animal species II are added and in the third step only anti Fc fragment of animal species II antibody conjugate is added.
e) Protein-A based DAS assay: Before the first step, two extra steps are introduced i.e adding of protein A in the microtitre plate, incubating and washing followed by adding specific IgG, incubating and washing. In the third step instead of anti Fc- antibody enzyme conjugate, Protein A enzyme conjugate is added.
f) Protein A based DAC assay: In the third step, instead of anti-Fc antibody enzyme conjugate, Protein A enzyme conjugate is added.

Dot immuno Binding Assay (DIBA), Electroblot immunoassay (EBIA or western blotting) and Immunosorbent Electron Microscopy (ISEM) are the important other immunological techniques for virus detection. Some of the protocols not covered in the practical notes are detailed below.

Immunosorbant electron microscopy

1. Extract 5-7 g leaf tissue in 18 ml of 200mM phosphate buffer (pH 6.0) containing 1 % Na2 SO3.
2. Filter, centrifuge for 10 min at low speed and discard pellet.
3. Add 1 ml 33% Triton X-100, mix well, and layer over 5 ml 30 % sucrose in 100mM phosphate buffer pH 7.2.
4. Centrifuge for 1 hr at 35000 rpm in Beckman Type 50.2 rotor.
5. Discard supernatant and rinse sides of tube with distilled water.
6. Resuspend pellet in 100 ul of 10 Mm phosphate buffer ph 7.2 containing 0.85 % NaCl.
7. Centrifuge for 8-10 min at 12000-15000 rpm in standard microfuge, discard pellet, retain partially purified extract for ISEM observation.
8. Dilute antiserum 1/1000 in 10 nM Tris-HCL (pH 7.4.) Place EM grid on a 10 µl drop of diluted antiserum. Incubate in a petridish moist chamber for 15-30 min at room temperature.
9. Place EM grid on a drop of partially purified extract, incubate overnight at 4°C.
10. Rinse with 10-20 drops of 2% sodium phosphotungstate (PTA), PH 6.8.
11. Examine the grid under Electron Microscope.

Double antibody sandwich ELISA (DAS-ELISA)

1. Coat ELISA plates with primary antibody (200µl / well) diluted in coating buffer and incubate the plates for 2 to 4 hrs at 37 °C or over night at 5 °C.
2. After incubation, the discard the contents and wash with PBS-T for about 3 minutes. Repeat wash thrice.
3. Add antigens or samples (200µl / well) and incubate 3-6 hr at 37°C or over night at 4°C
4. Wash the plate three times as in step 2 above.
5. Add 200µl of enzyme labeled (alkaline phosphatase) anti rabbit IgG (secondary antibody) diluted in antibody / conjugate dilution buffer to each well. Incubate at 37ºC for 3-6 hr or over night at 4°C
6. Wash the plate three times as in step 2 above.
7. Add 200µl of freshly prepared substrate (p-.nitrophenyl phosphate 0.6- 1.0 mg/ml of substrate buffer) to each well. Incubate at room temperature for 1- 2 hr or till the color development.
8. Stop the reaction (if necessary) with 50 µl of 3 M sodium hydroxide to each well.
9. Assess enzyme-mediated color reaction either by visual observation or measuring the absorbance at 405 nm using ELISA reader.

Triple antibody sandwich - enzyme linked immunosorbent assay (TAS-ELISA) detection

1. Coat ELISA plates with purified IgG of rabbit polyclonal antibody (100µl / well) diluted in coating buffer and incubate for 3 hrs at 37 °C
2. After incubation, the discard the contents and wash with PBS-T for about 3 minutes. Repeat wash and soak operation thrice, then empty plate and shake out residual liquid draining on a paper towel.
3. Add antigens 100µl / well diluted in extraction buffer, and incubate 2-3 hr at 37°C or over night at 4°C

4. Wash the plate three times as in step 2 above.
5. Add 200µl of blocking solution (5% dried skim milk powder dissolved in PBS-Tween - PVP) to each well. Incubate at 20ºC for 30min. to block polystyrene well reactive surfaces.
6. Drain the plate. Do not wash.
7. Add 100µl monoclonal antibody diluted in PBS-T - PVP to each well. . Incubate at 37ºC for 2-3 hr.
8. Wash the plate three times as in step 2 above.
9. Add 100µl of enzyme labeled (alkaline phosphatase) anti rabbit IgG (secondary antibody) diluted in antibody / conjugate dilution buffer to each well. Incubate at 37ºC for 3-6 hr or over night at 4°C
10. Wash the plate three times as in step 2 above.
11. Add 150µl of freshly prepared substrate (p-.nitrophenyl phosphate 0.6- 1.0 mg/ml of substrate buffer) to each well. Incubate at room temperature 1- 2 h or as long as necessary to observe reaction.
12. Stop the reaction (if necessary) with 50 µl of 3 M sodium hydroxide to each well

Access enzyme-mediated color reaction either by visual observation or intensity was read at 405 nm using ELISA reader

Nucleic acid based plant disease diagnosis

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Agriculturally important field crops and horticultural crops are infected with many fungal, bacterial, viral, phytoplasma, viroid and other phloem limited non-culturable bacterial pathogens. Among the obligatory plant pathogens, viral pathogens are very serious and require timely detection for quality planting material production, certification programmes and for quarantine purposes. Most of the viral pathogens affecting plant species contain RNA as their genome and the rest also contain DNA as their genome. As per the present data available, approximately 60 % known plant viruses contain single stranded positive sense RNA as their genome, 24% are ss DNA viruses, 4% each for ds DNA and ds RNA viruses,4%ss RNA with negative sense strand and only ss RNA with reverse transcriptase. Of late PCR based approaches are being used for detection of the virus, for which isolating the NA from infected plant is very important for detection of pathogens present in any plant species. In order to apply PCR based detection for these pathogens, a good protocol to isolate pathogen genome is necessary. The sensitivity and specificity of virus detection depends upon the quality of DNA/RNA obtained from infected plants. Isolating the NA from different kinds of tissues is an art and science. In case of Citrus, the woody nature prohibits applying simple methods of extraction of NA. Method described by Kobayashi *et al* .,(1998) was found superior than Dellaporta method, Soidum sulphite method and GenElute Plant genomic DNA miniprepkit, Sigma for the detection of *citrus yellow mosaic badna virus* from two citrus speices, acid lime and pummelo. Similarly vegetable species like Bhendi (*Abelmoshcus esculentus*) contains mucilaginous polysaccharides which interfere in the NA extraction. In case of RNA isolation from the fruit tissues of banana, EDTA and 2-mercaptoethonol have been used to remove the polyphenols and the polysaccharides can be precipitated with 0.1 vol of ethanol and $1/30^{th}$ vol of 3 M sodium acetate leaving the RNA in the solution. In this lecture notes the basic NA isolation aspects has been described

Basically, the NA isolation procedure from any plant cell contain three major steps, (1) removal of envelopes (cell walls/membranes) around the NA, (2) separation of NA from all other cell components (cell wall debris and metabolic substances), and (3) maintenance of integrity of NA during the procedure, *i.e.*, protection from nucleases and mechanical shearing. The age and the kind of the source tissue greatly affect the quality and yield of isolated DNA. Young, healthy and tender tissues, especially partially expanded leaves make an ideal choice as they can yield good quality and quantity of DNA due to a larger number of cells and less deposition of starch and secondary metabolites. In contrast, DNA extracted from mature leaves is reported to be of poor quality and low yield due to the presence of high concentrations of polyphenols, tannins, polysaccharides and other secondary metabolites, For DNA extraction, plant material is usually either collected fresh and directly subjected to processing, or is stored at –18ºC, –80ºC or in liquid nitrogen as frozen samples before use. Freezing prevents nuclease activity that would otherwise degrade the DNA in thawed tissue.

List of commonly used chemicals and its purpose in the extraction of NA

Chemical used	Purpose
CTAB	CTAB helps in precipitating DNA by forming a complex with it in a low ionic strength environment, and, at high salt concentration, it forms insoluble complexes with proteins and most acidic polysaccharides, leaving the nucleic acids in the solution, which then can be easily isolated
Guanidium isothiocyanate or along with Phenol	Powerful denaturant for proteins and inactivates RNase. Dissolves DNA, RNA and protein
PVP/PVPP	To eliminate polyphenolics as they act as adsorbents of polyphenols, especially at low pH. They form complexes with the polyphenolic compounds through hydrogen bonding, allowing the polyphenolics to be separated from the DNA, thereby reducing their levels in the product
NaCl	Suitable for the removal of polysaccharides from DNA solutions by increasing their solubility in ethanol.
β-mercaptoethanol	Prevents the polymerization of tannins that hinder the NA isolation process in a manner similar to polysaccharides
Spermidine	Used to precipitate DNA. Binds to phosphate backbone of NA
Triton X 100	Specifically lyses chloroplast and mitochondria but not Nuclei
proteinase K	Breaks a variety of peptide bonds and rapidly inactivate DNases and RNases in cell lysates
Solvents Phenol: chloroform	To remove protein, pigments and polyphenols
SDS	Removes protein
Lithium Chloride	Precipitate the RNA
Ethanol and Na acetate/ Potassium acetate	Precipitates the DNA and RNA will be in the solution
Diethyl pyro carbonate(DEPC)	Removes the RNase

Extraction or homogenisation

It is always better to use fresh young leaf samples for isolating the NA (RNA/ DNA) or freeze dried or frozen at -86^{0}C samples can also be used. Proper homogenization is necessary as it also reduces viscosity caused by high molecular weight compounds like complex carbohydrates. Efficient disruption of cell wall membranes and organelles is required to release all the RNA present in the sample. Frequent thawing of frozen samples would degrade the NA, hence handling the sample before homogenization is very crucial in successful isolation of NA from infected samples. Liquid nitrogen is the best to grind and homogenize any infected plant samples using a flash freezing procedure because it is rapid, and any type of tissues (both soft and hard tissue) can be used. It prevents cross contamination when multiple samples are being processed. Repeated removal of samples from storage and sub-sampling will reduce the quality of RNA. Immediately after grinding the tissues using pestle and mortor with LN2, the extraction buffer containing high salt (lysis buffer) is added which contain guanidine isothiocyanate to inactivate the RNases and in this step the viscosity of the extract reduces and makes the RNA to bind to the silica gel membrane. Besides the physical disruption methods for tissue homogenization, convenient chemical disruption methods are also available. This approach though expensive, circumvents the possibility of DNA getting sheared due to mechanical force applied. Amount of tissue for grinding varies from plant to plant and types of tissues.

Frequently encountered problems in NA isolation and remedies

Presence of proteins, polysaccharides, polyphenolics and secondary metabolites create difficulties while isolating the NA from infected tissues. In case of healthy tissues phenol accumulation will be much lesser that that of plant samples having pathogen infection. In case of medicinal plants, the secondary metabolites hinder the extraction of NA. In case of banana, betel wine, black pepper and mango, phenolics and polysaccharides create trouble in isolating the NA.

Polyphenols

Polyphenols are responsible for degradation of genomic DNA while isolating from plants. Polyphenols are extremely variable in their occurrence and type. During cell lyses, polyphenols come out of the vacuoles and are readily oxidized by cellular oxidases. The oxidized polyphenols undergo irreversible interactions with nucleic acids and causes enzymatic browning of the DNA pellet, thereby rendering it useless for most downstream processes. Polyvinyl pyrollidone (PVP) and polyvinyl polypyrollidone (PVPP) are the most commonly used chemicals to eliminate polyphenolics as they act as adsorbents of polyphenols, especially at low pH. PVP/PVPP forms complexes with the polyphenolic compounds through hydrogen bonding, allowing the polyphenolics to be separated from the DNA, thereby reducing their levels in the product. Antioxidants such as β-mercaptoethanol, BSA, sodium azide, ascorbic acid, DTT, sodium sulfite, sodium iso-ascorbate, *etc*., along with PVP are commonly used to deal with problems related to phenolics. β-Mercaptoethanol in particular is widely used, and prevents the polymerization of tannins that hinder the isolation process in a manner similar to polysaccharides. In plants with high levels of polyphenolics, such as tomato, grapevine, *Arabidopsis*, canola, cocoa, *etc*., the use of a specific inhibitor of polyphenol oxidases, such as DIECA is also reported. Glucose has also been used as reducing agent in cotton (*Gossypium hirsutum*) to avoid contamination and browning by polyphenolics.

Proteins / RNA

A substantial amount of proteins and RNA may get precipitated when isolating the DNA. RNA is usually purged using DNase free pancreatic RNase; if this is unavailable, lithium chloride can be effectively used for the purpose. Proteins are generally removed using chemicals like SDS, DTT and β-mercaptoethanol, which destroy the structural organization of proteins. Protein-hydrolyzing enzymes like proteinase K are also suitable for the purpose as they break a variety of peptide bonds and rapidly inactivate DNases and RNases in cell lysates, which facilitates the isolation of high molecular weight DNA. Conventionally proteins are removed by extracting with organic solvents like phenol and chloroform. The major drawback of this procedure is the caustic and toxic properties of phenol and chloroform, in addition to a considerable loss of the sample that is seen after phenol extraction. Grinding of plant materials in an SDS extraction buffer containing proteinase K may be required in case of protein-rich tissues, *e.g*., seeds.

RNase

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. RNases cannot be inactivated easily and even minute amounts are sufficient to destroy RNA. Isolating the RNA is much difficult than DNA due to the contaminating widely present RNases in the working environment. First and foremost thing is to decontaminate all the consumables is necessary for isolating the good quality RNA from the infected samples. Most of the plant viral genomes are RNA, hence care should be taken for isolating the RNA for the purpose of detection. RNA preparations from infected plant should be checked for its quality by running in the agarose gel

Commercial DNA/RNA isolation kits

There are many commercial kits are available for isolation of NA (RNA/DNA). Two widely used methods for RNA isolation are 1) TRizol method and 2)Pine Tree method. For DNA isolation Delloporta method, Potassium acetate method, Sarkosyl method and a many crop specific methods are also available. A remarkable advancement in this approach is the availability of commercial DNA isolation kits that are easy to use and provide fast and better results. These are equipped with mechanical cell disruptors (for fine grounding of tissue), lysis buffers, RNase, *etc*. Proteins and polysaccharides are removed by salt precipitation and centrifugation. The majority of these kits use high-quality resins or disposable chromatographic columns, which have different elution protocols. The resins rely on mixed ion-exchange/adsorption interaction for purification, use silica gel to which the DNA binds in a reversible manner in presence of chaotropic salts and elutes out in the column effluent when rehydrated by washing with aqueous buffers. Large scale automatable DNA mini-prep kits are offered by several companies: Qiagen, Sigma, Promega, Epibio, Cartagen, Roche, Epicentre biotechnologies, *etc*. Thus, pure DNA can be obtained within approximately1 h using these ready to use kits, but most of these are not cost effective enough to be used on a general laboratory scale

Assessment of quality of NA

DNA/ RNA preparation should be less in viscosity and clear in the solution. Spectrometric analysis of the NA is the cheapest and rapid method available for assessing the quality and quantity of NA (DNA/RNA) isolated. For DNA or RNA preparations the ratio of absorbance at 260/280 nm should be 1.8 and if it is lesser than 1.8 it indicates contamination with proteins and if the ratio is more than 2.0 indicate the contamination of DNA with the RNA . A ratio bet In order to know the impurities of polysaccharides and polyphenolics in the DNA preparations, additional absorption readings at 230 nm and 270 nm is necessary. The ratio A260/A230 must be greater than 1.8 and ratioA260/A270 should lie between 1.2 and 1.3 in DNA preparations free from contamination of polysaccharides and polyphenolics, respectively. The yield of DNA can be determined by using the assumption that an absorbance of 1 at 260 nm corresponds to 50 µg double-stranded DNA in 1 ml. Most of the viral NA is single stranded RNA or DNA, quantification using this method might give a false value.

General remarks on handling RNA

- Create and maintain an RNase free environment
- Proper microbiological, aspectic technique should be always used when working with RNA.
- Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination. Change gloves frequently and keep tubes closed whenever possible
- Keep isolated RNA on ice when used for downstream applications
- Use of sterile RNase free environment polypropylene tube
- Non disposable plastiware should be thoroughly rinsed with 0.1M NaOH, 1mM EDTA followed by RNA free water
- Chloroform resistant plastic ware can be rinsed with chloroform to inactivate RNase
- Glassware should be cleaned with a detergent, thoroughly rinsed with distilled water and treated with DEPC.Fill glassware with 0.1% DEPC in water, allow to stand over night @ 37 ºC and then autoclave to eliminate residual DEPC.
- Electrophoresis tank should be cleaned with detergent solution (0.5% SDS) thoroughly rinsed with RNase free water and then rinsed with ethanol allowed to dry.

- Water and other solution should be treated with 0.1% DEPC (Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hrs @ 37 ºC). Autoclave to remove any trace of DEPC.
- When preparing Tris buffer, treat water with DEPC first and then dissolve Tris to make the appropriate buffer and autoclave for 15 min to remove any residual of DEPC.

DNA & RNA isolation: Trouble shooting

Problem	Possible cause	Suggestion
RNA isolation		
Low RNA yield	• Insufficient disruption and homogenisation • Too much starting material • RNA is not solubilized completely	• Freezing and powdering the tissue in liquid nitrogen before disrupting • Reduce the amount of starting materials • To increase solubilization, pipette RNA pellet repeatedly in SDS or DEPC-treated water. Heat sample at 55°C for 10 to 15 min. Do not allow RNA pellet to dry completely.
Low 260/ 280 value	• To avoid contamination during the measuring the purity of RNA • The amount of sample used for homogenization may have been too small • There may have been contamination of the aqueous phase with the Phenol Phase • The final RNA pellet may not have been completely dissolved • Indicates possible polysaccharides carryover	• Use 10mM TrisCl (pH 7.5), not RNase free water, to dilute the sample before measuring purity • • Use appropriate amount of sample material • Be sure not to carry any of the organic phase with the RNA sample. Precipitate the RNA again with ethanol • To increase solubilization, pipet RNA pellet repeatedly in SDS or DEPC-treated water. Heat sample at 55°C for 10 to 15 min. Do not allow RNA pellet to dry completely. • Potassium acetate precipitation of polysaccharides: • Precipitation of polysaccharides with 30%

Problem	Possible cause	Suggestion
		ethanol at low salt concentration • Precipitation of polysaccharides with 20% ethanol and 0.5 M potassium acetate
RNA degradation	• Improper storage of RNA • Frozen tissue thawed in absence of buffer • RNase contamination	• Store isolated RNA at -70°C, not -20°C. • Add frozen tissue to buffer • Be certain not to introduce any RNases during the procedure or later handling.
DNA isolation		
Low DNA yield	• Insufficient disruption and homogenisation • Too much starting material • The final DNA pellet may not have been completely dissolved	• Freezing and powdering the tissue in liquid nitrogen before disrupting • Reduce the amount of starting materials • Completely dissolve the DNA pellet
If the A260/ 280 <1.70	• Phenol may not have been sufficiently removed from the DNApreparation.	• Try one or move wash of the DNA pellet with 10% ethanol-0.1 M sodium citrate solution
RNA contamination		• Use RNase enzyme during DNA isolation

Polymerase Chain Reaction (PCR) based plant disease diagnosis:

Polymerase Chain Reaction (PCR) is a widely used molecular biology technique that allows the selective amplification of a specific DNA sequence, enabling the generation of large quantities of that DNA segment. The method was first developed by Kary Mullis in 1983 and has since become a cornerstone in various applications, including genetic research, diagnostics, forensic analysis, and DNA cloning.

Principle of PCR:

PCR is based on the principle of in vitro DNA amplification, where a specific DNA region is replicated exponentially through a series of temperature-dependent enzymatic reactions. The key components of PCR include:

1. **DNA Template:** The target DNA sequence that needs to be amplified.
2. **Primers:** Short single-stranded DNA sequences designed to anneal to complementary sequences flanking the target region.
3. **DNA Polymerase:** Enzyme responsible for synthesizing a complementary DNA strand by extending the primers.
4. **Nucleotides (dNTPs):** Building blocks (adenine, thymine, cytosine, guanine) needed for DNA synthesis.
5. **Buffer Solution:** Provides an optimal chemical environment for the PCR reaction.
6. **Magnesium ions (Mg^{2+}):** Co-factor for DNA polymerase activity.

PCR occurs in cycles and involves three main steps:

1. Denaturation:

The reaction begins with the denaturation step, where the reaction mixture is heated to a high temperature (usually 94-98°C). This causes the DNA double helix to unwind, breaking the hydrogen bonds between complementary bases and resulting in the separation of the two DNA strands.

2. Annealing:

The reaction temperature is lowered to allow the primers to anneal to their complementary sequences on each DNA strand. This step typically occurs at temperatures between 50-65°C, depending on the melting temperature (Tm) of the primers.

3. Extension (Elongation):

DNA polymerase synthesizes a complementary strand by extending the primers using the original DNA strands as templates. This step occurs at a temperature optimal for the DNA polymerase enzyme (usually around 68-72°C).

These three steps constitute one PCR cycle. By repeating the cycle multiple times (usually 20-40 cycles), the targeted DNA region is exponentially amplified, resulting in a significant increase in the amount of the specific DNA sequence.

PCR Protocol:

Here is a general protocol for a standard PCR reaction along with the essential chemicals required**:**

Materials and Equipment:

- PCR machine (thermocycler)
- DNA template (containing the target sequence)
- Forward and reverse primers (short single-stranded DNA sequences flanking the target region)
- DNA polymerase enzyme
- Nucleotide mix (dNTPs: Adenine, Thymine, Cytosine, Guanine)
- PCR buffer (containing salts to provide optimal conditions for the enzyme)
- Magnesium chloride ($MgCl_2$, required for DNA polymerase activity)
- Distilled water
- Thermal cycler tubes
- Microcentrifuge tubes
- Thermal cycler program parameters (denaturation temperature, annealing temperature, extension temperature, and cycle number)

PCR Steps:

1. Set up the PCR reaction mix:

In a microcentrifuge tube, prepare a master mix containing the following components for each reaction:

- DNA template (1-100 ng/µL)
- Forward primer (0.1-1.0 µM)
- Reverse primer (0.1-1.0 µM)
- DNA polymerase (1-2.5 units) - Nucleotide mix (0.2-0.5 mM each dNTP)

- PCR buffer (1X concentration)
- $MgCl_2$ (1.5-3.0 mM)

2. Add distilled water to reach the desired reaction volume. Mix gently.

3. Aliquot the reaction mix into individual PCR tubes.

4. Add the DNA template:

- Pipette the DNA template into each reaction tube. Ensure proper mixing by pipetting up and down.

5. Place the PCR tubes in the thermal cycler:

- Secure the tubes in the thermal cycler block according to the manufacturer's instructions.

6. Program the thermal cycler:

- Define the PCR program with the following parameters:
- Denaturation: 94-98°C for 20-30 seconds
- Annealing: 50-65°C for 20-40 seconds (varies based on primer melting temperatures)
- Extension: 68-72°C for 20-120 seconds (depends on the length of the target sequence)
- Repeat the denaturation, annealing, and extension steps for a defined number of cycles (typically 20-40 cycles)

7. Run the PCR reaction:

- Start the thermal cycler, and the machine will cycle through the specified temperature steps for he defined number of cycles.

8. PCR product analysis:

- Analyze the PCR products using gel electrophoresis, DNA staining, and imaging systems.

Essential Chemicals:

1. DNA template: Contains the target sequence to be amplified.

2. Primers: Short, single-stranded DNA sequences complementary to the target region.

3. DNA polymerase: Enzyme responsible for synthesizing a complementary DNA strand.

4. Nucleotide mix (dNTPs): Individual deoxyribonucleotide triphosphates (dATP, dTTP, dCTP, dGTP) used for DNA synthesis.

5. PCR buffer: Provides optimal conditions for the DNA polymerase reaction.

6. Magnesium chloride ($MgCl_2$): Enhances the activity of DNA polymerase.

7. Distilled water: Used to adjust the reaction volume and create the master mix.

Following this protocol allows for the amplification of specific DNA sequences, making PCR a powerful tool in diagnostics of plant diseases.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) based plant disease diagnosis:

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a molecular biology technique that combines reverse transcription of RNA into complementary DNA (cDNA) with subsequent PCR amplification. It is widely used to study gene expression by quantifying the amount of RNA transcripts present in a sample and detecting RNA viruses.

Principle of RT-PCR:

The principle of RT-PCR involves two main steps: reverse transcription and polymerase chain reaction.

1. Reverse Transcription (RT):

- The RNA template is first reverse transcribed into complementary DNA (cDNA) using a reverse transcriptase enzyme. Reverse transcription involves the synthesis of a complementary DNA strand from an RNA template. The enzyme reverse transcriptase synthesizes the cDNA strand by adding nucleotides complementary to the RNA sequence.

2. Polymerase Chain Reaction (PCR):

- The cDNA generated in the reverse transcription step serves as the template for PCR amplification. PCR then proceeds as in conventional DNA PCR, with denaturation, primer annealing, and DNA synthesis (extension). The result is the exponential amplification of the cDNA, allowing for the detection and quantification of the original RNA.

Here is a general protocol for RT-PCR:

Materials and Equipment:

- RNA sample
- Reverse transcriptase enzyme
- Oligo(dT) primers or random hexamers
- RNase inhibitor
- PCR reagents (DNA polymerase, dNTPs, buffer)
- Primers specific to the cDNA target
- Thermal cycler
- PCR tubes

RT-PCR Steps:

1. RNA Extraction:

- Isolate total RNA from the biological sample using an appropriate RNA extraction method.

2. Reverse Transcription:

- Mix RNA with reverse transcriptase, oligo(dT) primers or random hexamers, and RNase inhibitor.
- Incubate the reaction mixture to allow reverse transcription of RNA into cDNA.
- Optionally, heat-inactivate the reverse transcriptase.

3. PCR Amplification:

- Use the cDNA as a template for PCR amplification.
- Prepare a PCR reaction mix containing DNA polymerase, primers specific to the cDNA target, dNTPs, and PCR buffer.
- Set up the PCR program with denaturation, annealing, and extension steps.

4. PCR Analysis:

- Analyze the PCR products using gel electrophoresis, real-time PCR, or other suitable methods.
- Quantify the amount of cDNA present in the sample.

Key Components of RT-PCR:

1. RNA Template:

- The RNA sample containing the target RNA sequences to be reverse transcribed and amplified.

2. Reverse Transcriptase:

- The enzyme responsible for synthesizing a complementary DNA strand from an RNA template.

3. Oligo(dT) Primers or Random Hexamers:

- Short single-stranded DNA sequences used as primers for initiating cDNA synthesis during reverse transcription.

4. RNase Inhibitor:

- An enzyme inhibitor used to protect RNA from degradation by RNases during the reverse transcription step.

5. DNA Polymerase:

- Enzyme responsible for synthesizing the complementary DNA strand during the PCR amplification step.

6. Primers:

- Short single-stranded DNA sequences that anneal to specific regions of the cDNA, flanking the target sequence for PCR amplification.

7. dNTPs:

- Deoxyribonucleotide triphosphates (adenine, thymine, cytosine, guanine) required for DNA synthesis during PCR.

RT-PCR is a powerful technique for studying gene expression, detecting RNA viruses, and quantifying RNA levels in various biological samples. Its ability to convert RNA into DNA and amplify specific targets makes it a valuable tool in molecular biology and diagnostics.

LAMP based plant disease diagnosis

"LAMP" which stands for Loop-mediated Isothermal Amplification is a simple, rapid, specific and cost-effective nucleic acid amplification method solely developed by Eiken Chemical Co., Ltd. It is characterized by the use of 4 different primers specifically designed to recognize 6 distinct regions on the target gene and the reaction process proceeds at a constant temperature using strand displacement reaction. Amplification and detection of gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65°C). It provides high amplification efficiency, with DNA being amplified 109-1010 times in 15-60 minutes. Because of its high specificity, the presence of amplified product can indicate the presence of target gene.

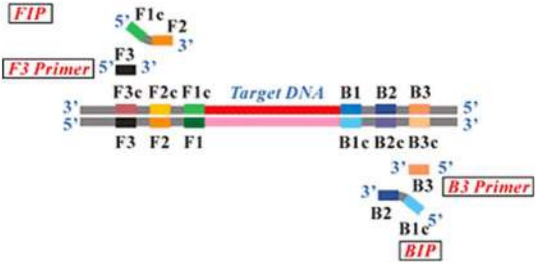
Characteristics

- There is no need for a step to denature double stranded into a single stranded form.
- The whole amplification reaction takes place continuously under isothermal conditions.
- The amplification efficiency is extremely high.
- By designing 4 primers to recognize 6 distinct regions, the LAMP method is able to specifically amplify the target gene.

- The total cost can be reduced, as LAMP does not require special reagents or sophisticated equipment's.
- The amplified products have a structure consisting of alternately inverted repeats of the target sequence on the same strand.
- Amplification can be done with RNA templates following the same procedure as with DNA templates, simply through the addition of reverse transcriptase.

Design of primers

Design 4 types of primers (described in detail below) based on the following 6 distinct regions of the target gene: the F3c, F2c and F1c regions at the 3' side and the B1, B2 and B3 regions at the 5' side.



FIP	:	Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end.
F3 Primer	:	Forward Outer Primer consists of the F3 region that is complementary to the F3c region.
BIP	:	Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.
B3 Primer	:	Backward Outer Primer consists of the B3 region that is complementary to the B3c region.

Main points of primer design

Proper primer design is crucial for performing LAMP amplification. The above primer regions can be determined by using the PrimerExplorerV5 (a special software to design LAMP primers) after considering the base composition, GC contents and the formation of secondary structures. Tm value can be obtained by Nearest Neighbour method.

The following is the main points of primer design:

1. Distance between primer regions

- The distance between 5' end of F2 and B2 is considered to be 120-180bp, and the distance between F2 and F3 as well as B2 and B3 is 0-20bp.
- The distance for loop forming regions (5' of F2 to 3' of F1, 5' of B2 to 3' of B1) is 40-60bp.

2. Tm value for primer regions

About 60-65°C in the case of GC rich and Normal, about 55-60°C for AT rich.

3. The stability of primer end

The dG calculated on 6bp from the following end regions should be less than -4kcal/mol, 5' end of F1c/B1c and 3' end of F2/B2 as well as F3/B3.

4. GC contents

About 50-60% in the case of GC rich and Normal, about 40-50% for AT rich.

5. Secondary structure

Primers should be designed so as not to easily form secondary structures. 3' end sequence should not be AT rich or complementary to other primers.

6. Others

If the restriction enzyme sites exist on the target sequence, except the primer regions, they can be used to confirm the amplified products.

Rapid DNA Extraction Protocol for Lamp Technique

1. Take 100mg of a young tomato leaf and cut it into small pieces and then grind it using a porcelain mortar and pestle in 400 µl of the CTAB buffer. Add more buffer until it reaches a final volume of 1200 µl in order to have enough homogenate to place into microfuge tube. Harvest the homogenate into 1.7 ml microfuge tubes.
2. Spin (13,500 rpm, 4 min, RT) using a microcentrifuge.
3. Transfer the supernatant into a new microfuge tube and add an equal volume of isopropanol (500 µl in our study) and mix gently by inversion.
4. Spin (13,500 rpm, 4 min, RT) using a microcentrifuge. Discard the supernatant Let the pellet air-dry.
5. Dissolve the DNA in 50 µl ddH_2O and store it at 4 °C for immediate use or − 20 °C for long-term storage.

Typical LAMP Protocol

Incubate the following reaction at 65°C for 30–60 minutes.

Component	25 µl Reaction	Final Conc
10X ThermoPol Buffer	2.5 µl	1X (contains 2 mM $MgSO_4$)
$MgSO_4$ (100 mM)	1.5 µl	6 mM (8 mM total)
dNTP Mix (10 mM)	3.5 µl	1.4 mM each
FIP/BIP Primers (25X)	1 µl	1.6 µM
F3/B3 Primers (25X)	1 µl	0.2 µM
LoopF/B Primers (25X)	1 µl	0.4 µM
Bsm DNA Polymerase, Large Fragment (8,000 U/ml)	1 µl	320 U/ml
Betaine	1 µl	0.8 M
DNA Sample	variable	> 10 copies or more
Nuclease-free Water	to 25 µl	
Total Reaction Volume		25 µl

General Guidelines:

1. A LAMP Primer Mix can be prepared with all 4 or 6 (with Loop) primers. A 25X Primer Mix should contain: 40 µM FIP, 40 µM BIP, 5 µM F3, 5 µM B3, 10 µM LoopF, 10 µM LoopB in TE or water.
2. Reactions should be setup on ice. If room temperature setup is desired, use *Bst* 2.0 WarmStart® DNA Polymerase (NEB #M0538).
3. If analyzing via agarose gel electrophoresis or other method requiring opening LAMP reaction vessels, setup secondary analysis area and equipment to avoid contamination.
4. Running a no-template control is strongly recommended to ensure amplification specificity.

5. If optimization is desired, try titrating Mg^{2+} (4–10 mM final) or *Bst* DNA Polymerase, Large Fragment (0.04–0.32 U/µl), or changing reaction temperature (50–68°C).

Quantitative PCR (qPCR) based plant disease diagnosis:

Quantitative Polymerase Chain Reaction (qPCR) is a powerful molecular biology technique used for the quantitative analysis of nucleic acids. It allows precise measurement of the amount of DNA or RNA present in a sample. qPCR is commonly used in gene expression studies, viral load quantification, and various other applications where accurate quantification of nucleic acids is essential.

Principle of qPCR:

The principle of qPCR is based on the real-time monitoring of the PCR amplification process. Unlike traditional PCR, qPCR allows for the continuous monitoring of the fluorescence signal during each PCR cycle. The fluorescence is generated by the binding of fluorescent dyes or probes to the amplified DNA or RNA. As the target DNA or RNA is amplified, the fluorescence intensity increases, and the cycle at which this fluorescence signal reaches a detectable threshold (Ct value) is proportional to the initial amount of the target sequence.

qPCR Protocol:

Materials and Equipment:

- DNA or cDNA template
- qPCR reagents (including DNA polymerase, primers, and probes)
- Fluorescent dyes or probes
- Thermal cycler with a real-time detection system (such as a fluorometer)
- qPCR tubes or plates

Steps:

1. Primer and Probe Design:

- Design specific primers ad fluorescent probes targeting the gene of interest.

2. Preparation of Reaction Mix:

- Prepare a master mix containing qPCR reagents, including DNA polymerase, primers, probes, and other necessary components.

3. DNA or cDNA Template Addition:

- Add the DNA or cDNA template to the reaction mix.

4. qPCR Cycling Conditions:

- Set up the qPCR program with denaturation, annealing, and extension steps, typically with a higher number of cycles compared to conventional PCR.

5. Real-Time Monitoring:

- Monitor the fluorescence signal during each PCR cycle in real-time. The real-time data are used to generate amplification curves.

6. Ct Value Determination:

- The Ct (Cycle threshold) value is the cycle number at which the fluorescence signal crosses a predefined threshold. The Ct value is inversely proportional to the initial amount of the target sequence.

7. Data Analysis:

- Analyze the qPCR data using specialized software to calculate the relative expression levels or absolute quantification of the target nucleic acid.

Key Components of qPCR:

1. Fluorescent Dyes or Probes:

- Fluorescent dyes, such as SYBR Green, bind to double-stranded DNA and emit fluorescence during amplification. Alternatively, fluorogenic probes, such as TaqMan probes, release fluorescence when they are cleaved during the PCR process.

2. Primers:

- Short single-stranded DNA sequences that flank the target region. Primers are essential for the initiation of DNA synthesis.

3. Probes:

- In addition to primers, some qPCR assays use fluorescent probes that hybridize to the target sequence, generating a fluorescence signal during amplification.

4. DNA Polymerase:

- Enzyme responsible for DNA synthesis during the PCR process.

5. Template DNA or cDNA:

- The nucleic acid sample containing the target sequence to be quantified.

Quantitative PCR provides accurate and reliable quantification of nucleic acids, making it an essential tool in various research areas, including gene expression studies, pathogen detection, and clinical diagnostics. The real-time monitoring aspect allows for efficient and precise measurement of nucleic acid concentrations in real-time during the PCR process.

Nanotechnology in plant disease diagnosis

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Introduction

Technology is generally regarded as the utilization or application of science to benefit society. Nanotechnology is an emerging technology, which is no longer just a vision for the future as it was generally seen at the end of 20th century. Instead, nanotechnology is a ubiquitous technology with a potential to impact on every aspect of modern human civilization. An incredibly diverse range of areas will be affected, such as agriculture, communication, energy generation/transmission, computers, environmental monitoring, food manufacturing/processing, health care, personal care, space travel, robotics, but probably the biggest impact will be in diagnostics or clinical detection.

It is an emerging technology seeking to exploit distinct technological advances of controlling the structure of materials at a reduced dimensional scale approaching individual molecules and their organised aggregates or supramolecular structures. Basically, the nanometre-length scale is creating possibilities for novel materials that can be used for the construction of devices and systems. Application in life sciences research, particularly at the cell level sets the stage for an exciting role of nanotechnology in disease diagnosis and unraveling the host – pathogen interaction.

Conventional pathogen detection methodologies, which include antibody based detection assays and those assays that amplify nucleic acids for detection, have fundamentally reached their limits of specificity and sensitivity. Advances in plant disease research are placing increasingly stringent demands on diagnostic tests to enhance sensitivity, specificity, thresholds of detection, sample throughput, reduced sample volumes, assay time, cost, and complexity. Advancements in these areas are needed to improve detection, diagnosis, and treatments. It is becoming clear that new pathogen detection methodologies involving nanotechnology will likely be central to this effort.

Challenges

Because of their small size, simple biology, and obligate intracellular life cycle, viruses present significant detection challenges. Since the 1940s, there have been three general approaches to detect viruses:

- analysis of the host organism's response to the virus, especially antibody serology;
- detection of a virus's molecular fingerprints, including viral proteins and viral nucleic acids; and
- direct sensing of whole viral particles.

Continuous technical improvements in each method have improved their speed, detection limit, accuracy, and cost-effectiveness. In this review, how nanotechnology offers improvements over existing diagnostic and detection assays, and the translation from basic research to commercial availability is discussed.

Problem

Viral diseases cause serious crop losses and affect the quality of products, while the use of virus-free seeds and planting stocks results in a substantial increase in agricultural crop production (Waterworth

& Hadidi, 1998; Strange& Scott, 2005). Due to the lack of effective treatment protocols, the main approach for the production of virus-free seeds and planting stocks is rejection of infected and selection of healthy plants. The effective ELISA- and PCR-based methods have been developed for laboratory detection of most commercially important phytopathogenic viruses. In recent years, the DNA microarray diagnostics method has become widely adopted, providing the capability of parallel detection of all pathogens in a single sample of the crop culture tested (Hadidi *et al*., 2004; Boonham *et al* 2007). All these methods of instrumental analysis possess both high sensitivity and productivity, making them fit for wide use in planting material certification and quarantine control, as well as in monitoring viral infections. However, these methods are complicated and laborious; they require qualified personnel and expensive equipment, limiting their use to well-equipped facilities and laboratories. Thus, the general body of crop plants is still beyond the scope of phytosanitary and quarantine control. Hence there is an urgent need to make available of easy to use robust diagnostic techniques by utilizing the nanotechnologies for plant virus diagnosis. In the field of clinical diagnosis, the advancement has already taken place by employing the nanotechnology and there is a scope to modify them to suit in plant virus diagnosis need to be explored.

Origin of concept

There is a constant need to improve the performance of current diagnostic assays as well as develop innovative testing strategies to meet new testing challenges. The use of nanoparticles promises to help promote *in vitro* diagnostics to the next level of performance. There are three major areas where nanotechnology has been integrated into the next generation of diagnostic techniques: (i) to improve assay sensitivity, specificity, and limits of detection (LOD); (ii) to increase sample throughput; and (iii) to reduce assay complexity and cost. Nanoparticles often require less analyte to register a response, which is largely due to the small area of the sensing surface. Smaller sensing areas generally allow higher-density arrays to be fabricated, and this feature maximizes the number of analytes (e.g., pathogens or biomarkers) that can be interrogated in a single test without increasing sample requirements. Assay complexity and cost can also be significantly reduced by nanoparticles / nanosensors that eliminate sample processing steps, such as nucleic acid amplification (e.g., PCR), and/or provide "label-free" (e.g., fluorescent dyes) detection platforms. However, it is important to note that several such devices self-described as "label free" often require a recognition element, such as an antibody or oligomers for selective detection.

The broad range of nanotechnologies applied to pathogen detection can be categorized according to the technologies, i.e., nanoarrays, nanofluidics, and nanotransducers (Table 1). Each of these types of nanotechnologies, while fundamentally different, has been shown to improve sensitivity, specificity, and throughput while decreasing analysis time and sample volume.

Quantum dots (QDs), gold nanoparticles (AuNPs), and superparamagnetic nanoparticles are the most promising nanostructures for *in vitro* diagnostic applications. These nanoparticles can be conjugated to recognition moieties such as antibodies or oligonucleotides for detection of target biomolecules. Nanoparticles have been utilized in immunoassays, immunohistochemistry and DNA diagnostics.

Table 1. Nanotechnologies with potential applications in molecular diagnostics

Nanotechnology on a chip
Microfluidic chips for nanoliter volumes: NanoChip
Optical readout of nanoparticle labels
NanoArrays
Protein nanoarrays
Nanoparticle technologies
Gold particles
Nanobarcodes
Magnetic nanoparticles: ferrofluids, supramagnetic particles combined with MRI
Quantum dot technology
Nanoparticle probes
Nanopore technology
Measuring length of DNA fragments in a high-throughput manner
DNA fingerprinting
Haplotyping
Cantilever arrays
Multiple combined tests (such as protein and DNA) to be performed on the same disposable chip
Prostate specific antigen binding to antibody
DNA nanomachines for molecular diagnostics
Nanoparticle-based immunoassays
DNA–protein and nanoparticle conjugates
Nanochip-based single-molecular interaction force assays
Resonance light scattering technology
Nanosensors
Living spores as nanodetectors
Nanopore nanosensors
Nanosensor glucose monitor
Optical biosensors: surface plasmon resonance technology
Probes Encapsulated by Biologically Localized Embedding (PEBBLE) nanosensors
Photostimulated luminescence in nanoparticles
Quartz nanobalance DNA sensor
SEnsing of Phage-Triggered Ion Cascade

From Jain (2005)

Available nanotechnologies for diagnosis

Nanotechnology offers many technological advances for pathogen detection. The use of nanoparticles as labels in conjunction with novel detection technologies has led to improvements in sensitivity and multiplexing capabilities (Jain 2005; Rosi & Mirkin, 2005). Metallic nanoparticles composed of gold or silver have many optical and electronic properties, derived from their size and composition (Nath *et al*, 2008). When coupled to affinity ligands, these nanomaterials have found important applications as chemical sensors. For example, gold nanoparticles conjugated with specific oligonucleotides can sense complementary DNA strands, detectable by color changes (Eghanian *et al*, 1997; Mirkin *et al*., 1996). Other nanoparticles including fluorescent quantum dots and carbon nanotubes have been used

in various applications including DNA detection, and the development of immunoassays for the detection of pathogens (Bruchez *et al*, 1998; Edgar *et al*, 2006; Baptista *et al*, 2006; Alivisatos *et al*., 2005).

The properties of the nanomaterials used for pathogen detection can be tailored by changing the size, shape, composition and surface modification of the nanomaterial. Particularly, their electronic, spectroscopic (emissive, absorptive), light scattering and conductive properties can be modified by engineering the nanoparticles' structural parameters, including their size, composition, self assembly and binding properties (Rosi & Mirkin, 2005).

Nanoparticles are typically in the size range of 1–100 nm (1 nm= 10^{-9} m) (Liu, 2006), and can have different shapes and compositions. Their very small size imparts physical and chemical properties that are very different from those of the same material in the bulk form. These properties include a large surface to volume ratio, enhanced or hindered particle aggregation depending on the type of surface modification, enhanced photoemission, high electrical and heat conductivity, and improved surface catalytic activity (Liu, 2006; Garg *et al* 2008; McNeil 2005; Rosi & Mirkin, 2005, Shrestha *et al*., 2006). Nanoparticles are also structurally robust and their physical properties are tailorable by variation of particle size, shape and composition. Their nano-size is within the typical size range of biomolecules and cellular organelles. This would allow a nearly one-on-one interaction between the nanoparticle and the biomolecule of interest (Azzazy *et al*., 2006, 2007; Jain, 2005). The properties of nanoparticles make them of high potential for use in *in vitro* diagnostics where they promise increased sensitivity, speed, and cost-effectiveness.

Other important nanotechnologies which can be used in plant virus diagnosis are fluorescent polymeric nanoparticles, magnetic nanoparticles, nanochips, nanoarrays and fibre optic based biosensors.

Techniques and tools

Effective monitoring of viral infections requires rapid and sensitive methods of detection available in both laboratory and field conditions. One of the promising solutions for overcoming this challenge is immunochromatographic assay (ICA), based on the interaction between the target virus and immunoreagents (antibodies and their conjugates with colored colloidal particles or nanoparticles) applied on the membrane carriers (teststrips). When the test strip is dipped into the sample being analyzed, the sample liquid flows through membranes and triggers immunochemical interactions resulting in visible coloration in test and reference lines [von Lode, 2005; Price & Kricka,2007)].

In the last few decades, rapid lateral diffusion immunochromatographic assay platforms requiring only a single handling step have become popular for point-of-care uses, and have made disease diagnosis possible in remote settings. In these assays (Fig. 1), a membrane strip serves as the solid support that carries a surface-immobilized antigen line or spot for target capture. The strip is also impregnated on one end with a loosely bound detection molecule conjugated with dyes or nanoparticles (such as colloidal gold). As a fluid sample is placed on the sample pad, the detection molecule binds to the target antibody in the sample, and the complex is carried forward to the antigen line/spot by capillary force. Binding of the target antibody to the antigen on the capture line then leads to a visible color change. These assays are rapid (~15 min) and easy to operate (single-step), require small amounts of blood or other biological samples (ca a few hundred microliters) and can be deployed in low-resource, field settings. As a result, commercial rapid-assay kits are available for detecting numerous viruses.

Express immunochromatographic test-strip assays were developed for detection of five plant viruses varying in shape and size of virions: spherical carnation mottle virus, bean mild mosaic virus, rodshaped tobacco mosaic virus, and filamentous potato viruses X and Y. Multimembrane composites (test strips) with immobilized polyclonal antibodies against viruses and colloidal gold-conjugated antibodies were used for the analysis. The immunochromatographic test strips were shown to enable the detection of viruses both in purified preparations and in leaf extracts of infected plants with sensitivity from 0.08 to 0.5µg/ml for 10 min. The test strips may be used for express diagnostics of plant virus diseases in field conditions (Byzovza *et al*., 2009). Similar format can be developed for the viruses infecting tuber crops which can be used for field level diagnosis.

A major challenge for field diagnostics that employ antibodies as targeting ligand is the need to maintain the antibody's structure and prevent thermal denaturation. Considering this challenge, gold nanoparticles and fluorescent π-conjugated polymer constructs have been used for the fluorescent-based identification of microorganisms without the need of antibodies (Phillips *et al*., 2008). In this technique, the electrostatic interaction between cationic gold nanoparticles and anionic polymers led to fluorescence quenching (Phillips *et al*., 2008) (Fig. 2). However, in the presence of bacteria, the negatively charged bacterial cell wall caused displacement of the nanoparticles' polymer (Phillips *et al*., 2008). Hence, the interaction between the nanoparticles and bacteria and the concomitant dissociation of the polymer from the nanoparticles induced the release of the polymer's quenched fluorescence, leading to enhanced fluorescence emission. A library of three nanoparticle preparations was prepared and distinct fluorescence emission patterns were observed for each organism, including *E. coli, B. subtilis, L. lactis and S. coelicolor* (Phillips *et al*., 2008). Subsequent quantitative analysis for pattern recognition through linear discriminant analysis led to the construction of a signature plot, having each microorganism's characteristic fluorescence emission (Phillips *et al*., 2008). Consequently, this approach can be utilized for the affordable and robust identification of microorganisms without the need for heat labile antibody-conjugated probes. It should be noted that as this method is at its infancy, the detection threshold was high (OD600=1; 1×10^9 colony forming units). However, with further optimization and the use of more responsive polymeric conjugates that employ a diversified association with the nanoparticles, a higher sensitivity should be achieved for diagnosis of plant viruses.

Commercialization

A number of foreign companies produce test strips for detection of plant viruses: Spot Check LF (Adgen Ltd., UK), Pocket Diagnostic (Forsite Diagnostics Ltd., UK), and Immunostrips (Agdia, United States). Results obtained with these strips indicate that virus infection can be detected within a few minutes. Among the substantial advantages of this approach are its high sensitivity, ease of both sample preparation, and the analysis itself.

Prospects

Since tuber crops are vegetatively propagated and viral diseases are major problem, the disease causing agents are easily carried through propagating material. Hence disease diagnosis is very important in certification programme followed for producing quality disease free planting material in tuber crops. For effective virus indexing, reliable and quick diagnostic kit development is important. In this case nanotechnology can play a vital role for sensitive diagnosis of tuber crop viruses by developing lateral flow assay devices or immune-strips using nanoparticles.

Conclusions

Nanotechnology has the potential to make significant contributions to disease detection, diagnosis and prevention. Tools are important and integral parts for early detection. Novel tools and tools complementing existing ones are envisaged. It offers opportunities in multiple platforms for parallel applications, miniaturisation, integration, and automation.

Since enormous losses are being incurred in different crops due to viral infection or different kinds of plant pathogens, quick and effective disease diagnosis should be available for field level diagnosis. In order to utilize the advancement taken place in the clinical diagnosis using nanotechnology, efforts should be taken to use the same for plant virus or plant pathogens diagnosis by providing more funds and research programmes on this developing field.

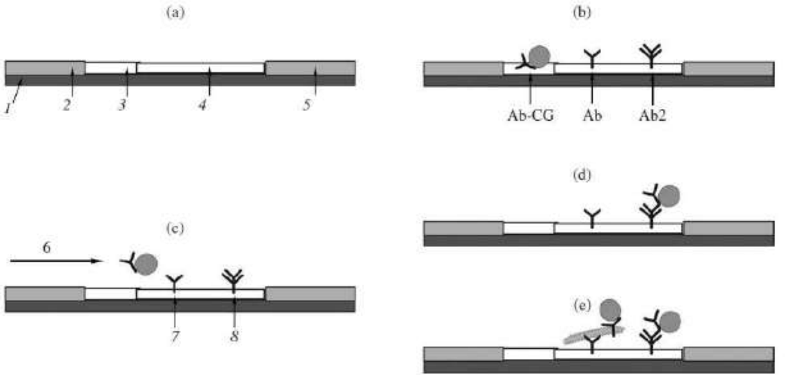

Fig.1 Lateral flow device for virus detection

a—test-system composition: *1—plastic mount, 2—absorption membrane; 3—conjugate mount; 4—working membrane; 5—absorption pad.*

b—reagents introduced*: Ab—virus-specific antibodies; Ab2—* antibodies against rabbit IgG; Ab-CG colloidal gold conjugated to virusspecific antibodies.

c—A schematic representation of analysis:- *6—spreading of liquid front; 7—test line; 8—control line. d—Test result in the absence of the virus: one colored band in the control line area*

e—Test result in the presence of the virus: two colored bands in both test and control line areas.

-From Byzova *et al* (2009)

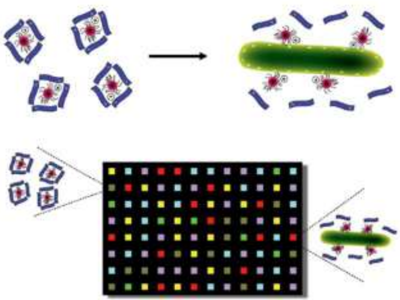

Fig.2. Fluorescence-based detection of bacteria using cationic gold nanoparticles and anionic fluorescent π-conjugated polymers. In the presence of the bacterial anionic cell wall, there is displacement of the polymer leading to fluorescence emission. Discrete fluorescence emission patterns corresponding to different microorganisms can be obtained in a high-throughput format. Adapted from reference [Phillips *et al*., 2008)

Nutritional Disorders in Tropical Tuber Crops: Symptoms and Management

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Plants require 17 essential elements for their growth and yield. Among these, the primary nutrients like nitrogen (N), phosphorus(P), potassium(K), secondary nutrients like calcium (Ca), magnesium (Mg), sulphur (S) and micronutrients like iron (Fe), copper (Cu), manganese (Mn), zinc (Zn) and boron (B) are important for tuber crops like cassava, sweet potato, elephant foot yam, yams , taro and tannia. In Kerala, tuber crops are mostly grown in laterite (Ultisols) and sandy loam soils (Entisols) which are acidic with less availability of major nutrients like N and K, very low availability of Ca and Mg and B. Nutrients like P, S, Fe, Cu, Mn are not limiting in these soils. However, Zn deficiency is encountered in some parts of Kerala.

Since the biological efficiency of tropical tuber crops are very high producing tuber yield to the tune of 20-100 t ha^{-1}, the replenishment of the nutrients uptake need to be done through application of manures and fertilizers from external sources needs to be carried out through different nutrient management practices. If proper nutrient replenishment is not done, the crop will manifest different types of nutritional disorders in the form of visible symptoms in different plant parts especially leaves and tubers. Usually, in tuber crops, the inadequate availability of nutrients like N, P, K, Ca, Mg, Zn, B in soil could cause nutrient deficiency disorders and are explained clearly with symptoms and their management.

Nutrient deficiency symptoms and their management in different crops

1. Nitrogen

Cassava Elephant foot yam Lesser yam

Symptoms

- General stunted growth and yellowing affecting mostly the lower leaves
- In severe cases nechrosis and drying of the older leaves and incidentally the whole plant

Management

- Application of organic manures and NPK fertilizers as per the recommended package of practices (PoP)
- If severe yellowing is noticed and if there is no moisture content in the soil, foliar application of 0.1% urea can be done to save the crop from complete loss

2. Phosphorus

Sweet potato

Symptoms

- Purplish discolouration of older leaves and these discoloured leaves will turn yellowish and later dry and shed

Management

- Application of recommended dose of P (25 kg ha^{-1}) or as per soil test data basally even in high P soils
- In severe P deficiency, foliar application of 19:19:19 @ 0.5-1% can correct the symptom manifestation

3. Potassium

Cassava Sweet potato Elephant foot yam Tannia

Symptoms

- Symptoms appear in the old lower leaves as drying of the margins and tips
- In severe cases, complete drying of the lower leaves resulting in the death of the plant

Management

- Application of organic manures and NPK fertilizers as per the recommended PoP
- In the case of symptom initiation only, apply muriate of potash (MOP) in the soil provided there is moisture.
- In later stages of symptom manifestation, foliar application of potassium sulphate (SOP) @0.5-1% will rectify the problem

4. Calcium

Cassava Sweet potato Tannia

Symptoms

- Ca being immobile in the plant system, symptoms only in younger leaves
- The tip of leaves from middle to top turn round in appearance with slight curling of the leaflets depending upon the variety

Management

- Application of lime/dolomite in acid soils @1-2 t ha^{-1} to avoid the symptom manifestation
- If symptoms appear, apply calcium nitrate ($CaNO_3$) as foliar spray @ 0.5-1% to manage the symptoms so that the new emerging leaves can be saved from the deficiency

5. Magnesium

Symptoms

- Interveinal chlorosis of the lower older leaves is the typical symptom of Mg deficiency
- Symptom appear as yellow discolouration of the laminar area between veins where the veins remain dark green
- In severe cases, the margins and tips of the leaves turn nechrotic and dry

Management

- Basal soil application of dolomite @1-2 t ha^{-1} or magnesium sulphate ($MgSO_4$) @ 20 kg ha^{-1} after top dressing
- In severe cases of symptom manifestation, foliar application of $MgSO_4$@0.5-1% can be done depending upon the sensitivity of the crop to sulphate (SO_4) injury

6. Boron

Cassava Sweet potato

Symptoms

- Boron being immobile, symptom manifestation is seen on the emerging tender top portion
- Appear as rosette/broom like with small sprouts in clusters or bunches bearing reduced/crinkled leaves giving a distorted appearance to the plant adversely affecting the growth of the plant
- In tubers, cracking of the skin and rind of the tubers with the flesh turning to woody

Management

- Soil application of either borax or boric acid based on soil test data (general recommendation of borax @ 10 kg ha^{-1}
- After Once the symptom is seen, foliar application of $CaNO_3$ (0.5%) along with solubor (0.05-0.1%) (combined) at fortnightly intervals till the plant recoup

7. Imbalanced Nutrient Application

Symptoms

- Application of higher levels of N and Pwithout K application result in situation similar to this
- Shedding of healthy leaves at the middle of the plant and drooping down of the leaves with petioles bending down
- In severe cases, leaves above the middle portion may become yellow, dry and fall down

Management

- Balanced application of NPK as per PoP or soil test
- Soil application of MoP at the initial stages of symptom manifestation
- Foliar application of sulphate of potash (SoP) @0.5-1% to save the new emerging leaves from expressing symptoms

8. Lime Induced Iron Chlorosis

Symptoms

- Usually seen in alkaline soils with high pH (above 8)
- The symptom is manifested as iron deficiency induced through excess lime (Lime induced Fe deficiency)
- Uniform chlorosis of the entire leaves of the plant with stunted growth and drying in severe cases
- Extreme situations result in complete devastation of the crop

Management

- On visual observation of the same, spraying a mixture of 1% ferrous sulphate along with 1% zinc sulphate can rectify the problem
- Application of S @10-20 kg ha^{-1} as elemental sulphur or gypsum or use of sulphur containing fertilizers like ammonium sulphate, single super phosphate or potassium sulphate basal can prevent the manifestation of the same in alkaline soils

9. Toxicity Disorders in Tuber Crops due to Boron

- These type of symptoms were usually encountered with boron in cassava and elephant foot yam due to excess application of B through soil and foliar.

Cassava Elephant foot yam

Symptoms

- Drying and shedding of the leaves. But will recoup after 15-30 days if there is sufficient moisture in the soil
- Distorted growth of the plant with irregular and dwarf stature of the crop with tapering of the psuedostem, thin, pale and unhealthy appearance of the crop
- Later though the plant dry up it will come up and grow as a distorted plant yielding very poor

Management

- While applying B in both soil and plant, care should be taken to restrict the dosage of foliar spray concentration to up to 0.1% and applications should be on the critical growth stages like maximum vegetative growth (4-5 MAP), tuber bulking (6-7 MAP) and one month after that (7-8 MAP)
- Soil application must be based on soil test for B
- Apply B in soil if the soil status is below 0.5 ppm

In the present times of climate change, different types of symptom expressions in plants due to nutrients have become as serious as pests and diseases. Drastic decline in soil fertility and productivity due to continuous monoculture without replenishment of the depleted soil nutrients is one of the reasons for the occurrence of these nutritional disorders. In many a situations, the symptoms due to nutritional disorders may akin to that of pests and disease symptoms and may be difficult to distinguish too. In such circumstances, though we can have first hand diagnosis through visual observation, the confirmation can be made through both soil and plant analysis.

Integrated Molecular and Bioinformatics Approaches for Disease diagnosis in Plants

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Modern plant pathology relies on bioinformatics approaches to create novel plant disease diagnostic tools. In recent years, a significant amount of biological data has been generated due to rapid developments in genomics and molecular biology techniques. Integrated molecular and bioinformatics approaches have revolutionized disease diagnosis in plants by offering rapid, accurate, and scalable methods for identifying pathogens and understanding plant-pathogen interactions. As the amount of data grows exponentially, there is a parallel growth in the demand for tools and methods in data management, visualization, integration, analysis, modelling and prediction. Bioinformatics plays an essential role in today's plant pathology with regards to the development of new plant diagnostic tools.

The areas of sequence analysis include sequence alignment, sequence database searching, motif and pattern discovery, gene and promoter finding, reconstruction of evolutionary relationships and genome assembly and comparison. Structural analyses include protein and nucleic acid structure analysis, comparison, classification and prediction. The functional analysis includes gene expression profiling, protein–protein interaction prediction, protein sub cellular localization prediction, metabolic pathway reconstruction and simulation.

These methods and tools include,

(i) Bioinformatics Pipelines: Specialized bioinformatics tools and pipelines are developed to analyse large-scale sequencing data efficiently. These pipelines facilitate the identification of pathogen sequences, their comparison with known databases, and the prediction of potential virulence factors or effectors.

- *Pipeline Construction:* Customized bioinformatics pipelines are developed using scripting languages like Python or Perl, integrating various tools and algorithms for processing sequencing data, performing quality control, and analysing results.
- *Workflow Management:* Workflow management systems like Snakemake or Nextflow streamline the execution of bioinformatics pipelines, allowing for reproducible and scalable analyses across different computing environments.

(ii) Metagenomics: Metagenomic approaches involve the sequencing of DNA directly from environmental samples (e.g., soil, plant tissues) without the need for isolation and cultivation of pathogens. This allows for the detection of all microorganisms present in a sample, including unculturable or unknown pathogens.

- *Sequence Assembly:* Metagenomic sequencing data is assembled using specialized algorithms such as MEGAHIT or MetaSPAdes, which can handle complex mixtures of DNA sequences from various organisms.
- *Taxonomic Profiling:* Computational tools like Kraken or MetaPhlAn classify assembled sequences into taxonomic groups, allowing for the identification of pathogens and other microorganisms present in the sample.

- *Functional Annotation:* Tools such as HMMER or BLAST are used to annotate genes and predict their functions, providing insights into the metabolic pathways and potential virulence factors of identified pathogens.

(iii) Variant Analysis: Bioinformatics methods are employed to analyze genetic variations within pathogen populations. This information is crucial for tracking the emergence of new strains or variants with altered virulence or resistance profiles.

- *Variant Calling and Annotation:* Computational algorithms such as GATK (Genome Analysis Toolkit) or SAMtools are employed to identify single nucleotide polymorphisms (SNPs), insertions, deletions, and other genetic variations within the sequenced genomes. Variants identified from genomic data are annotated with information about their effects on genes and proteins using tools like ANNOVAR or SnpEff, facilitating the interpretation of their functional significance.

(iv) Expression Profiling: Transcriptomic analysis helps in understanding the gene expression patterns of both the pathogen and the host during infection. Differential gene expression analysis identifies key genes involved in plant defense responses or pathogen virulence.

- *Differential Expression Analysis:* Transcriptomic data obtained from RNA sequencing (RNA-seq) is analyzed using tools like DESeq2 or edgeR to identify genes that are differentially expressed between infected and healthy plants, providing insights into the molecular mechanisms of plant-pathogen interactions.
- *Gene Co-expression Networks:* Computational methods such as weighted gene co-expression network analysis (WGCNA) are used to identify modules of co-expressed genes associated.

(v) Pathogen Identification: Bioinformatics tools such as BLAST (Basic Local Alignment Search Tool) and Hidden Markov Models (HMMs) are used to compare pathogen sequences obtained from sequencing data with databases of known pathogens. This helps in accurate identification of the causative agents of diseases, with specific biological processes or pathways related to disease resistance/susceptibility.

(vi) Phylogenetics: Phylogenetic analysis enables the reconstruction of evolutionary relationships between different pathogen isolates. This information aids in understanding the origin, spread, and diversification of pathogens, which is essential for disease management strategies.

- *Sequence Alignment and Phylogenetic Inference:* Multiple sequence alignment algorithms like MAFFT or ClustalW are used to align sequences from different isolates, and phylogenetic trees are constructed using methods such as maximum likelihood or Bayesian inference to infer evolutionary relationships.
- *Molecular Clock Analysis:* Computational techniques like BEAST or MEGA are employed to estimate the divergence times between different lineages based on sequence data, providing insights into the evolutionary history of plant pathogens.

(vii) Machine Learning and Data Integration: Machine learning algorithms are employed to integrate multi-omics data and predict disease outcomes or identify diagnostic biomarkers with high accuracy. Machine learning algorithms such as support vector machines (SVMs) or random forests can be trained on sequence features to classify pathogens and distinguish them from non-pathogenic organisms.

- *Feature Selection:* Machine learning techniques such as recursive feature elimination (RFE) or LASSO regression are applied to select informative features from multi-omics data for disease diagnosis and prediction.

- *Ensemble Methods:* Ensemble learning approaches such as random forests or gradient boosting are utilized to integrate heterogeneous data sources and improve the accuracy of disease classification models.
- *Data integration of omics data and hyperspectral Images :* Bioinformatics plays a critical role in data integration, analysis, and model prediction, as well as in managing the massive amounts of data resulting from new, high-throughput approaches. Shoaib et al., 2023 propose a novel explainable gradient-based approach EG-CNN model for both omics data and hyperspectral images to predict the type of attack on plants in this study. Theu used gene expression, metabolite, and hyperspectral image data from plants afflicted with four prevalent diseases: powdery mildew, rust, leaf spot, and blight.

(viii) Database Development: Comprehensive databases are developed to store and share genomic, transcriptomic, and phenotypic data related to plant pathogens and their interactions with host plants. Databases Provide Abundant Gene and Pathway Information to Study Plant Biology These databases facilitate data mining and knowledge discovery for researchers worldwide.

- *Data Curation:* Comprehensive databases like NCBI GenBank or Ensembl Plants curate and organize genomic, transcriptomic, and phenotypic data related to plant pathogens, providing valuable resources for researchers to access and analyze.
- *Web Interfaces and APIs:* Web-based interfaces and application programming interfaces (APIs) are developed to facilitate data retrieval, visualization, and analysis, allowing researchers to explore and query large-scale datasets efficiently.

By leveraging these computational techniques, integrated molecular and bioinformatics approaches enable researchers to unravel the complex mechanisms underlying plant diseases and develop targeted strategies for disease diagnosis, management, and crop improvement. Modern molecular and bioinformatics technologies have made understanding host-pathogen interactions easier. Plants have many ways to protect themselves from microbial diseases, such as physical barriers, PAMP detection, and R genes that recognize pathogen-effector proteins and turn on effector-triggered immunity. Plant pathogen genome databases provide genomic and phenotypic data on plant pathogen species and information on plant-pathogen interactions. Map-based or positional gene cloning is improving our understanding of plant-pathogen interactions, with R genes being used to develop resistance to pathogens. Plant genomes typically contain several hundred nucleotide-binding site-leucine-rich repeats (NLRs), with their number, arrangement, and domain combinations varying by species.

In the era of big data, bioinformatics faces opportunities and challenges for its application to agriculture. Learning and developing more bioinformatics tools will help integrate all existing bioinformation resources and provide support for effective breeding and plant resistance analysis.

Role of AI and IoT in Plant Disease Diagnosis

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Abstract

Artificial Intelligence (AI) has emerged as a transformative tool in modern agriculture, offering innovative solutions to the persistent challenge of managing plant diseases. The relentless expansion of global agriculture demands efficient solutions to mitigate the economic and environmental impacts of plant diseases. AI technologies, coupled with data-driven approaches, have empowered researchers, farmers, and policymakers with new strategies for safeguarding crops. Key applications include:

1. Early Disease Detection: AI, particularly computer vision and machine learning, enables the rapid and accurate identification of disease symptoms in crops. By analyzing images of leaves, stems, or entire plants, AI algorithms can detect subtle changes in color, texture, and morphology indicative of diseases. This early detection allows for timely intervention and reduced crop losses.

2. Disease Prediction Models: AI-driven predictive models harness historical disease data, environmental variables, and disease progression patterns to forecast disease outbreaks. By considering factors such as weather conditions, soil quality, and previous disease occurrences, these models empower farmers to make informed decisions about disease management and crop protection strategies.

3. Precision Agriculture: AI-driven precision agriculture techniques optimize the use of resources, such as pesticides and water, by targeting specific areas affected by diseases. Smart spraying systems, guided by AI algorithms, reduce the environmental impact and cost of chemical applications while maximizing their effectiveness.

4. Genomic Analysis: AI facilitates the analysis of plant and pathogen genomes to identify disease-resistant crop varieties through genetic markers. This approach streamlines breeding programs, enabling the development of more resilient and disease-resistant plants.

5. Mobile Apps and Chatbots: AI-powered mobile applications and chatbots provide farmers with accessible tools for identifying and managing plant diseases. Users can submit images of affected plants for analysis, receiving real-time information and recommendations for disease control.

6. Environmental Sustainability: AI-driven disease management practices promote sustainable agriculture by reducing the indiscriminate use of pesticides and minimizing environmental impact. This aligns with global efforts to create more eco-friendly and resource-efficient farming practices.

As AI continues to evolve, its integration into agriculture offers the promise of increased crop yields, reduced production costs, and a more sustainable food supply chain. Nevertheless, challenges such as data privacy, accessibility, and the need for farmer education must be addressed to fully harness AI's potential in managing plant diseases.

Keywords: Artificial Intelligence, Disease management, Data analytics

Introduction

Agriculture, the foundation of human sustenance, faces the ever-looming threat of plant diseases, which can devastate crop yields and jeopardize food security. In recent years, the convergence of cutting-edge technology and agriculture has given rise to innovative solutions for tackling this

challenge. At the forefront of this transformation is Artificial Intelligence (AI), a field that is revolutionizing how we detect, diagnose, and manage plant diseases. In this comprehensive article, we delve deep into the multifaceted applications of AI in plant disease management, exploring the technological advancements, the potential benefits, and the challenges that lie ahead.

I. Early Detection and Diagnosis:

1.1 Visual Symptom Recognition:

Artificial Intelligence is making remarkable strides in early disease detection through image recognition. AI algorithms are trained on vast datasets of plant images, allowing them to differentiate between healthy and diseased plants with astonishing accuracy. This technology can identify subtle visual symptoms, such as leaf discoloration, lesions, or deformities, that may go unnoticed by the human eye. With AI's ability to rapidly process and analyze images, it serves as an invaluable tool for identifying diseases in their nascent stages.

a. Tomato

b. Cassava

Fig 1. Plant disease symptoms

1.2 Machine Learning and Deep Learning:

At the heart of AI's visual symptom recognition capabilities lie machine learning and deep learning techniques. Convolutional Neural Networks (CNNs), a class of deep learning models, have proven to be particularly effective in image classification tasks. These models excel at feature extraction, enabling them to discern intricate patterns and variations in plant health. As more data becomes available, AI-driven disease detection systems continue to improve in accuracy and reliability.

1.3 Drones and Remote Sensing:

The integration of AI with drones and remote sensing technologies has further expanded the scope of early disease detection. Drones equipped with high-resolution cameras and AI algorithms can survey vast agricultural fields quickly and comprehensively. This aerial perspective allows for the identification of disease hotspots, enabling farmers to take targeted actions, such as isolating affected areas or applying treatments precisely where needed.

Fig 2. Camera drone collecting aerial images

II. Disease Identification

2.1 Precision in Disease Identification:

Beyond early detection, AI contributes significantly to disease identification. When a disease is detected, AI can classify and identify the specific pathogen or disease type accurately. This precise identification is critical for selecting the most appropriate treatment or management strategy, as different diseases may require different approaches. The ability of AI to swiftly categorize diseases minimizes the risk of misdiagnosis and ensures that farmers employ effective solutions.

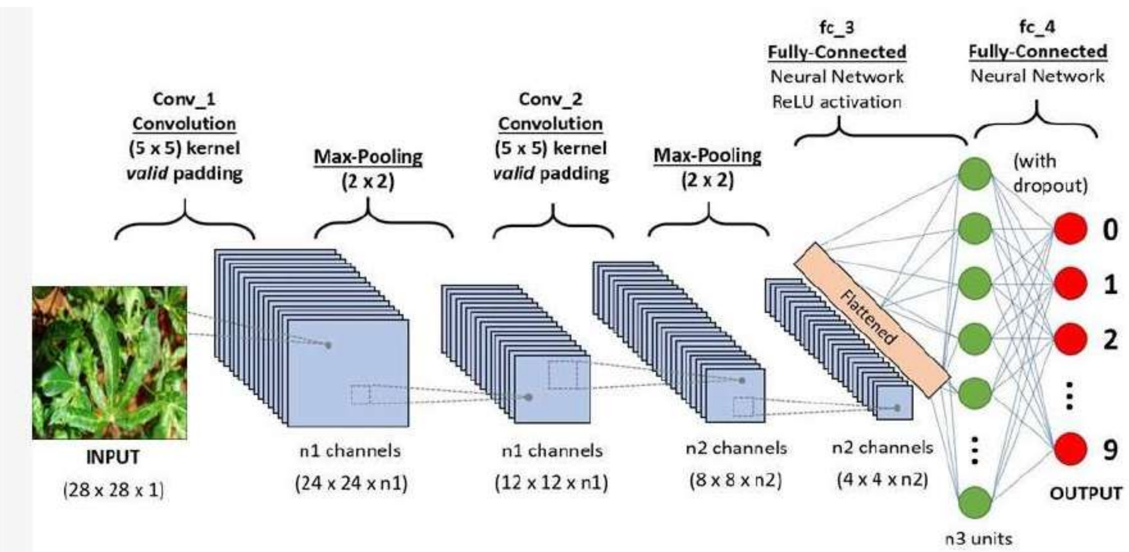

Fig 3. CNN model for disease identification

III. Disease Forecasting

3.1 Predicting Disease Outbreaks:

AI extends its utility to proactive disease management through forecasting. By leveraging historical data on weather patterns, crop rotations, and disease prevalence, machine learning models can predict the likelihood of disease outbreaks in specific regions and during particular timeframes. This foresight empowers farmers to implement preventive measures, reducing the need for reactionary responses and minimizing crop losses.

3.2 Climate-Driven Models:

The relationship between climate and disease dynamics is complex but well-documented. AI-driven climate models can analyze vast datasets and uncover hidden correlations between climate factors and disease prevalence. This knowledge allows farmers to anticipate disease risks associated with changing weather patterns and adapt their practices accordingly.

IV. Prescription Farming

4.1 Tailored Recommendations:

AI-driven prescription farming is a game-changer in optimizing resource usage and minimizing environmental impact. By considering real-time data on environmental conditions, soil quality, and plant health, AI algorithms generate personalized recommendations for farmers. These recommendations may include precise instructions on when and how to apply pesticides, fertilizers, or irrigation, maximizing the efficiency of resource allocation.

4.2 Sustainable Agriculture:

Sustainability is at the core of prescription farming. AI helps reduce overuse of chemicals and water, addressing environmental concerns. The targeted application of resources not only minimizes the ecological footprint but also leads to cost savings for farmers, making agriculture more economically sustainable.

V. Crop Monitoring

5.1 Drones and Robots:

AI-powered drones and robots have emerged as indispensable tools for crop monitoring. These autonomous devices can navigate fields, scanning plants for disease symptoms and assessing overall plant health efficiently and systematically. Real-time monitoring enables farmers to detect issues early and respond promptly, preventing the unchecked spread of diseases.

5.2 Hyper-Spectral Imaging:

Hyper-spectral imaging, combined with AI, offers a deeper level of crop analysis. This technology captures detailed spectral data from plants, providing insights into their biochemical composition and health status. AI algorithms can process this data to identify subtle changes in plant physiology that may signal disease presence, even before visible symptoms appear.

VI. Data Integration

6.1 Holistic Insights:

AI excels at data integration, aggregating information from various sources, such as weather data, soil quality assessments, historical disease records, and real-time sensor data. This holistic view of the agricultural ecosystem provides farmers with comprehensive insights into the factors influencing disease dynamics. It enables more informed decision-making and a deeper understanding of the complex interactions at play in the field.

VII. Decision Support Systems

7.1 Informed Decision-Making:

AI-driven decision support systems are transforming the way farmers approach disease management. These systems analyze an array of variables, including weather conditions, soil health, crop history, and disease risks, to provide actionable insights and recommendations. Farmers armed with this

information can make informed choices about planting times, crop varieties, and disease management strategies.

VIII. Genetic Engineering

8.1 Accelerating Research:

AI accelerates genetic research in plants, unlocking the potential for disease-resistant crops. By analyzing plant genomes, AI can identify genes associated with disease resistance. This knowledge expedites the development of genetically modified crops that are inherently more robust against specific diseases, reducing the need for chemical interventions.

IX. Early Warning Systems

9.1 Real-Time Alerts:

AI-powered early warning systems provide farmers with real-time alerts regarding potential disease risks. These systems combine data from multiple sources, including weather forecasts, disease models, and on-ground sensors. When conditions favor disease development, farmers receive timely notifications, allowing them to implement preventive measures before diseases become widespread.

X. Challenges and Ethical Considerations

While AI holds immense promise in managing plant diseases, several challenges and ethical considerations must be addressed:

10.1 Data Privacy and Ownership: Data privacy issues may arise when collecting and sharing agricultural data. Farmers must have control over their data and understand how it will be used.

10.2 Accessibility: The adoption of AI technology may be limited in regions with inadequate infrastructure or resources. Ensuring equitable access to AI-driven solutions is crucial.

10.3 Ethical Use of AI: Ethical considerations surrounding AI in agriculture include transparency in decision-making, accountability for AI-driven recommendations, and addressing potential biases in data and algorithms.

10.4 Data Quality: The effectiveness of AI models depends on the quality of data used for training. Ensuring data accuracy and reliability is paramount.

Conclusion

Artificial Intelligence is revolutionizing plant disease management, providing innovative tools and solutions to farmers worldwide. From early detection and disease identification to proactive forecasting, prescription farming, and genetic engineering, AI's multifaceted applications offer hope for more resilient and sustainable agriculture. As AI continues to evolve and mature, it holds the promise of increasing food production while minimizing environmental impact. However, it is essential to address challenges related to data privacy, accessibility, ethics, and data quality to ensure that the benefits of AI in agriculture are accessible to all and contribute to a more food-secure and sustainable future.

Course Materials for Practical Sessions

Isolation of Nucleic acids from infected plants – DNA & RNA

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Total DNA isolation from cassava mosaic diseased plants (CTAB method)

1. Place 100 mg of diseased cassava leaf tissue in a plastic bag and freeze it in liquid nitrogen.
2. Grind with (10 volume) 1ml of pre- heated (60^0C) extraction buffer 2% (W/V) CTAB (Cetyl trimethyl ammonium bromide), 1.4M NaCl, 0.2% (V/V) 2-mercaptoethanol, 20 µM EDTA, 100 µM Tris HCl, pH 8.0.
3. Take 1ml of the sample in a microcentrifuge tube and incubate at 60^0C for 30 min.
4. Centrifuge at 10,000 rpm for 5 min. To the supernatant add 4 µl RNase and keep it for 40 min at 30^0C.
5. Add equal volume (750 µl) of chloroform: isoamyl alcohol (24:1) mixture to the sample and mix it gently.
6. Centrifuge at 12,000 g for 10 min.
7. Transfer the top aqueous layer to a fresh microcentrifuge tube
8. Add 0.8 volume of isopropanol and incubate at -20^0C for at least one hour.
9. Centrifuge at 12,000 g at 4^0C for 15 min.
10. Discard the supernatant and wash the pellet in 0.5 ml (500 µl) of 70 % ethanol
11. Centrifuge for 5 min at 12,000 g and decant ethanol and dry the pellet.
12. Suspend the pellet in 50 ml of sterile distilled water (or 1X TE buffer) and store at -20^0C.

Total DNA isolation from cassava mosaic diseased plants (Miniprep method)

H1Geno MB HiPurA® SuperPlant DNA Purification Kit –Protocol (product code- MB571)

Kit contents

Product Code	Reagents provided	50 Preps
DS0200	SuperPlant Extraction Buffer	55 ml
DS0070	Additive-II	5 ml
DS0071	Additive-III	1.25 g
DS0003	RNase A Solution (20 mg/ml)	1.25 ml
DS0019	Wash Solution Concentrate (WSP)	30 ml
DS0040	Elution Buffer (ET) [10mM Tris-Cl, pH 8.5]	6 ml
DBCA03	HiElute Miniprep Spin Column (Capped) [in DBCA016 Collection Tube]	50nos
DBCA016	Collection Tube (Uncapped), Polypropylene (2.0 ml)	50 nos
PW1139	Collection Tube, Polypropylene (2.0 ml)	100 nos

Materials needed but not provided in kit:

- Chloroform: Isoamylalcohol (24:1) (Product Code: MB115)
- Ethanol (96-100%)
- Mortar and pestle
- Liquid nitrogen
- Tabletop Microcentrifuge (with rotor for 2.0 ml tubes)
- 65°C water bath or heating block

General Preparation Instructions

- Grinding of the plant material can be done using mortar and pestle. Midrib should be removed from the material before grinding, as midrib is a major source of carbohydrate contamination.
- SuperPlant Extraction Buffer: Immediately prior to use, add 90 µl of Additive-II and 18 mg of Additive-III in 900 µl of SuperPlant Extraction Buffer. Preheat the solution to 65°C.
- Dilute Wash Solution Concentrate (WSP) (DS0019) as follows: 30ml WSP provided with the kit is to be diluted with 70 ml of (96-100 %) Ethanol

Sample Preparation

Finely cut the leaf material before grinding. Weigh 200 mg of the finely cut plant material and grind properly using a mortar and pestle in liquid nitrogen to a fine powder. Allow the liquid nitrogen to evaporate. DO NOT ALLOW THE SAMPLE TO THAW (keep samples on ice if needed). Proceed immediately to the DNA isolation protocol.

Protocol

NOTE: Additive-II and Additive-III to be added to SuperPlant Extraction Buffer (DS0200) before extraction.

1. To 200 mg of the ground material add 900 µl of SuperPlant Extraction Buffer (DS0200) (preheated to 65°C) and transfer the sample to a capped 2.0 ml collection tube. Mix by vortexing
2. Incubate the samples for 60-90 minutes with occasional inversion at 65°C.
3. Add 1 ml of Chloroform: Isoamylalcohol (24:1) and mix gently by inversion for 5 minutes.
4. Centrifuge the samples at 14,000 rpm for 10 minutes at room temperature (15-25°C).
5. Transfer the top aqueous layer (containing DNA) into a fresh 2.0ml collection tube (not provided) and add 20 µl of RNase A Solution (20 mg/ml) (DS0003). Incubate for 5 minutes at room temperature (15-25°C).
6. Add equal volume of Ethanol (96-100%) to the lysate obtained from the above step and mix by pipetting.
7. Load lysate in HiElute Miniprep Spin Column (Capped) [DBCA03] Add 650 µl of the lysate, including any precipitate, which may have formed, to the column placed in a uncapped 2.0ml collection tube. Centrifuge for 1 minute at 8000 rpm. Discard the flow-through.
8. Repeat the above step with the remaining sample. Discard the flow-through liquid and reuse the 2.0 ml collection tube (uncapped).
9. Wash (Prepare the diluted Wash Solution (WSP) (DS0019) as indicated in General Preparation Instructions) Add 500 µl of diluted Wash Solution (WSP) and centrifuge for 1 minute at 8000 rpm. NOTE: Discard the flow-through and reuse the 2.0 ml collection tube (uncapped).

10. Add another 500 µl of diluted Wash Solution (WSP) to the column and centrifuge for 2 minutes at a maximum speed 14,000 rpm. Discard the flow-through and reuse the same collection tube.
11. Centrifuge the tube with column for an additional 2 minutes at a maximum speed 14,000 rpm to dry the membrane.
12. DNA Elution
13. Place the column in a new 2.0ml collection tube (uncapped) and pipette 100 µl of the Elution Buffer (ET) (DS0040) directly onto the column without spilling to the sides. Incubate for 5 minutes at room temperature (15-25°C). Centrifuge at 6000 rpm) for 1 minute to elute the DNA. Transfer the elute to a new capped 2.0ml collection tube for DNA storage.

Storage of the elute with purified DNA: The elute contains pure genomic DNA. For short- term storage (24-48 hours) of the DNA, 2-8°C is recommended. For long-term storage, -20°C or lower temperature (-80°C) is recommended. Avoid repeated freezing and thawing of the sample which may cause denaturing of DNA. The Elution Buffer (ET) will help to stabilize the DNA at these temperatures.

Quantification of DNA samples

The DNA samples will be quantified with Nanodrop using 1 µl of DNA sample. Place 1µL of DNA onto the pedestal. Close the lid and click measure, to record the concentration and purity. Purity is measured under the 260/280 column and a good purity ranges from 1.80-2.00. The 260/230 ratio is a second of measure of purity of the samples as the contaminants absorb at 230nm. The 260/230 ratio should be higher than the 260/280 ratio, as it is usually between 2 and 2.2. Lower ratio of 260/280 will be an indication of contamination. The DNA samples will be quantified in terms (ng/µl). Repeat for each sample.

Total RNA Isolation from cassava leaves – Lithium Chloride method

Homogenization:

- Grind 100 mg of tissue sample in liquid nitrogen using motar and pestle pre treated with 0.1 % DEPC and homogenize in 1ml CTAB which is prewarmed at 65 º C for 10 min (1 ml/50-100 mg) and transfer to 2ml microfuge tube
- Incubate the homogenate at room temperature for 5 min.

Phase Separation:

- Centrifuge the homogenate at 15000 rpm for 15 min and transferred to 2ml tube
- Equal volume of Chloroform: Isoamyl alcohol C:I (24:1) added to the supernatant
- Centrifuge at 20000 g for 15 min at 4º C

RNA precipitation:

- Transfer the aqueous phase to a fresh tube
- To the aqueous phase, add 0.25vol of ice cold 10M Lithium Chloride added, mixed well and incubated overnight at -20 ºC

Ethanol Wash:

- Centrifuge at 30000 g for 30 min at 4º C and the pellet washed with 75% ethanol by centrifugation at 10000g at 4 ºC and washing repeated.

RNA Solubilization:

- Remove the ethanol and air-dry the RNA pellet for 3-5 min.
- Dissolve RNA in sterile distilled water (RNase free)
- Incubate at 37 º C for 10-15 min until complete dissolution of pellet
- Store at -80º C for further use

Reagents

- 0.1 % DEPC treated water: Take 100 µl DEPC (Diethyl pyro carbonate) in 100 ml of distilled water , stir over night and autoclave
- Tri-reagent (Sigma)
- Chloroform
- Isopropanol
- 75% ethanol: Take 75 ml of absolute ethanol and make up to 100 ml with distilled DEPC treated water

Quantification of RNA samples

The RNA samples will be quantified with Nanodrop using 1 µl of RNA sample. Place 1µL of RNA onto the pedestal. Close the lid and click measure, to record the concentration and purity. Purity is measured under the 260/280 column and a good purity ranges from 1.80-2.00. The 260/230 ratio is a second of measure of purity of the samples as the contaminants absorb at 230nm. The 260/230 ratio should be higher than the 260/280 ratio, as it is usually between 2 and 2.2. Lower ratio of 260/280 will be an indication of contamination. The RNA samples will be quantified in terms (ng/µl). Repeat for each sample.

Direct antigen coated ELISA (DAC-ELISA)

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DAY 1

1. Collect samples in plastic bags and identify the samples with numbers. Make a composite sample from each plant to be tested by taking one leaf from the top, middle and bottom levels. Always keep one known positive and negative controls for comparison of results.
2. Extract 100 mg of plant tissue in 1 ml antigen extraction buffer and centrifuge the extracted sap at 8000 rpm for 2 minutes.
3. Collect 200 µl of the supernatant and coat each well of microtitre plate. Maintain two or three wells (replicates) for each sample. Avoid using wells on the top row, bottom row and rows on the extreme right and left for sample loading. Cover the plate with aluminium foil and incubate at 37°C for 3 hrs.
4. Empty the wells and wash the plate thrice by filling the wells with PBS-T allowing 5 min for each wash. Remove the wash solution and drain out the excess liquid on the walls of the wells by tapping the plate in inverted position on a paper towel spread on the bench.
5. Add 200 µl blocking solution (5 % skimmed milk powder dissolved in PBS-T) to each well. Incubate at 37°C for 30 minutes.
6. Wash the plate thrice as in step 4 above.
7. Add 200 µl of detection antibody (diluted to the required working concentration (1:6000)) in antibody buffer (PBST-PVP buffer) to each well. Incubate at 4^0C overnight.

DAY 2

8. Wash the plate thrice as in step 4 above.
9. Add 200 µl aliquot of alkaline phosphatase conjugated anti-rabbit IgG (available commercially) diluted in enzyme conjugate buffer(PBST-PVP-Albumin) (as per the dilution mentioned by the manufacturer(1:7000)) per well. Incubate at 37°C for 2 hrs.
10. Wash the plate thrice as in step 4 above.
11. Add 200 µl freshly prepared substrate p-ntitrophenyl phosphate [PNPP] (1 mg PNPP in 1 mL substrate buffer) to each well. Incubate at room temperature until the yellow colour is clearly visible in the positive controls and take readings based on absorbance value at 405nm after regular intervals(30 min, 1 hr, 2hr, Overnight).

Note: Since PNPP is sensitive to light, substrate addition has to be carried out in dark.

12. Assess results by visual observation or by measurement of absorbance of the hydrolysed substrate at 405 nm wavelength in a microtitre plate reader. Record samples whose A_{405} values are more than double the A_{405} value of healthy control as positive for detecting virus.

Reagents

1. **PHOSPHATE BUFFERED SALINE. 10X PBS** (pH 7.4)

Na_2HPO4	-	1.19g /2.38 g ($Na_2HPO_4.12H_2O$ - 2.90 g/l)
KH_2PO4	-	0.20g / 0.4g
KCL	-	0.2g / 0.4g
NaCl	-	8.00g / 16g
H_20	-	1 L / 2 L

2. **WORKING SOLUTION 1X PBS**

   100 ml + 900 ml d.H2O of 10x

3. **WASH BUFFER (PBST-T)**

1 X PBS	-	1000 ml (1 litre)
Tween-20	-	0.5 ml (or 0.05 µl Tween-20 in 100 ml)

4. **ANTIBODY BUFFER (PBS-T-PVP)**

PBS-T	-	100 ml
PVP (40,000 mwt)	-	2 gm

   (i.e. Wash buffer + 2% PVP)

5. **ENZYME CONJUGATE BUFFER**

   2% PVP+ 0.2% Albumin in 1x PBST.

6. **BLOCKING BUFFER**

   PBST-PVP + 5% dried skimmed milk powder.

7. **SUBSTRATE BUFFER**

   97 ml Diethanloamine

   600ml H_2O

   0.2 g Sodium azide (NaN_3)

   Adjust pH to 9.8 with HCL and make up to 1L with water.

Note: Store the substrate buffer at 4°C and warm to room temperature before use.

PCR based Diagnosis of Plant Viral Diseases

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Polymerase Chain Reaction (PCR) is widely used for the detection of plant viruses due to its sensitivity, specificity and ability to amplify small amounts of target DNA. The a basic outline of how PCR-based detection of plant viruses progress through various steps such as Sample Collection, Nucleic acid extraction (RNA/DNA), Reverse Transcription (in case of RNA viruses), PCR reaction and analysis of the amplicons on the agarose gel.

I. Diagnosis of Cassava mosaic geminivirus infection by PCR

In this session we are trying to detect *Sri Lankan Cassava mosaic virus* (SLCMV) causing Cassava mosaic disease (CMD) in cassava. Sri Lankan Cassava mosaic virus & Indian cassava mosaic virus are the two species, infecting cassava in the Indian subcontinent. These viruses can be detected by PCR based diagnostic methods. In this experiment, we are detecting the SLCMV through the application of its coat protein (CP) gene using specific primers CP(F) and CP(R) Total DNA is isolated using standard protocol followed in the lab and using 50ng of DNA the PCR reactions are set up as follows.

Reaction Components	Volume (µL)
Nuclease free H_2O	36.5
Sample DNA	5
Taq buffer (10x)	5
dNTP (10mM)	1
CP (Forward)	1
CP (Reverse)	1
Taq DNA polymerase (2.5U)	0.5
Total	50

The reaction mixture is prepared in 0.2ml tubes are then placed in a Thermal Cycler with the following cycling programme for specific amplification of the CP gene of the virus. Along with the samples to be tested, a DNA from uninfected (Healthy) plant and non-template control has to be kept, for comparison.

Temperature profile for the application of CP gene of SLCMV.		
Initial Denaturation	95°C for 2 minutes	1
Cycle denaturation	94°C for 20 Seconds	34 cycles
Annealing	54°C for 30 seconds	
Extension	72°C for 1 minute	
Final Extension	72°C for 7 minutes	1
Hold	4 to 12°C	

After completion of the program, subject the PCR product to Agarose (1%) gel electrophoresis and view the amplified DNA band under a gel documentation system. In the case of SLCMV infected plants, an amplified product of 780bp can be observed, whereas in the case of healthy and non-template control, no bands can be observed.

II. Reverse Transcription PCR for the Detection of Poty Viruses in Crops

Potyviruses are the largest family of plant viruses that can infect a wide range of crops, including potatoes, tomatoes, peppers, and soybeans. They are single-stranded RNA viruses and are transmitted primarily by aphids in a non-persistent manner. Potyviruses can cause various symptoms in plants, including mosaic patterns on leaves, stunted growth and reduced yields. Management strategies for potyviruses include proper diagnosis, usage virus-free planting material, controlling aphid populations, and practicing crop rotation.The*Potyvirus* group can be detected by PCR based method. The procedure described in this session is achieved by a two-step. Initially, the total RNA is isolated from the leaves of yam and reverse transcribed using suitable reverse transcriptase enzyme to generate complimentary DNA (cDNA). Using this cDNA, as template another round of PCR is performed using *Potyvirus* group specific primers and the amplicons are analyzed on agarose gel.

If denaturation of template RNA is desired, use the following protocol.

Mix RNA sample and primer $d(T)_{23}VN$ in a sterile RNase-free microfuge tube.

Components	Volume(µL)
Total RNA	Upto 1ug
d(T) 23 VN (50µM) or Random Primer Mix (60 µM)	2 µl
10mM dNTP	1 µl
Nuclease free water	to 10 µl

Denature sample RNA/primer for 5 minutes at 65°C. Spin briefly and put promptly on ice.Add the following components.

Components	Volume (µL)
10X M-MuLV Buffer	2 µl
MuLV RT (200U/ µl)	1 µl
RNase Inhibitor (40U/ µl)	0.2 µl
Nuclease free H_20	6.8 µl

The above reaction mix and incubated at 42^0C, for one hour. Inactivate the reaction by incubating at 65^0C.The cDNA is later used to set up the PCR reaction using the species specific forward and reverse primers as follows

Components	Volume (µL)
Nuclease free water	6
2X PCR Master mix	10
Forward Primer	1
Reverse Primer	1
cDNA	2
Total	20

The reaction mixture is then subjected to PCR as per the temperature profile given below.

Reactions were run in a thermal cycler under the following amplification cycles.	
95^0C for 2 minutes	
95^0C for 30 seconds	34 cycles
55^0C for 30 seconds	
72^0C for 50 seconds	
72^0C for 8 minutes	
Hold at 4°C	

The amplified PCR products have to be analysed on 1% agarose gel. The approximate size of the amplicon is approximately 400bp. Presence of this specific band observed on the gel confirms the presence of *Potyviurs* group of viruses in the tested plants.

Real Time qPCR

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Real-time qPCR amplifies specific DNA or RNA sequences of target pathogens using fluorescent probes. These probes bind to the amplified target, and the emitted fluorescence signal is measured in real-time. The amount of fluorescence correlates directly with the amount of target DNA/RNA, allowing for both detection and quantification of the pathogen.

Based on detection method real time PCR can be classified into,

SYBR Green qPCR which uses a fluorescent dye, SYBRGreen that binds to any double-stranded DNA, making it cost-effective but less specific. It requires careful primer design to avoid nonspecific amplification.

II) Probe-based qPCR uses specific probes that bind only to the target sequence, offering higher specificity and sensitivity. However, it is more expensive due to the need for probe design and synthesis.

Based on quantification method real time PCR can be classified into,

I) Relative quantification which compares the expression level of a target gene to a reference gene, providing information on fold changes but not absolute copy numbers.

II) Absolute quantification uses a standard curve to determine the absolute number of target molecules present in a sample, requiring accurate standards and careful calibration.

Advantages of Real-Time qPCR for Pathogen Detection includes high sensitivity, specificity, speed, simultaneous quantification and detection and multiplexing (detection of multiple pathogens in a single reaction).

Objective: To detect and quantify the SLCMV DNA A in infected cassava sample by real time PCR using SYBR Green dye by absolute quantification (standard curve method).

Materials and Methods

Plastic wares (autoclaved aerosol filter pipette tips, PCR plate or PCR strips), pipettes, spectrophotometer, Real time PCR machine(Biorad CFX96 /ABI Step one plus / Eppendorf)

Reagents include SYBR Green qPCR Master-mix, Forward and ReversePrimers

(qPCR Primers can be designed using any primer designing tool (NCBI/ Primer 3 plus), Nuclease Free water, Total DNA isolated from SLCMV infected and healthy samples

Protocol

To quantify copy numbers of SLCMV DNA-A molecules through qPCR, a calibration curve was generated. Standard DNA of known concentration is prepared from plasmid harbouring a full length copy of SLCMV DNA A. To determine the initial concentration needed for standard preparation, copy number of plasmid should be calculated first. The molecular weight of each plasmid was determined from plasmid concentration (ng) and length (bp). The conversion from mass to molecules was done assuming an average molecular weight of a deoxyribonucleotide (330kDa) and the number of base pairs in the DNA using the formula

$$\textbf{Plasmid Copy Number } /\mu\text{l} = \frac{(\text{ amount(ng) x [6.022 x 1023]})}{(\text{length(bp) x } [1 \times 109] \text{ x } 650)}$$

where,

Amount is the concentration of DNA (plasmid+insert) per microlitrein nanograms (ng/µl); 6.022 x 10^{23} is Avogadro's constant; Length is the length of DNA (plasmid+insert) fragment in base pairs; 1 x 10^9 is the convertion factor in nanograms.

qPCR reaction

Set up a real time PCR assay for absolute quantitation of SLCMV viral loads in infected and control cassava samples using standards having known copy numbers. For each DNA sample, three replicate reactions were prepared. Non-template controls were also used in the set up.

Steps:

1. Quantify the plasmid DNA using spectrophotometer and calculate the copy number of plasmid per microlitre using theformula.
2. Make 10 fold serial dilution for the plasmid DNA to obtain standards with 10^8 to 10^3 copies
3. Set up the experiment design in the real time machine software (Biorad CFX Maestro) .
4. Define the reactions asStandard, DNA templates as "Unknown" and water templates as "NTC".
5. Prepare the master mix for sufficient reactions as given below

Sl.No	Component	Unknown-DNA (100ng/ µl)template Volume (µl)	Non-Template control (NTC)- water template Volume (µl)
1	2X SYBR GreenqPCR Master Mix	6.25	6.25
2	qPCR Forward primer	0.25 µM	0.25 µM
3	qPCR Reverse primer	0.25 µM	0.25 µM
4	Water	4.75	4.75
5	Template DNA	1.0	1.0
	Total	**12.5**	**12.5**

1. Mix well and centrifuge briefly
2. Pipette it out 11.5 µl of the PCR master mix to each well (standards, unknown samples and non-template control)
3. Add 1 µl DNA template from the standards, unknown samples or water to each well separately.
4. The PCR reactions were carried out in a final volume of 12.5 µL following an initial activation for 3 min at 95°C, followed by 40 cycles of amplification (denaturation for 15 sat 95°C, annealing for 20 s 53°Cand extension for 20s at 72°C) in the Biorad CFX 96 real time machine.

To determine the melting temperatures of the amplified products after SYBRGreenqPCR, the temperature was raised from 55 °C to 95 °C and the fluorescence was detected for 10 s after each 0.2 °C

5. Analyze the output data after the PCR using the real time PCR software

Results

From each reaction, the threshold cycle value (Ct) was established as the cycle number at which fluorescence was detectable over the threshold value calculated by the software for cycles 2–10. For absolute quantification, the number of viral molecules (SLCMV DNA A) was determined from standard curves generated from standard plasmid containing DNA A.

A standard curve was plotted for the Ct values vs log concentration of standards using analysis software. From the standard curve, the concentration of the unknown samples (genome copies) was determined using the Ct values.

General real time PCR Practices

- Wear a clean lab coat and clean gloves.
- Maintain separate areas and dedicated equipment and supplies for standard preparation, unknown sample preparation, PCR setup, PCR amplification and analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Avoid air bubbles while dispensing PCR master mix and templates
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipette or aerosol-resistant pipette tips
- Clean lab benches and equipment periodically with 10% bleach solution.

Rolling Circle Amplification (RCA)

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Rolling circle amplification (RCA) using the bacteriophage Phi29 DNA polymerase allows for a reliable diagnosis of geminiviruses and presumably all viruses with small single-stranded circular DNA genomes.

The enzyme possesses both, polymerase and strand displacement-activity, thus allowing circular DNA to be replicated nearly unlimited extent using rolling circle amplification mechanism. Neither specific primers are needed to start reaction, nor expensive equipment. In a single reaction, all infecting circular DNA components are amplified, including defective subviral DNA and presumably the circular satellite DNAs which accompany several geminiviruses of Old World and are important pathogenic determinants.

Most infectious clones of geminiviruses consist of partial tandem-repeats of viral genomes in the vectors, which usually involve tedious multi-step assemblies of genomic fragments in the construction process. Partial digestion of multimeric viral genomes produced by RCA, followed by direct cloning into appropriate vectors further simplified the procedure.

Methodolgy

- Amplification of circular DNA was performed using10-20ng of total nucleic acid as template.
- The reaction mixture comprised of 2µl of Phi 29 DNA polymerase buffer (10X), 2µl of exo-resistant random hexamer primers (500µM) and 2 µl of dNTPs (10mM).
- The template DNA was denatured for 3 min at 94ºC and cooled down to room temperature.
- After cooling 4 µl of Pyrophosphatase (0.1 U/ µl) and 0.7 µl of Phi 29 DNA polymerase (10U/ µl) were added and incubated for 18-20 h. at 30ºC followed by heat inactivation at 65ºC for 10 min.
- The products were used for construction of multimeric clones as concatameres. Gel electrophoresis of RCA product shows uniform smear of DNA as large amount of replicative concatamers are generated by RCA.

Restriction analysis of RCA product

500 ng of rolling circle amplified DNA was subjected to digestion with different endonucleases to identify the unique restriction sites which can be used for cloning. Restriction of RCA, with common enzymes yielded 2.7 or 1.3 kb fragments which were presumed to represent full length genomic DNA components (either DNA-A or DNA-B) and the satellite DNA (alpha or beta).

The advantages are:

- No expensive devices are necessary
- Simple handling
- Detection of all infecting circular DNA components without any knowledge of sequence information in a single step
- Low costs per reaction

Loop mediated isothermal amplification (LAMP) PCR for detection of virus

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Introduction

LAMP is a nucleic acid amplification method to amplify a specific DNA region under isothermal conditions. This technique was developed as an attempt to overcome some drawbacks of the conventional PCR, a method that requires the acquisition of a high-cost equipment such as thermal cycler. In conventional PCR, the necessity of a high precision in heating/cooling ramps and temperatures produces occasional losses of appropriate specificity for the identification of the selected targets. In addition, the polymerase enzyme is quite sensitive to inhibitors usually present in nucleic acid extracts, especially isolated from plant matrices. Conversely, the isothermal amplification by LAMP does not require any specific equipment, while it provides high specificity owing to the use of four to six primers that recognize between six and eight independent regions, all of them addressing a specific target region. In addition, the robustness of the enzyme used in the LAMP methodology reduces the inhibitors problems. The benefit of LAMP to be easily adapted for point-of-care analysis makes the technique a valid method for surveys or quarantine programs where rapid, reliable, and specific analysis is required.

Principle

LAMP technique can be divided into three stages: the production of the starting material for the reaction, cyclic amplification, and elongation combined with re-cyclization. The most important step in the first stage is to produce an artificial template in the form of single-strand DNA with a dumbbell-like structure. The subsequent stages involve an exponential amplification of the self-priming template and the replacement of the strands, which yields a mixture of double-strand DNA.Three pairs of primers are used in LAMP namely internal primers (forward internal primer (FIP) and backward internal primer (BIP)),external primers (forward primer (F3) and backward primer (B3)) and loop primers(loop primer forward (FL) and loop primer backward (BL)). The internal primers are long (45–49 bp) and complementary to two distant locations on the template (on the sense strand and the antisense strand). The external primers are shorter (21–24 bp) and bind with the template more slowly than the internal primers. The internal and external primerscombined with the Bst DNA polymerase create the dumbbell-like DNA structure. This structure serves as a template for further amplification. Adding loop primers, which are complementary to the dumbbell-like DNA, increases the number of starting points during the LAMP reaction up to a total of eight amplified DNA sequences. Thus, the loop primers significantly improve the efficiency and sensitivity of the reaction and reduce the time it takes by 50%. Furthermore, the loop primers activate only once when the artificial template has been created, which considerably increases the selectivity of the reaction.

Requirements

Chemicals: Nuclease free water, Buffer, MgSO4, dNTP, primer sets, Betaine, Bst II polymerase, Template DNA

Plastic wares/ Others: Autoclaved PCR tubes (0.2 mm) and tips, micropipettes etc.

Protocol

1. Thaw all the chemicals required for LAMP PCR in a mini cooler.
2. Prepare the master mix for sufficient reactions as given below.

Sl. No.	Components	Reaction volume (µl)
1	Nuclease free water	14.5
2	Isothermal amplification buffer	2.5
3	100 mMMgSO4	1.5
4	10 mMdNTP	0.5
5	Forward primer (F3)	0.5
6	Backward primer (B3)	0.5
7	Forward inner primer (FIP)	0.5
8	Backward inner primer (BIP)	0.5
9	Forward loop primer (FL)	0.5
10	Backward loop primer (BL)	0.5
11	5 M Betaine	1
12	Bst II polymerase	1
13	Template DNA	1
	Total	**25**

3. Mix well, spin down and place the reaction mix in a thermal cycler.

   PCR program:

   63° C - 1 hour

   80°C - 10 min

   12°C - hold
4. Visualize results by running the PCR product in 2 % agarose gel.