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Crop Disease Diagnosis

Disease diagnosis is very important for developing effective strategies for disease management. Without diagnosis, there can be no disease management. Crop disease diagnosis is an art as well as a science (Holmes et al., 2000). The diagnostic process involves the recognition of symptoms (which are associated with disease) and signs (which are not outwardly observable) and requires intuitive judgment as well as the use of scientific methods. Several conventional techniques are followed to diagnose disease incidence. These techniques include visual inspection and recognition of symptoms and isolation and examination of crop pathogens using microscopy. Such techniques are time-consuming and may not be able to detect latent infections. Several diagnostic assays have been developed for early and rapid diagnosis. These include immunoassays, nucleic acid probe-based methods, and PCR-based techniques. The use of these techniques in the diagnosis of fungal, bacterial, viral, viroid, and phytoplasma diseases is described here.

DIAGNOSIS BASED ON DISEASE SYMPTOMS

Some pathogens produce characteristic symptoms that can be easily recognized in the field. Fungal diseases such as powdery mildews, rusts, downy mildews, and smuts show characteristic symptoms. Some bacterial diseases such as canker and crown gall can be recognized based on symptoms. A few virus diseases produce characteristic symptoms such as bunchy top, rosette, witches'-broom, phyllody, and flower color-breaking. Diseases should be diagnosed at early stages of their development, so that epidemics can be effectively prevented. The early stages of many diseases are inconspicuous and it may not be feasible to make a rapid visual assessment until the level of disease is sufficiently high. Further, even at advanced stages of symptom development, some diseases cannot be recognized with certainty. For example, symptoms such as chlorosis, mosaic, leaf drooping, yellowing, dwarfing, stunting, necrosis, root rots, wilts, fruit rot, dieback, leaf blight, and bud rot can be caused by several types of fungal, bacterial, viral and phytoplasma pathogens. In these cases, the appearance of fruiting bod-

ies of the pathogens on leaf, stem, and fruit surfaces must be checked. However, sporulation of the pathogen occurs only at a very late stage of infection. Sometimes saprophytes may develop on these lesions, making diagnosis a difficult task. Hence, the pathogen has to be isolated in pure culture and identified using a microscope. Sometimes selective media may be required to isolate pathogens and induce sporulation for identification. Many fungal pathogens grow slowly in media and may be lost too readily for identification. If the pathogen is a bacterium, virus, or phytoplasma, more complicated tests have to be conducted to identify them. Several biochemical tests must be conducted to identify bacterium. Routines for extraction and purification of viruses require professional proficiency and skill. In fact, conventional plant-pathological techniques need high expertise for routine identification. In the case of latent infections in vegetative planting materials, seeds, and fruit, conventional methods may not be useful to diagnose infection.

DIAGNOSIS BY IMMUNOLOGICAL TECHNIQUES

Immunodiagnostic assays provide a fast method of confirming visible symptoms as well as detecting pathogens that cannot be easily identified by other methods. They permit early detection of plant pathogens and accurate identification of pathogens. Because many fungicides are specific only to certain pathogens or groups of pathogens, immunodiagnosis will be useful in the selection of the most appropriate treatment. Viruses, bacteria, and fungi (especially those spreading as a sterile mycelium) can be readily detected by these methods.

Immunoassays depend on the development of antibodies specific to the particular pathogen. Cells of living animals, particularly mammals, have the ability to recognize binding sites on proteins, glycoproteins, lipopolysaccharides, and carbohydrate molecules that are not present in their bodies (i.e., foreign to that animal). Such molecules, known as antigens, stimulate the immune system of the animal and this leads to the production of specific antibodies, each of which specifically recognizes and binds to its complementary antigen. The role of an immunoassay is to reveal the presence of specific complexes between the antibody and an antigen that are unique to the pathogen (Fox, 1998). The antibodies produced in an animal body can recognize the microbial antigen, which is present on cell walls or found attached with them. In other words, the antibodies can recognize the plant pathogen by recognizing the antigen specific to the pathogen. In principle, immunoassays are based on the fact that antibodies react specifically with

the homologous antigen. However, the reaction is not easy to detect. Several techniques have been developed to exploit this reaction in immunoassays.

Production of Polyclonal Antibodies

Rabbits, rats, mice, sheep, or goats are used to produce antibodies. For the production of antibodies to fungal pathogens, mycelia (obtained from sterile cultures grown in the laboratory) are freeze dried. They are then finely ground with a pestle and mortar and used as antigens. Surface washings from fungal mycelia are also used as antigens. These antigens are emulsified with an equal volume of an adjuvant, such as Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the second or subsequent injections. Other types of adjuvants such as Quil A are also used. Bacterial antigens can be prepared by first killing the bacteria with heat or formalin and then suspending them in phosphate-buffered saline. The bacterial cells are disrupted by grinding, sonication, or enzyme action to produce soluble cytoplasmic antigens. Virus antigens can be prepared from infected host tissues by differential centrifugation or precipitation with ammonium sulfate.

These antigens (otherwise called immunogens) are then injected into rabbits (or other animals) to induce complementary antibodies. The antigen emulsion is injected under the skin at the back of the neck of the animals. One or more booster injections with the emulsion are given several weeks after first injection. Antiserum is obtained by taking blood from the ears of rabbits or from the tail veins of mice.

Antibodies have a basic common structure, which consists of two heavy and two light chains held together by disulfide bonds. However, they differ in the ability of their tips to bind to sites on different "foreign" protein, glycoprotein, lipoprotein, lipopolysaccharide, and carbohydrate molecules (antigenic substances). Such antigenic substances stimulate the animals' immune systems to produce an assortment of specific antibodies, each of which specifically recognizes and binds almost exclusively to its complementary epitope (antibody reaction site) on the antigen. This interaction between an antibody and an antigen depends on pairing of an individual epitope of the antigen in three dimensions between the contours of the tips of the side chains of a particular antibody. Several antigens have been detected in viral, bacterial, and fungal pathogen preparations. The antisera raised against these antigens may contain a mixture of antibodies directed toward various epitopes of the pathogens. These antisera, which contain polyclonal antibodies, are called "polyclonal antisera."

Production of Monoclonal Antibodies

Kohler and Milstein (1975) were the first to produce monoclonal antibodies. Monoclonal antibodies (MCAs) recognize a single epitope only and can therefore differentiate between related pathogens. Monoclonal antibodies that are specific at genus, species, pathovar, and strain levels are available. MCAs are homogenous and therefore free from the variability common to polyclonal antisera. Once a hybridoma cell line is obtained, an endless supply of the same MCA can be produced. The method of preparation of monoclonal antibodies is given here:

An antigen (immunogen) is prepared from cultures of fungal and bacterial pathogens or from the sap of virus-infected plants as described for the preparation of polyclonal antibodies. Mice, usually females of the BALB/c strain, are immunized by intraperitoneal injection with the antigen preparation. The preparation is given at about two week intervals until antiserum from trial bleeds gives a high titer against the immunogen. The final booster injection is given without an adjuvant about three to four days before fusion. The mice are killed and their spleens are removed. β-Lymphocytes are isolated from the spleens of immunized mice. Meanwhile, a potentially immortal line of myeloma cells is obtained from an animal similar to the animal used for immunization (mostly mouse), subcultured, and grown for fusion with the spleen cells to form hybridoma cells. Viable spleen cells are mixed at a ratio of 1:10 with viable myeloma cells. This cell mixture is washed by centrifugation at 60 g for 10 min and the pellet loosened by tapping the tube. One ml of polyethylene glycol 4000 (PEG 4000) is then added dropwise over a period of 1 minute to the pellet, stirred for another minute, and slowly diluted by the addition of a tissue culture medium. Dulbecco's modified Eagle's medium is generally used as a tissue culture medium. Penicillin-streptomycin solution, L-glutamine (200 mM), phenol-red solution, and controlled processed serum replacement are added to the medium. The cells are washed and resuspended in fresh HAT (hypoxanthine, aminopterin, thymidine) medium (a selective medium made by supplementing the complete tissue culture medium with 0.01 M hypoxanthine, 0.0016 M thymidine, and 0.00004 M aminopterin) and dispensed into 96-well tissue culture plates. The plates are kept in an incubator at 37°C with 5 percent CO_2. After ten to 16 days, aliquots of the supernatant are removed from the wells containing clones and screened by ELISA (enzyme-linked immunosorbent assay). Only hybridomas producing antibodies specific to the antigen are saved and grown on a large scale in vitro. Selected hybridoma cells are grown in bulk. The antibodies are purified on a sepharose column. Once a useful MCA has been generated, the selected hybridoma cells can be immortalized by storing them in liquid nitrogen. Whenever needed, MCAs can be produced indefinitely and in unlimited quantities.

Both polyclonal and monoclonal antibodies are used in various immunodiagnosis techniques.

Agglutination Test

This test can be carried out in slides or in test tubes. In the slide agglutination test, drops of antigen and diluted antiserum containing antibodies are mixed together on a glass microscope slide. Agglutination is observed by eye or microscope (if ambiguous). In the test-tube agglutination test, the antigens are mixed with antibodies in test tubes, and the aggregation of antigens and antibodies is monitored with a binocular microscope.

Precipitation Test

In this test, aliquot dilutions of antigen are layered over equal volumes of antiserum diluted in normal serum in capillary, or other small tubes. The test is regarded as positive if there is precipitation at the interface (Fox, 1998). When the antigens are layered over the antibodies, the antigens are precipitated out of solution by the antiserum when antigen and antibodies are related.

Latex-Agglutination Test

In this test, the antibodies are first adsorbed onto the much larger latex particles and mixed with the antigen preparation (sap of diseased tissues). An opalescent suspension containing antigen particles will settle out, producing a clear supernatant by granulation or flocculation in a positive test.

Ouchterlony Immunodiffusion Test

In this test, a petri dish containing 1 percent agar is used. A central well (usually 0.5 cm in diameter), surrounded by several satellite wells, is cut into the agar. The antibody is placed on the central well, whereas the antigen is placed on surrounding wells. The antibodies and antigens are allowed to approach each other by diffusion in agar. In the positive test, a precipitin band, usually visible to the naked eye, forms at the leading edge where diffusing antigen and antibody molecules meet.

Immunoelectrophoresis

By this method, mixtures of antigens are separated before immunodiffusion. A narrow trough is cut in a layer of thin gel parallel to an electric current that passes close to the antigens along the length of the gel. Each antigen moves in a separate wave at a characteristic rate according to its distinct charge. As a result, proteins separate into bands. Once the proteins have separated sufficiently, the current is switched off and antiserum is added to the trough cut in the gel. Precipitin arcs composed of complexes of antibodies and antigens form where the individual electrophoresced antigens have reached.

Antigen-Capture Indirect ELISA

Infected plant sap is used as an antigen. Many variations of ELISA exist, such as antigen-capture indirect ELISA, direct sandwich ELISA, double antibody sandwich ELISA (DAS-ELISA), triple antibody sandwich ELISA, and $F(ab')_2$-based ELISA.

In the antigen-capture indirect ELISA method, the wells are coated with the antigen. The remaining sites are blocked by a blocking agent, and a specific primary antibody is added. A secondary antibody-enzyme conjugate binds to the primary antibody. This is followed by the addition of an enzyme substrate leading to a colored product in proportion to the concentration of pathogen. This methodology involves coating immunoplates with the antigen and then centrifuging them at 600 g for 5 min at 4°C. The plates are post-fixed by incubation of 100 µL per well of 0.25 percent glutraldehyde solution for 5 to 7 min at room temperature. The fluid is flicked off by inverting the plate, and the plates are washed three times with either tris-buffered saline (TBS) Tween (TBST) or phosphate-buffered saline (PBS) Tween (PBST) followed by a short period of drying by laying the plates on a paper tissue. About 200 µg blocking solution (2 percent casein in either TBST or PBST) is absorbed to the remaining sites in the well for 1 h at room temperature. After one washing, the antibody in TBST or PBST is added at 50-100 µL per well. After 1 to 2 h incubation at 37°C and three further washings, antimouse immunoglobulin IgG alkaline phosphatase conjugate diluted in TBST or PBST containing casein (2 percent) is incubated for 1 to 2 h at 37°C (50-100 µL per well). The excess fluid is removed by flicking the plate, followed by washing with TBST or PBST for three to five times. Phosphatase tablets are freshly dissolved in diethanolamine (DEA) buffer, pH 9.6, and this substrate solution is pipetted into the wells. Substrate conversion is quantified by measuring absorbance at 405 nm with a plate reader (after at least 30 min incubation in the dark at 37°C).

Direct Sandwich ELISA

In the direct sandwich ELISA method, 96-well immunoplates are coated with the specific antibody (polyclonal or preferably monoclonal antibody) and incubated successively with the antigen containing sample followed by a second enzyme-labeled specific antibody that is directly conjugated with an enzyme. This leads to a colored product in proportion to concentration of pathogen.

Double Antibody Sandwich ELISA

In the double antibody sandwich ELISA method, a specific capture antibody is immobilized onto a solid surface (Huttinga, 1996), such as the wells of a microtiter plate. The infected plant tissue sample is added, and unbound material is washed away. Bound antigen is detected by the addition of a detecting antibody that has been conjugated with an enzyme, and unbound material is again washed away. The presence of the detecting antibody is determined through the addition of a substrate for the enzyme. The amount of color that develops is proportional to the amount of antigen present in the sample. The intensity of the color can be recorded by automated equipment (Miller, 1996).

Triple Antibody Sandwich ELISA

In the triple antibody sandwich ELISA method, a specific antibody produced in one animal species is bound to the solid substrate (96-well plates), while a second specific antibody produced in another animal species binds to the bound antigen. The "sandwich" is detected by an antibody-enzyme conjugate that binds to the second antibody (Miller, 1996).

$F(ab')_2$-Based ELISA

In $F(ab')_2$-based ELISA, $F(ab')_2$ fragments from target-specific antibodies are used as the capture reagent. A specific second antibody produced in the same animal species binds to antigen already bound to the $F(ab')_2$ fragments. The sandwich in turn is detected by the addition of a general detecting antibody-enzyme conjugate that reacts specifically with the Fc portion of the second antibody (Miller, 1996).

Dipstick ELISA

ELISAs can be carried out on membranes made of nitrocellulose, nylon, or other materials. In the dipstick assay, nitrocellulose dipsticks surface-coated with a capture antibody are used. Dipsticks on which specific MCAs have been bound are dipped into the test specimen, allowing ELISA reactions to occur in situ. Flexible plastic dipsticks are quick and easy to use in the field to test sap squeezed onto the coated surface. Any pathogen (particularly viruses) present is entangled and detected following incubation with the antibody-enzyme conjugate, washing, and then the substrate, further washing, and finally the stopping solution. If the specified pathogen is present, the dipstick becomes colored, whereas if free of pathogen it remains colorless.

Dot-Blot ELISA

In this assay system, ELISA reactions are carried out on nitrocellulose membranes. A drop containing the specific monoclonal antibody is absorbed as a "dot," onto which a drop of the test sample is later added and blotted (Fox, 1998).

Direct Tissue Blot Immunoassay

This assay is also called tissue print ELISA, immunoprinting, or direct tissue print immunoassay. In this assay, stems and leaf petioles are cut with a razor blade, and the cut surface is pressed gently and evenly to the nitrocellulose membrane. These blots are allowed to dry for 10 to 30 min, incubated with the monoclonal antibodies for 2 h, and rinsed with PBST buffer for 10 min. The blots are labeled with an enzyme conjugate, goat antimouse Ig (H + L), for 1 to 2 h at 37°C, rinsed with PBST-polyvinylpyrrolidone (PVP) buffer, and rinsed again with TTBS (20mM tris (hydroxymethyl) aminomethane, 500 mM sodium chloride, and 0.05 percent Tween 20), each for 10 min. The blots are incubated with freshly prepared NBT-BCIP (nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate in sodium carbonate buffer) substrate for 5 to 20 min. After stopping the reaction by putting the blots in water in a petri dish, the blots are observed under a light microscope. Development of an area with an intense purple color located at phloem tissue cells is considered a positive reaction (Lin et al., 2000).

In Situ Immunoassay (ISIA)

In this assay, sections 100 to 200 μM thick are cut from stems, petioles, or veins of diseased plants. The sections are transferred to 24-well plastic plates and fixed with 70 percent ethanol for 5 to 10 min. After the alcohol is removed using a pipette, the sections are incubated with specific monoclonal antibodies for about 30 min. The sections are washed with PBST-PVP for 5 to 10 min, and incubated with enzyme-labeled secondary antibodies (alkaline phosphatase conjugated goat antirabbit IgG) at 37°C for 30 to 60 min. The sections are washed again with PBST-PVP and with TTBS buffer. Sections are then incubated with freshly prepared NBT-BCIP substrate mixture for 5 to 15 min. After stopping the reaction by removing the substrate solution, the sections are transferred to a glass slide and observed under a light microscope. The development of a purple color in the phloem tissue cells is considered as a positive reaction (Lin et al., 2000).

Recombinant Enzyme-Linked Immunosorbent Assay

In this assay, monoclonal antibodies are subcloned from hybridoma cell lines and expressed in *Escherichia coli* as single-chain variable fragment antibody (scFv) fusion proteins. The scFv technology allows the cloning of variable genes from preexisting monoclonal antibodies' cell lines, linking them with a flexible peptide as an scFv. These constructs are expressed in bacteria as soluble proteins or fused with the capsid proteins of filamentous bacteriophages. In addition, antibody genes are expressed fused with other proteins such as alkaline phosphatase or with amphipathic helices (Terrada et al., 2000).

Phage Display Technology

Recombinant antibody technology, in combination with phage display technology, provides a useful approach for the production of target-specific antibodies without the use of laboratory animals and time-consuming immunization protocols (Griep et al., 2000). Recombinant antibodies are raised from a human combinatorial antibody library. Helper phages, which contain the entire phage genome but lack an efficient packaging signal, are used to "rescue" phagemids from the combinatorial library. When both the helper phage and single-chain variable fragment encoding the phagemid vector are present within the same bacterium (e.g., *Escherichia coli*), phages are assembled that carry scFv-antibodies (phage antibodies [PhAbs]) on their surface and contain the scFv-encoding phagemid vector. Consequently,

within PhAbs, the genotype and phenotype are linked. To select for antigen specificity, PhAbs rescued from the combinatorial antibody library are allowed to bind to immobilized target antigens (panning). Washing removes PhAbs that lack affinity for the target antigens. Bound PhAbs are eluted, and selected PhAbs are applied to three sequential rounds of panning to further enhance the percentage of target-reactive PhAbs (Griep et al., 2000). PhAbs are selected to major structural proteins of the test virus. The scFv-encoding genes are retrieved and expressed in *E. coli* as ready to use antibody-enzyme fusion proteins. After subcloning, the encoding DNA sequences in the expression vector pSKAP/S, which allowed the scFvs to be expressed as alkaline phosphatase fusion proteins. The antibodies are used as coating and detecting reagents in a DAS-ELISA (Griep et al., 2000).

Latex-Protein A Agglutination Assay

In this assay, inert particles such as latex beads are used to detect antigen/antibody complexes. Protein A, a cell surface protein from *Staphylococcus aureus* that binds nonspecifically to the heavy chains of mouse and rabbit IgG antibodies, is used to link the particles coated with the specific antibodies by shaking the two together. Samples of plant extracts are tested by incubation with coated particles. If the test pathogen is present in the sample, the antigens form bridges linking the particles together, resulting in agglutination.

Immunofluorescence

Two methods of immunofluorescence are used to diagnose plant diseases. In the direct immunofluorescence method, specific antibodies bound to their target antigens are detected by using second antibodies conjugated with fluorescent dyes such as fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate. Fluorescence, indicating the presence of the target antigen, is visualized microscopically. The microscope should have a special device for fluorescence using ultraviolet light (fluorescence microscopy). In direct immunofluorescence, the fluorescent dye is conjugated directly to the specific antibody (Salinas and Schots, 1994). Usual protocols involve extraction of the pathogen fraction from the tissue of individual or composite plants that is fixed to a well of a multiwell glass microscope slide by flaming or acetone treatment. Fixed preparations are stained directly by conjugated antibody or by the indirect procedure in which the primary antibody is not conjugated but a secondary conjugated antibody is bound to the primary one (De Boer et al., 1996).

Immunosorbent Electron Microscopy

This assay system is mostly used for the diagnosis of virus diseases. Electron microscope grids coated with carbon strongly adsorb protein, and when they are floated on a drop of antiserum containing antibodies to the pathogen, the antibodies become attached. The grids are then floated on a drop of the sap of an infected plant. After staining, the pathogen (particularly virus particles) adsorbed to the antibodies can be seen under a transmission electron microscope.

Immunosorbent Dilution-Plating Technique

In this method, bacterial pathogens are trapped on antibody-coated petri dishes or inoculation rods so they can be incubated on selective media. Any unbound bacteria are removed by washing. The colonies that form are detected by drying the agar and staining by immunofluorescence.

Western Blotting

This technique involves the transfer of proteins or glycoproteins on polyacrylamide gels electrophoretically onto a membrane or solid phase and the probing of such membrane-bound antigens with a specific antibody solution, based on the covalent binding of the antibody and its antigen (Fox, 1998). In the electrophoresis, different proteins move apart to form distinct bands. The larger molecules migrate more slowly than the smaller molecules. The protein in these bands can be transferred by blotting to a strip of porous nitrocellulose material. Afterward, specific polyclonal or monoclonal antibodies are used to bathe nitrocellulose for about an hour. An enzyme/substrate system (phosphatase or peroxidase conjugate), similar to an ELISA reaction, is also used to locate the position of bound antibodies (Fox, 1998).

Immunogold Assay

In the immunogold assay, following the electrophoresis of proteins on SDS polyacrylamide gel, proteins are transferred to a nitrocellulose membrane. Monoclonal antibody specific to a protein of the test pathogen is conjugated to gold particles and applied on the nitrocellulose membrane. The signal appears as a band and can be visualized on the membrane directly.

Radioimmunoassay

In the radioimmunoassay method, the protein is transferred to a nitrocellulose filter after electrophoresis, and a monoclonal antibody is added to it. MCA binds to a specific protein, and a radioactive detector antibody is added. The band is visualized indirectly by preparing an autoradiograph.

NUCLEIC ACID PROBE-BASED METHODS

Both DNA and RNA probes are used for crop disease diagnosis. DNA is double-stranded and the two strands are held together via specific hydrogen bondings between the base pairs. Pairing occurs with great specificity: Adenine (A) pairs only with thymine (T), and guanine (G) only with cytosine (C). The interaction of the two single-stranded molecules is known as hybridization. Several physical conditions can denature the DNA into single strands. High temperature and strongly alkaline pH denature double-stranded DNA. By manipulating the physical conditions, a strand can anneal with another strand of the target DNA. Nucleic acid hybridization, or reassociation, is a process by which complementary single-stranded nucleic acid anneals to double-stranded nucleic acid. This process occurs because of the hydrogen bonds between the two strands of the DNA. The pairing can also take place between DNA and RNA or RNA and RNA, and the pairing can occur between adenine (A) and uracil (U) in RNA instead of thymine (T) in DNA and guanine (G) and cytosine (C). Single-stranded DNA and RNA can act as probes. A diagnostic nucleic acid probe may be defined as a nucleotide sequence, labeled with a reporter molecule, that is able to identify a target pathogen within a test sample by selectively hybridizing to the complementary sequence present within a microorganism's DNA (De Boer et al., 1996). Nucleic acid probes are sequences of nucleic acids that are labeled with a marker and used to detect complementary nucleic acid sequences in a sample. Probes, which can be either DNA or RNA, range in size from 15 to several thousand base pairs (bp). Hybridization protocols usually require the probe or target sequence to be immobilized on a solid surface, but the reaction can also occur in solution or in situ. The probe-target hybrids are visualized using autoradiography, colorimetric assays, or chemiluminescence.

Hybridization Formats

Nucleic acid hybridizations may be performed in solution or on a solid support. In a solid-support format, the target nucleic acid from the pathogen to be detected is immobilized on a nitrocellulose or nylon membrane. DNA

is first cut into fragments by a restriction enzyme, often followed by gel electrophoresis and denaturation (Southern, 1975). RNA is usually transferred by a similar technique known as Northern blotting. After it has been blotted onto the support, the target nucleic acid is fixed by baking or cross-linking under UV light. The labeled probe hybridizes to the target DNA. After the excess unbound probe has been removed by washing, the hybrid is detected by a suitable assay (Fox, 1998). The diagnostic probes are labeled with radioisotopes, primarily ^{32}P. Nonradioactive methods are also available for labeling DNA probes. Nonradioactive probes may be labeled enzymatically or chemically. Biotin, a vitamin, is commonly used for labeling, and the modified nucleotide is biotin-11-dUTP. Probes attached to biotin bind to avidin (a protein) extremely tightly. As a result, a more effective complex of many enzyme molecules can be produced by binding enzymes first to biotin and then to avidin, around which they cluster (Fox, 1998). The steroid digoxigenin may be employed in a similar way with antidigoxigenin enzymes for labeling. Sulfonation of cytosine residues by sulfite was one of the first hapten-based labeling systems (De Boer et al., 1996). The probe is detected using a monoclonal antisulfonate antibody followed by an alkaline phosphatase-conjugated antimouse IgG antibody. Another method of chemical labeling involves photobiotin, a compound in which biotin is bound to aryl azide by a linker arm. In the presence of light, aryl azide is converted into an aryl nitrene moiety that reacts with nucleic acid. Oligonucleotides are most efficiently labeled chemically by a reactive amine. A detectable group, such as alkaline phosphatase or horseradish peroxidase, is added to the amino-oligonucleotide after synthesis (De Boer et al., 1996).

Many commercial nucleic acid-based tests employ the sandwich assay, in which adjacent sections of the target nucleic acid are hybridized by two probes. One is the capture probe, which by hybridization links the target to the solid support to which this probe is already bound. The other probe is labeled with a reporter group, which may be a fluorescent molecule, an enzyme, or a radioactive atom that hybridizes with an adjacent section of the target nucleic acid. The reporter group with a fluorescent molecule can be readily distinguished, even in minute amounts (Fox, 1998).

Dot-Blot Diagnosis

When an extract or cell sap believed to contain the target nucleic acid is dotted on a filter, the method is called dot-blot. Dot-blot is a routine spot hybridization method. In this method, the DNA is not cut by restriction enzymes and fractioned. The DNA is applied as a small drop of sap extracted from an infected plant directly onto a nitrocellulose sheet. The DNA is then

dried and hybridized with the probe in a sealed plastic bag using a buffer extract (Maule et al., 1983). The nucleic acid is loaded into a multisample vacuum manifold, which is used to spot it onto the membrane.

Colony Blot

Colony blot can be useful to detect bacteria in infected tissues. Dilutions of the bacteria from an infected tissue are grown on nitrocellulose filters placed on suitable agar media. After incubation, the nucleic acids are simultaneously released and denatured with alkali or by incubation in a microwave oven. The filter is then washed and baked prior to hybridization as a colony.

In Situ Hybridization Assay

In this assay, host tissue sections are fixed to a microscope slide and exposed to probe DNA. In another type of assay, sections of cut plant tissue are pressed against the nitrocellulose filter, which is then treated with alkali to denature the DNA and the tissue print is fixed on the filter. Hybridization reveals the presence of the pathogen.

PCR-Based Methodology

The polymerase chain reaction provides a powerful and rapid technique to exponentially amplify specific DNA sequences by in vitro DNA synthesis (Henson and French, 1993). Three essential steps to a PCR include (1) melting the target DNA, (2) annealing two oligonucleotide primers to the denatured DNA strands, and (3) extending the primer via a thermostable DNA polymerase. Newly synthesized DNA strands serve as targets for subsequent DNA synthesis since the three steps are repeated up to 50 times. The specificity of the method derives from the synthetic oligonucleotide primers, which base-pair to and define each end of the target sequence to be amplified (Henson and French, 1993).

PCR uses a thermostable *Thermus aquaticus (Taq)* DNA polymerase to synthesize DNA from oligonucleotide primers and template DNA. The template DNA may be genomic, first-strand cDNA, or cloned sequences. Primers are designed to anneal to complementary strands of the template such that DNA synthesis initiated at each primer results in replication of the template region between the primers.

The PCR involves three distinct steps governed by temperature. DNA, primers, deoxynucleotides, buffer, and *Taq* polymerase are combined in a microcentrifuge and overlaid with mineral oil. The tube is placed in a thermocycler programmed to repeat a set of short incubations at predetermined temperatures. In the first step, the template DNA is denatured to separate the complementary strands. This is done at 95°C for 5 minutes. In the second step, the mixture is held at an annealing temperature to allow the primers to hybridize to their complementary sequences. This is done at 55°C for 1 min. A PCR primer may comprise two regions, a 3' (priming) region and a 5' (variable) region. The most important region in determining the efficiency of annealing and subsequent DNA synthesis during the PCR is the 3' region, which should be perfectly complementary to the template sequence. The priming region should normally be 20 to 25 bases long. The *Taq* polymerase stabilizes these base-paired structures and initiates DNA synthesis. In the last step, the reaction is heated to about 72°C for 1 to 5 minutes. This process leads to a *Taq* polymerase-directed DNA synthesis. The cycle is repeated by keeping the reaction tubes in a thermal cycler for more than 20 times. In the first cycle each template gives rise to a newly synthesized complement. Thus, the number of copies of the target region is doubled. Similarly, in each subsequent cycle, the DNA concentration corresponding to the target region is almost doubled. About 20 cycles of PCR would produce 10^6-fold amplification of the target DNA.

Initially, very low numbers of DNA molecules can be multiplied enormously by PCR. Only a few nanograms of the initial template DNA is necessary for amplification. Both dsDNA and ssDNA can be amplified by PCR. It is possible to amplify RNA by reverse transcription (RT) into a cDNA copy by RT-PCR. Synthetic oligonucleotides (primers) that are complementary to the end sequences can be produced. Because the PCR process does not depend on the use of purified DNA, the host tissue extract can also been used for diagnosis.

The PCR product is analyzed by agarose gel electrophoresis. The PCR product from a defined band can be recovered from agarose gel. The DNA generated in a PCR can be reamplified and used for sequencing. DNA sequencing reactions are performed using commercially available kits of T7 polymerase or Sequenase.

RAPD (random amplified polymorphic DNA) PCR is an important diagnostic tool. Oligonucleotides of arbitrary sequence are used as primers for amplification. Shorter sequences on the order of 100 to 1,000 base pairs are most efficiently amplified and easily resolved by agarose electrophoresis. Hence, DNA sequences within a few hundred base pairs are usually chosen as primer annealing sites. In these cases, pairs of sequences complementary to the primer may be close to one another and arranged with 3' ends pointing toward one another. Under these circumstances, annealing of the primer to the target genome will result in the production of an amplified fragment af-

ter appropriate thermal cycling. RAPD PCR with DNA of infected plant tissues is performed as follows: In a microcentrifuge tube, genomic DNA (25 to 50 ng), deoxynucleoside triphosphate (dNTP) (200μm), primer (200 pmol), PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 1 mg/mL gelatin, 0.1 percent Tween-20, 0.1 percent NP), and 1 unit *Taq* polymerase are mixed, spun briefly, and overlaid with 50 μL mineral oil. Forty-five PCR cycles are performed at 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min. The reaction mixture (10 μL) is run through a 1 percent agarose gel in tris borate ethylene diamine tetra-acetic acid (TBE) buffer (89mM Tris, 89 mM boric acid (pH 8.3), 2.5 mM EDTA [ethylene diamine tetra-acetic acid]).

Any DNA or RNA sequence that is specific for a particular organism can be used for detection of that organism. Potential targets specific to pathogens include specific sequences from mitochondrial DNA fragment, pathogen-specific plasmid sequences, and short, interspersed repetitive elements present in bacteria. Ribosomal genes and the spacers between them possess conserved as well as variable sequences, and can be amplified and sequenced with universal primers based on their conserved sequences. Genes for 5.8S, 18S, and 26S (28S) fungal nuclear ribosomal RNA genes (rDNA) are used as primers (Henson and French, 1993).

Considerably greater sequence variation is found in the internal transcribed spacer (ITS) regions between the rRNA genes within an rRNA repeat unit (rDNA). Even more sequence differences are in the nontranscribed spacer (NTS) regions between the rDNA repeat units, and still more are in the intergenic spacer regions or noncoding sequences that occur within the rDNA repeat unit of some fungi (Henson and French, 1993). Any organism that has rDNA repeats may be specifically detected by selecting primer sequences based on variable spacer regions.

Immuno-PCR and Immunocapture PCR

Disease diagnosis methods that combine antibody binding and PCR are highly sensitive and can detect microbial antigens in addition to their nucleic acids. In the immuno-PCR method, a DNA fragment is molecularly linked to antigen-antibody complexes. Protein A and streptavidin portions of the linker molecule bind the antibody and DNA, respectively. Antigen present in a sample binds the specific antibody, which, in turn, binds the linker molecule. The latter is bound to a nonspecific, biotin-labeled DNA sequence that is subsequently amplified by PCR. Immuno-PCR is 10^5 times as sensitive as the enzyme-linked immunosorbent assay for the detection of antigen (Sano et al., 1992).

Immunocapture PCR is another sensitive diagnostic assay. Whereas immuno-PCR requires the antigen-specific antibody only, immunocapture PCR requires the antigen-specific antibody and nucleic acid sequence information from the microbe being detected.

Immunocapture-Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR)

In a typical IC-RT-PCR assay, PCR tubes are rinsed in PBST, soaked in ethanol for 15 min, and air dried. The tubes are coated with the specific virus antiserum in a coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; 3 mM NaN_3; pH 9.6) for 3 h at 37°C. The tubes are washed three times with PBST and blotted dry on a tissue. They are placed at –70°C for at least 10 min and the contents thawed at 94°C for 2 min to disassemble the antibody-bound virus and free the viral RNA from the protein coat. A total of 50 μL of RT-PCR mix (4 μL of 5× first-strand reverse transcription buffer; 2 μL of DTT; 2.6 μL of dNTP; 0.1 μL of RNasin RNase inhibitor; 0.25 μL of SuperScript RNase H- Reverse Transcriptase; 3 μL of 10 × PCR buffer; 4.2 μL of 25 mM $MgCl_2$; 1 μL of reverse primer; 0.5 μL of forward primer; 0.2 μL of *Taq* polymerase; and 32.75 μL of nuclease-free water) as added to each tube. The tubes are then treated as follows: 37°C for 1 h, 94°C for 2 min (35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s), and 72°C for 10 min. Following RT-PCR, the products are assessed by electrophoresis in 1.5 percent agarose gels and stained with ethidium bromide (Gillaspie et al., 2000).

Repetitive-Sequence-Based Polymerase Chain Reaction (Rep-PCR)

Rep-PCR is based on PCR-mediated amplification of DNA sequences located between specific interspersed repeated sequences in microbial genomes. These repeated elements are termed BOX, REP, and ERIC elements. Amplification of the DNA sequences between primers based on these repeated elements generates an array of different-sized DNA fragments from the genomes of individual strains. The separation of these fragments on agarose gels yields highly specific DNA fingerprints that can be either visually compared or subjected to computer-assisted pattern analysis (McDonald et al., 2000). The rep-PCR is useful in the identification of bacterial pathogens and disease diagnosis (Louws et al., 1999). Some fungal pathogens can also be identified by this technique (Jedryczka et al., 1999).

DIAGNOSIS OF CROP FUNGAL DISEASES

Several fungal pathogens survive in vegetative propagating materials and may serve as the primary inoculum source for the spread of the disease. Early diagnosis of the inoculum may help in eradicating the disease in the field. As many fungicides are specific only to certain pathogens or groups of pathogens, early diagnosis will be useful in the selection of the most appropriate treatment. Current detection of pathogenic fungi is based on visual inspection of characteristic symptoms. When sporulation is present, general confirmation can be made by light microscopy (Miller, 1996). The pathogen may be isolated by transferring diseased plant tissue to an agar medium in petri dishes. Sometimes a selective medium may have to be used. A bioassay is required to test the pathogenicity of isolates, particularly when closely related saprophytic species are common.

Several immunoassays have been developed to diagnose fungal diseases. Immunoassay kits are commercially available to detect species of *Rhizoctonia, Phytophthora,* and *Pythium* and *Septoria nodorum* (= *Stagonospora nodorum*), *S. tritici,* and *Pseudocercosporella herpotrichoides* (Miller, 1996). *Tilletia controversa* in wheat, *Phytophthora fragariae* in strawberry, and *Rhizoctonia* spp. in poinsettia are detected by DAS-ELISA (Miller, 1996). Indirect ELISA assays are used to detect *Spongospora subterranean* in potato (Harrison et al., 1994), *Colletotrichum acutatum* and *Phytophthora* spp. in strawberry, *Leptosphaeria maculans* in canola, and *Fusarium oxysporum* f. sp. *narcissi* in narcissus (Miller, 1996). *Verticillium dahliae* in potato is detected by immunofluorescence assays (Miller, 1996).

Gaeumannomyces graminis in wheat can be detected by Southern blot with cloned mitochondrial DNA labeled with ^{32}P or digoxigenin as a probe (Bateman et al., 1992). *Phytophthora capsici, P. cinnamomi,* and *P. palmivora* can be detected by PCR and dot-blot with an rDNA ITS (internal transcribed spacer) probe, labeled with ^{32}P (Lee, White, et al., 1993). *Phytophthora parasitica* and *P. citrophthora* can be detected by dot-blot with cloned chromosomal DNA, as a probe labeled with ^{32}P (Goodwin, English, et al., 1990; Goodwin, Kirkpatrick, et al., 1990). Southern blot with cloned chromosomal DNA as a probe, labeled with biotin, is useful in detecting *P. infestans* (Moller et al., 1993).

Fusarium culmorum can be detected by Southern blot with cloned genomic DNA as a probe, labeled with digoxigenin (Koopmann et al., 1994). *Leptosphaeria maculans* in rapeseed can be detected using RAPD-PCR with amplified chromosomal DNA as a probe, labeled with digoxigenin (Schafer and Wostemeyer, 1994). Dot-blot with cloned total DNA as a probe, labeled with ^{32}P is used to detect *Pyrenophora teres* and *Pyrenophora graminea* (Husted, 1994). Slot-blot with cloned genomic DNA as a probe, labeled with ^{32}P will be useful to detect *Pseudocercosporella herpotrichoides* in cereals (Nicholson and Rezanoor, 1994). *Pythium ultimum* can be detected by PCR and dot-blot using rDNA ITS as a probe, labeled with digoxigenin (Levesque et al., 1994).

Botrytis cinerea infection in pear stems can be detected by plating stem halves on a selective medium and by ELISA, which is a more sensitive method (Meyer et al., 2000). The PCR assay is highly sensitive and reproducible as a tool for the detection and identification of fungi when species-specific primers are carefully selected. Several fungal pathogens have been detected using this method, including *Verticillium* spp., *Fusarium* spp., *Rhizoctonia oryzae, Gaeumannomyces graminis, Magnaporthe poae, Leptosphaeria korrae,* and *Phiolophora gregata* (Fouly and Wilkinson, 2000).

Nuclear rDNA of fungi consists of small and large subunits, a 5.8S region, and an internal transcribed spacer region(s). Each subunit and region base sequence is variable among the genera and species of fungi. This variability can be used to detect fungal pathogens. For example, the ITS region of *Gaeumannomyces* is highly variable among its species and less variable among its varieties. However, the small subunit of nuclear rDNA is distinctly variable among the varieties of *G. graminis*. This small subunit of nuclear rDNA (18S rDNA) is used for the detection of *G. graminis* varieties using PCR amplification (Fouly and Wilkinson, 2000). A PCR-based diagnostic assay has been developed to detect *Rhynchosporium secalis* in barley (Lee et al., 2001). RAPD analysis is useful to characterize *P. infestans* isolates from potato and tomato (Mahuku et al., 2000). Species-specific primers were designed based on sequence data of a region consisting of the 5.8S RNA gene and internal transcribed spacers 1 and 2 of *R. secalis* (Lee et al., 2001). An oligonucleotide primer set, RS8 and RS9, was used in detecting *R. secalis*. This primer amplified a 264 bp fragment from the DNA of all *R. secalis* isolates. This is used to detect *R. secalis* in infected barley tissues (Lee et al., 2001). A species-specific PCR has been developed to detect black sigatoka and yellow sigatoka leaf-spot pathogens in banana (Johanson et al., 2000). The Rep-PCR technique is used to detect species of *Fusarium, Stagonospora, Septoria, Tilletia,* and *Leptosphaeria* (Jedryczka et al., 1999; McDonald et al., 2000).

DIAGNOSIS OF CROP BACTERIAL DISEASES

Several diagnostic techniques have been developed to diagnose fire blight of apple and pear. The available methods include isolation of the pathogen on the semiselective media of Miller and Schroth, Crosse and Goodman agar, or crystal violet-cycloheximide-thallium nitrate (CCT) agar

(Merighi et al., 2000). A minimal medium 2-copper sulfate agar was developed to specifically identify *Erwinia amylovora* (Bereswill et al., 1998). Isolation followed by pathogenicity tests will also be useful to detect the pathogen. Miller (1983) described an immunofluorescent microscopic method for the detection of *E. amylovora*. The double-antibody sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA) was developed to detect the pathogen (Gorris et al., 1996). An analysis of fatty acid methyl esters by gas chromatography (GC-FAME) is also used to detect *E. amylovora* (van der Zwet and Wells, 1993). *E. amylovora* can be detected by nested PCR, PCR dot-blot, and reverse-blot hybridization methods (McManus and Jones, 1995). PCR techniques are extensively used to detect the bacterium (Bereswill et al., 1995; Guilford et al. 1996). A PCR-ELISA was developed by Merighi et al. (2000) to detect the pathogen. A PCR-based method detected *E. amylovora* effectively in pear (Sobiczewski et al., 1999). A Rep-PCR technique is used to identify several bacterial pathogens (Louws et al., 1999).

An immunofluorescence test is used to detect *Clavibacter michiganensis* subsp. *michiganensis* in tomato, *C. michiganensis* subsp. *sepedonicus* in potato, and *Erwinia chrysanthemi* in carnation (De Boer et al., 1996). ELISA is useful in detecting *C. michiganensis* subsp. *michiganensis* in tomato, *C. michiganensis* subsp. *sepedonicus* in potato, *E. chrysanthemi* in carnation, *Pantoea stewartii* in carnation, *Xanthomonas campestris* pv. *campestris* in cabbage, and *Pseudomonas savastanoi* pv. *phaseolicola* in bean (De Boer et al., 1996). *X. translucens* pv. *undulosa* in wheat and *X. vesicatoria* in pepper and tomato are detected by dot immunoassay (De Boer et al., 1996).

Nucleic acid probes have been employed to detect some bacterial pathogens. A dot-blot assay has been employed to detect *Erwinia carotovora, Xanthomonas axonopodis* pv. *citri, X. axonopodis* pv. *phaseoli, Pseudomonas syringae* pv. *tomato,* and *Clavibacter michiganensis* subsp. *michiganensis.* Colony blot assay is used to detect *Agrobacterium tumefaciens, E. amylovora, Pseudomonas savastanoi* pv. *phaseolicola,* and *P. syringae* pv. *morsprunorum* (De Boer et al., 1996). PCR is used to detect *A. tumefaciens* (Dong et al., 1992), *Clavibacter michiganensis* subsp. *sepedonicus* (Schneider et al., 1993), *Ralstonia solanacearum* (Seal et al., 1992), and *X. axonopodis* pv. *citri* (Hartung et al., 1993). PCR and RFLP-based techniques are useful in detection of *A. vitis* in grape (Burr and Otten, 1999).

DIAGNOSIS OF CROP VIRAL DISEASES

A number of different serological techniques have been developed for detecting *Citrus tristeza virus* (CTV). These include ELISA (Rocha-Pena and Lee, 1991), sodium dodecyl sulfate (SDS)-immunodiffusion (Gransey et al., 1978), immunoelectron microscopy, radioimmunosorbent assay (Rocha-Pena and Lee, 1991), immunogold assay, Western blot assay (Rocha-Pena and Lee, 1991), dot immunobinding assay (Rocha-Pena et al., 1991), direct tissue blot immunoassay (Lin et al., 2000), and in situ immunofluorescence (ISIF) (Brlansky et al., 1988). Specific monoclonal antibodies produced by hybridoma technology are used for ELISA. In spite of the high serological variability of CTV, a mixture of two monoclonal antibodies (3DFI and 3CA5) is able to detect all CTV isolates tested. These antibodies were patented in 1984 and are considered an international reference for CTV diagnosis. With these commercially available monoclonal antibodies, approximately 2 million samples have been analyzed since 1990 (Terrada et al., 2000). Lin et al. (2000) developed an in situ immunobioassay that does not use fluorescent dyes. This assay is a simple and specific procedure that detects CTV in infected citrus plants in about 2 h.

Terrada et al. (2000) obtained single-chain variable fragment antibodies that bind specifically to CTV from the hybridoma cell lines 3DF1 and 3CA5. These scFv were genetically fused with dimerization domains as well as with alkaline phosphatase, and diagnostic reagents were produced by expressing these fusion proteins in *E. coli* cultures. The engineered antibodies were successfully used for CTV diagnosis in citrus plants by tissue print ELISA and DAS-ELISA. The fully recombinant ELISAs were as specific and as sensitive as conventional ELISAs performed with the parental monoclonal antibodies (Terrada et al., 2000).

ELISA and Western blots using monoclonal antibodies are recommended to identify grapevine leafroll-associated viruses. The monoclonal antibodies of these viruses are commercially available (Monis, 2000). High-quality polyclonal antisera have been raised against several members of the genus *Tospovirus* including *Tomato spotted wilt virus, Groundnut ringspot virus, Tomato chlorotic spot virus,* and *Watermelon silverleaf mottle virus,* and *Impatiens necrotic spot virus*. These antisera are now widely applied for *Tospovirus* detection with the aid of a DAS-ELISA (Griep et al., 2000). Polyclonal antisera, although widely used in routine diagnosis, are available in limited amounts, and their specificity varies from batch to batch. The antisera are being replaced by monoclonal antibodies, which can be produced indefinitely. Several monoclonal antibodies against tospoviruses have been developed. Griep et al. (2000) used recombinant antibody technology in combination with phage display technology to produce *Tomato spotted wilt virus*-specific antibodies. A panel of recombinant single-chain variable antibodies against the N protein and G1 and G2 glycoproteins of TSWV was retrieved from a human combinatorial scFv antibody library using the phage display technique. Antibodies were obtained after subcloning the encoding

DNA sequences in the expression vector pSKAP/S, which allowed the scFvs to be expressed as alkaline phosphatase fusion proteins. An antibody, N56-AP/S, at a concentration of 0.1 μg/ml, can detect as little as 1 ng of N protein of TSWV in a DAS-ELISA. The CL (mouse light-chain) ZIP (leucine zipper) fusion protein of scFv N56 was an effective coating and detecting reagent in a DAS-ELISA or detection of TSWV (Griep et al., 2000).

ELISA is commonly used to detect *Peanut stripe virus* and *Peanut mottle virus* in peanut seed and vegetative tissues. Gillaspie et al. (2000) developed an immunocapture reverse-transcription polymerase chain reaction (IC-RT-PCR) for detection of peanut virus diseases. Peanut tissue slices were extracted in a buffer and centrifuged, and a portion of the supernatant was incubated in a tube that had been coated with an antiserum to either PStV or PeMV. Following immunocapture of the virus, the tube was washed and the RT-PCR mix (with primers designed from conserved sequences within the capsid region of each virus) was placed in the same tubes. The IC-RT-PCR method was more sensitive than ELISA in the detection of peanut viruses (Gillaspie et al., 2000). *Prunus necrotic ringspot virus, Prune dwarf virus,* and *Apple mosaic virus* affect stone-fruit (plum, almond, apricot, cherry, and peach) trees. Nonisotopic molecular hybridization and multiplex reverse-transcription polymerase chain reaction methodologies have been developed to detect all these viruses simultaneously (Saade et al., 2000). For multiplex RT-PCR, a degenerate antisense primer was designed and used in conjunction with three virus-specific sense primers. The amplification efficiencies for the detection of the three viruses in the multiplex RT-PCR reaction were identical to those obtained in the single RT-PCR reactions for individual viruses (Saade et al., 2000). RT-PCR is the most sensitive test for the detection of *Banana bract mosaic virus* (BBrMV) in banana plants followed by the $F(ab')_2$ indirect DAS-ELISA and IC-PCR (Rodoni et al., 1999).

The coat protein gene of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) was amplified with primers based on a completely sequenced GRSPaV isolate. The protein expressed in *Escherichia coli* was used to raise an antiserum in rabbit. This antiserum was used to detect the virus in infected grapevine extracts by dot immunobinding (by spotting on polyvinyl difluoride membranes) or by Western blot. ELISA was ineffective in detecting the virus in grapevine (Minafra et al., 2000). Hailstones et al. (2000) developed a specific seminested RT–PCR assay that detects *Citrus tatter leaf virus* in citrus trees. The sensitivity of the assay is at least 500 times greater than that of ELISA-based methods and allows detection directly from field trees.

DIAGNOSIS OF VIROID DISEASES

Bidirectional electrophoresis is used to test chrysanthemums for the chrysanthemum stunt viroid. Molecular hybridization methods are also used to detect this viroid (Dinesen and van Zaayen, 1996). Primer pairs and nucleic acid preparations were used with RT-PCR to detect peach latent mosaic viroid (PLMVd) from stone fruits (Osaki et al., 1999). PCR methods are useful in detecting hop stunt viroid in hops, apple scar skin viroid and pear rusty skin viroid in apple and pear, citrus exocortis viroid in citrus, and grape viroids in grapes (Henson and French, 1993).

DIAGNOSIS OF PHYTOPLASMA DISEASES

Phytoplasmas are detectable microscopically in phloem by means of Dienes' stain (Sinclair et al., 1996). The DAPI (4',6-diamidino-2-phenylindole-2HCL) test is more useful in the diagnosis of phytoplasmas (Seemüller, 1976). DAPI binds to DNA and causes it to fluoresce under UV. When longitudinal sections of twigs, petioles, or small roots of ash yellows-affected *Fraxinus* and *Syringa* trees are treated with DAPI and examined with a fluorescence microscope, phytoplasmal DNA appears as blue-white fluorescent specks or aggregations in sieve tubes, whereas normal sieve tubes remain dark (Sinclair et al., 1996). The DAPI test is considered nonspecific because DNA of any organism fluoresces under the test conditions.

Several DNA-based techniques are available to detect phytoplasmas. PCR is highly useful in detecting several phytoplasmas. PCR primers that are commonly used are based on sequences in the 16S ribosomal RNA gene that are common to all phytoplasmas, but do not occur in plants (Lee, Hammond, et al., 1993). A DNA segment of characteristic size is amplified from any phytoplasma. The organism is then identified by using this initially amplified DNA segment as a template for further PCRs using primers that amplify DNA from only particular phytoplasmas. These primers are based on nucleotide sequences between the positions of the first primer pair on the 16S rDNA (Lee et al., 1994).

In another method, the initial PCR product is subjected to restriction fragment length polymorphism (RFLP) analysis, in which the amplified segment is digested with certain restriction endonucleases and separated into fragments by gel electrophoresis. Phytoplasmas in different groups have different RFLP profiles (Guo et al., 2000). Ash Y phytoplasmas can be distinguished from others by RFLP analysis of 16S rDNA with the restriction enzyme *Alu*I (Lee, Hammond, et al., 1993).

Phytoplasmas can be diagnosed by means of DNA hybridizations using probes that hybridize to group-specific sequences. Immunological tests were developed to identify phytoplasmas. Monoclonal antibodies to peach eastern X-disease agent have been developed and their use in disease detection has been demonstrated (Lin and Chen, 1985). Immunocapture PCR tests have also been developed to diagnose phytoplasma diseases (Sinclair et al., 1996).

PLANT CLINICS AND DIAGNOSTIC SERVICE LABORATORIES

Several plant clinics have been set up in the United States and many other countries, and they play an important role in crop disease diagnosis. Plant clinics exist typically as part of a plant pathology department of a state's land grant university, as a part of a state's department of agriculture, or as a private or commercial service. Clinics operate throughout North America as the primary source of diagnostic information and as the primary focal point for the submission of plant-disease specimens (Barnes, 1994). Extension-university-supported clinics and state department of agriculture clinics represent the backbone of disease diagnostic services. These clinics routinely use ELISA-based diagnostic procedures and PCR technology. Many clinics routinely use highly technical diagnostic procedures. The clinics provide clients with an accurate diagnosis, and the diagnostic report is sent by mail, fax, phone, modem, or computer network.

Several diagnostic service laboratories were established in the United States since the 1970s. They charge a nominal fee for diagnosis. Plant disease clinics have an instructional component, whereas diagnostic service laboratories simply diagnose plant diseases (Barnes, 1994).

DIGITALLY ASSISTED DIAGNOSIS

Diseases are recognized mostly by visual disease symptoms. Photographic images of plant disease symptoms and signs can be useful in diagnosis. Modern telecommunications systems permit individuals to share high-resolution digital images among multiple locations within seconds (Holmes et al., 2000). These digital-imaging and digital-image transfer tools are used for crop disease diagnosis. Digitally assisted diagnosis permits long-distance consultation and accurate diagnosis of plant disease problems. In the United States, the University of Georgia introduced in 1997 the Web-based delivery system for digitally assisted diagnosis, and

they named it Distance Diagnostics through Digital Imaging (DDDI). County extension offices are provided with a computer, digital camera, dissecting and compound microscopes, and have access to the DDDI system. In cases in which the disease shows unique symptoms, adequate background information is available, and a high quality digital image is obtained, accurate diagnosis can be made within minutes with the help of experienced technicians. It is now possible for the grower to send and receive images from anywhere in the world, including fields, using cellular modems (Holmes et al., 2000). Several Web sites, which describe digitally assisted diagnostics programs, are now available: <http://www.dddi.org>, <http://ddis.ifas.ufl.edu/>, <http://cf.uwex.edu/ces/ag/disdiag>, <http://www.ent.iastate.edu/rdi>, <http://www.ca.uky.edu/agcollege/plantpathology/PPAExten/digiimag.htm> (Holmes et al., 2000).

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