2
New Approaches to the Detection of Microbial Plant Pathogens

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Introduction

Both wild and cultivated plant species are subject to diseases to such an extent that about 80 000 diseases of plants have been recorded throughout the world, while the earliest records of plant diseases have been found in fossils about 250 000 000 years old. Plant diseases have affected the well-being of nations, for example the potato late blight (*Phytophthora infestans*) in Ireland (1845–60) (Cox and Large, 1960); Sigatoka leaf spot (*Mycosphaerella musicola*; Meredith, 1970) and Panama disease (*Fusarium oxysporum* form sp. *cubense*; Stover, 1962) in Central America (1900–65) and beet curly top of sugar beet (*Beta vulgaris*) (beet curly top virus) from 1916 to 1932 (Bennett, 1971). It is estimated that the annual world-wide losses due to plant diseases are as high as US$60 billion (Klausner, 1987; Agrios, 1988).

Plant diseases caused by microbial organisms can be classified into six major groups: viruses, viroids, fastidious prokaryotes [mycoplasma-like organisms (MLOs), spiroplasmas and rickettsiae-like organisms (RLOs)], bacteria, fungi and protozoa (Agrios, 1988). Clearly, the control of plant diseases depends on

Abbreviations: BCIP, 5-bromo-4-chloro-indoxyl phosphate; cDNA, complementary DNA; dATP, deoxyadenosine 5′-triphosphate; dNTP, deoxynucleoside 5′-triphosphates of adenine (dATP), cytosine (dCTP), guanine (dGTP) and thymine (dTTP); dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; HRP, horseradish peroxidase; IBA, immuno-blot assay; IgG, immunoglobulin G; ISEM, immunosorbent electron microscopy; MLO, mycoplasma-like organism; mRNA, messenger ribonucleic acid; NBT, nitro blue tetrazolium; PCR, polymerase chain reaction; PLRV, potato leafroll virus; pv, pathovar, i.e. pathogenic variety; RFLP, restriction fragment length polymorphism; RIA, radioimmunoassay; RLO, rickettsiae-like organism; rNTP, ribonucleoside 5′-triphosphates of adenine (ATP), cytosine (CTP), guanine (GTP) and uracil (UTP); SEM, scanning electron microscopy; ssDNA, single-stranded DNA; ss RNA, single-stranded RNA; Td, temperature at which DNA or RNA hybrids are 50% dissociated; TR-FIA, time-resolved fluoroimmunoassay; VLP, virus-like particle.

Biotechnology and Genetic Engineering Reviews – Vol. 7, December 1989
0264–8725/89/07/45–111$20.00 + $0.00

accurate and rapid detection and identification of the pathogens. In this context detection is the process of testing for the presence of pathogens, while identification is defined as specific identification of the causal agent.

Methods of pathogen detection are required for:

1. Determining the presence or absence of specific pathogens in crops, as an aid to correct management decisions (Wiese, 1982; Burr, Katz and Myers, 1987; Stall *et al.*, 1987).
2. Epidemiological studies to estimate incidence and aetiology of disease and related yield loss (Fry, Apple and Bruhn, 1983; Davis, 1987; Fujimoto and Vidaver, 1987; Hayward, 1987; Katz *et al.*, 1987).
3. Testing plant materials in breeding programmes (Davis, 1987).
4. Testing seeds and planting stocks for plant quarantine and certification schemes (Sletten, 1987; van Vuurde, 1987).
5. Determining the effectiveness of control measures, e.g. heat and chemical therapy, breeding for resistance, cultural methods (Grogan, 1981; Chu, Francki and Hatta, 1983; Chee, Pool and Bucher, 1984; Davis, 1987; Hayward, 1987).
6. Identifying and diagnosing new pathogens and developing specific probes to determine their incidence (Grogan, 1981; Chu, Francki and Hatta, 1983; Chu and Helms, 1988).
7. Application in research to quantify a pathogen and its components and to study pathogenesis and gene functions (Daniels *et al.*, 1987; Mills, Niepold and Bertoni, 1987; Salmond, 1987).
8. Studying taxonomic and evolutionary relationships, and classifying pathogens (Hamilton *et al.*, 1981; Davis *et al.*, 1983).

Traditionally, diagnosis of plant diseases has been based on recognizing characteristic symptoms presented by diseased plants and looking for the presence of pathogens on their surface (Kiraly *et al.*, 1970; McIntyr and Sands, 1977). This, together with other observations and evaluation of the environmental conditions, generally allows the causative agent to be classified as a virus-like organism, a bacterium, a fungus or some environmental factor. Successful diagnosis of many fungal and bacterial plant diseases depends on a knowledge of plant pathology and experience in detecting and identifying the pathogen on the surface. In considering pathogen detection, problems can arise in particular disease–host combinations:

1. Many pathogens, particularly bacteria and fungi, spend part of their life cycles as pathogens and the remainder as saprophytes.
2. Pathogens may not be present on the surface; and it is then necessary to look for the pathogen inside the diseased plant, e.g. seed-borne pathogens.
3. Symptoms can be obscure, e.g. latent infections in potato ring rot (De Boer and McNaughton, 1986; De Boer, 1987b).
4. The occurrence and prevalence of plant pathogens usually vary from season to season, depending on the abundance of pathogens, their vectors, environmental conditions and the susceptibility of crops and varieties grown.

5. Symptoms can be misleading. For example, virus-like symptoms may be produced by fastidious prokaryotes (Bove, 1984). *Xanthomonas campestris* pv. *carotae* causes bacterial blight of carrots (*Daucus carota*), producing black lesions similar to those caused by *Alternaria* sp. (Schaad, 1987). Also, symptoms caused by blueberry scorch virus are similar to symptoms caused by bacterial blight of blueberry, botrytis blossom blight or frost damage (Martin and Bristow, 1988). Furthermore, co-infection of plants by more than one kind of pathogen can alter the symptoms drastically (Banttari and Zeyen, 1970; Chen, Kiyakawa and Matsui, 1972).

In all these cases, special methods are required to isolate and/or detect these pathogens amidst the cell components and other saprophytes. This usually involves performing a series of diagnostic tests which vary according to the suspected pathogen. During the past few years, progress in molecular biology, biochemistry, immunology and in other fields has promoted the development of many new methods of pathogen detection and disease diagnosis (Huang and Pagano, 1977; Itakura, Rossi and Wallace, 1984; Donis-Keller *et al.*, 1986; Caskey, 1987; Kerr, 1987; Michelmore and Hulbert, 1987; Mullis and Faloona, 1987; Valentino, Eberwine and Barchas, 1987; Miller and Martin, 1988). All these methods can be classified into specific or non-specific methods.

Due to the nature of the different plant pathogens, diagnostic methods are better suited to some than others and have been used preferentially for their detection (Hamilton *et al.*, 1981; Fahy and Persley, 1983; Agrios, 1988). In this chapter, we will discuss the methods routinely used for the detection of plant pathogens and then describe recent developments in detection technologies that apply to plant pathogens.

Detection methods

In this review, ways of detecting plant pathogens are divided into specific and non-specific methods. Specific methods are those which are used to detect a particular species or group of pathogens after preliminary diagnosis suggests the presence of that pathogen. Specific methods are also used when the assay involves a known pathogen, for instance as in epidemiological surveys. On the other hand, non-specific methods include those used to detect unknown pathogens or when detection of a number of pathogens is desired, e.g. in plant quarantine and certification schemes [International Board for Plant Genetic Resources (IBPGR), 1988].

SPECIFIC METHODS

Serological methods

Antibodies. The earliest serological techniques in plant pathology used polyclonal antisera prepared by centrifugation of clotted blood of immunized animals. For classical enzyme-linked immunosorbent assays (ELISA), this is further refined to a serum fraction that is predominantly IgG, which is obtained

by ammonium sulphate precipitation, followed by passage over an ion-exchange cellulose column (Clark and Adams, 1977).

Although polyclonal antisera are still used regularly, the use of monoclonal antibodies in plant pathology is becoming progressively more routine. The production of monoclonal antibodies has already been well described (Kohler and Milstein, 1975; Galfre and Milstein, 1981; Halk and De Boer, 1985). Basically, an animal is immunized with the desired antigen and its spleen removed. The cells of the spleen are then fused with myeloma cells to form hybridomas which secrete antibodies. The spleen is composed largely of B-lymphocytes which are antibody-secreting cells. Each B-lymphocyte produces only one antibody specific to one epitope of the antigen. Thus, each individual hybridoma colony (originally from the fusion of one spleen cell and one myeloma cell) secretes homogeneous antibody. The appropriate clonal line is selected by measuring the strength of interaction of its antibody with the original antigen. These hybridoma cells produce antibodies continuously and can be propagated in culture almost indefinitely, or held in liquid nitrogen for long term storage.

As will be discussed below, many of the recent improvements in serological tests for plant pathogens are much more effective when monoclonal rather than polyclonal antibodies are used.

Targets for detection. It is almost routine procedure in plant virology to produce an antiserum to any newly identified and purified virus. This philosophy, however, is not widely held in bacteriology or mycology. As a result, there are American Type Culture Collection stocks of polyclonal (and many monoclonal) antisera available for most plant viruses that have been purified but relatively few antisera available for phytopathogenic bacteria or fungi. World-wide this has led to quarantine seed-testing, seed certification and virus indexing programmes based on the routine use of immunoassays for viruses (Hamilton, 1983; van Vuurde and Maat 1983; Mink and Aichlee, 1984; Torrance and Dolby, 1984; Raju and Olson, 1985; Lawson, 1986; Anonymous, 1987) whereas serological testing for bacterial or fungal pathogens in such schemes is not common (Anonymous, 1987). However, a number of research groups are beginning to produce antisera against important bacterial and fungal plant pathogens that are difficult to diagnose visually or are likely to be present in or on seeds or plant parts which require testing in certification schemes.

Monoclonal antibodies have been produced for many of the more important genera of plant pathogenic bacteria and some have been produced against fungi, MLOs and spiroplasmas (Lin and Chen, 1985a, 1985b, 1987). A major difficulty for producing specific antibodies against either bacteria or fungi has been the source of antigen. Whole cells, cell walls, soluble antigens, extracellular fractions and cellular fractions have all been used (Alvarez, Benedict and Mizumoto, 1985; De Boer and McNaughton, 1986, 1987; De Boer, 1987a; Hung, Wells and Chen, 1987), but there is no general consensus regarding the type of immunogen likely to result in the most specific polyclonal antiserum. Alvarez, Benedict and Mizumoto (1985) used whole cells of

Xanthomonas campestris pv. *campestris* and obtained monoclonal antibody lines that were specific at the genus, pathovar and strain levels. Similarly, Hardham, Suzaki and Perkin (1986) used zoospores and cysts of *Phytophthora cinnamomi* as immunogens and obtained monoclonal antibody lines that were isolate, species or genus specific. Interestingly, this work showed no obvious relationship between the epitopes recognized by the antibody and the specificity of the antibody (*Figure 1*).

It should be pointed out that although indexing or testing for viruses by immunoassay is routine and that this may soon be the case for seed-borne pathogenic bacteria and fungi, there are some problems to be resolved. Virus indexing cannot depend on serological testing alone. In virus-free schemes of potatoes (*Solanum tuberosum*), for example, all nuclear stock are tested by biological methods every few years. This checks against contamination by new viruses and against new serotypes of viruses that are not detected by the established immunoassays.

A possible way of indexing for most viruses is through serological detection of double-stranded RNA (dsRNA). Healthy plants generally do not contain any high molecular weight dsRNA, whereas all but a few plant viruses have their genetic material packaged as either dsRNA or single-stranded RNA (ssRNA). Even in the case of single-stranded RNA viruses, they must pass through replication intermediates, so their RNA can be detected in double-stranded form. Thus, a serological assay such as that described for the replicative form of cucumber mosaic virus (Gabriel, 1986) may ultimately become a powerful tool in plant quarantine. There are also monoclonal antibodies specific for dsRNA (Ouellette and Benhamou, 1987; Benhamau *et al.*, 1987).

A different problem arises with the serological detection of some fungi. Antibodies generally are made to only one or a few of the forms in the life cycle of a fungus, so the pathogen may pass undetected by being at a different stage in its life cycle at the time of assay.

A further possible application of antibody-based specific assays for bacteria or fungi is that of monitoring introduced biological control agents. A similar approach is being explored currently for the monitoring of *Metarhizium anisopliae* attack of *Adorphorus couloni* (Guy and Rath, 1989).

Precipitation methods. As mentioned above, serology-based diagnosis has been applied more widely for the detection of viruses than for fungal or bacterial plant pathogens. The first serological tests were based on observing the precipitate formed when antibodies complexed with viral antigens. These assays could be divided into three types: dilution endpoint techniques, agar diffusion techniques and electrophoresis techniques. The dilution endpoint techniques include the tube precipitin test (Hitchborn and Hills, 1968), the microprecipitin test (van Slogteren, 1955), the ring interface test (Whitcomb and Black, 1961), the flocculation or adsorption precipitin test (Bozicevich, Scott and Vincent, 1963; Cunningham, Tinsley and Walker, 1966; de Sequeira and Lister, 1969), latex agglutination, the quantitative precipitin test (Matthews, 1967) and the complement fixation test (Wright, 1963; Tremaine and Wright, 1967). Agar diffusion techniques include the single- (Shepard,

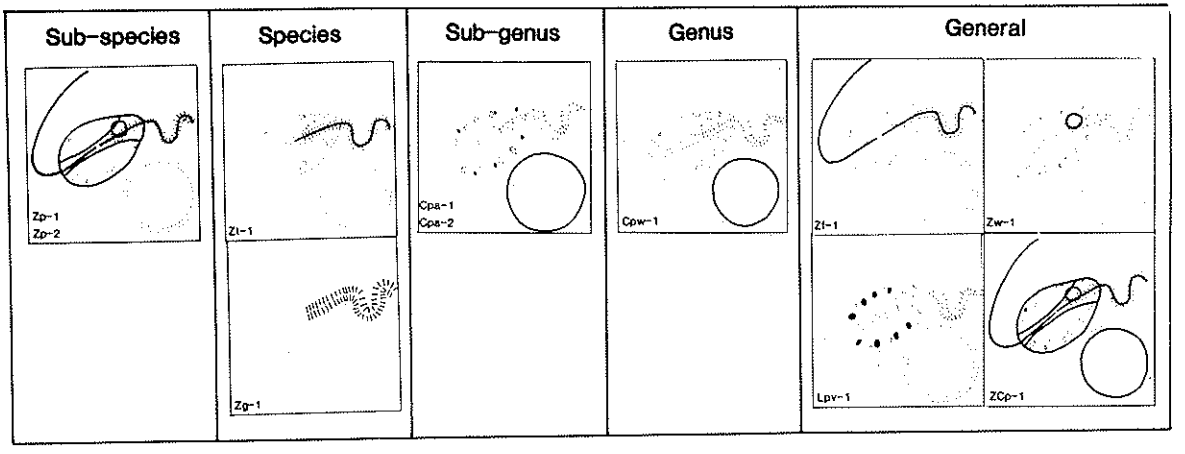

Figure 1. Specificities of monoclonal antibodies produced against *Phytophthora cinnamomi*. The diagram summarizes the binding patterns of monoclonal antibodies on zoospores and cysts of *P. cinnamomi*. Unlabelled surface or vesicular components are shown by dotted lines, and regions recognized by antibodies are indicated by the heavy lines. The code names in the bottom left corner of the squares refer to the monoclonal cell lines. This diagram was kindly provided by Dr A. Hardham and is based on the results of Hardham, Suzaki and Perkins (1986) and Hardham (unpublished data).

1970) and the double-diffusion test (Atabekov *et al.*, 1968). Electrophoretic techniques include immuno-electrophoresis (Atabekov *et al.*, 1968) and immuno-osmophoresis (John, 1965). Although all these methods identify the virus in question and some of them give quantitative results, they are techniques that use substantial amounts of antiserum and are not suited to rapid screening of large numbers of samples.

Immunomicroscopy. Immunosorbent electron microscopy (ISEM) was also one of the methods first used in virology. This technique, first described by Derrick (1972, 1973) involves the coating of electron microscope grids with specific antibodies and incubating these grids on extracts from the test plants. The antibodies specifically trap virus particles onto the grid. After negative staining, the particles are observed readily using an electron microscope. This technique is much more sensitive than any of the precipitation methods. Indeed, it is sufficiently sensitive to be effective on low-titre viruses such as the luteoviruses (Roberts, Tamada and Harrison, 1980; Waterhouse and Murant, 1981). It also uses small amounts of antiserum. However, large numbers of samples cannot be handled rapidly with ISEM.

The direct immunofluorescence assay has been used for the detection of fungi and plant pathogenic bacteria on plants or in soil samples for nearly 20 years (Choo and Holland, 1970; Malajczuk, McComb and Parker, 1975). This assay uses pathogen-specific antibodies conjugated with fluorescent dye molecules. Extracts from plant or soil samples are attached to microscope slides and then incubated with the fluorescent-tagged antibodies. The slides are then viewed using a fluorescence microscope (for the presence of labelled pathogen). The attributes of this technique are very similar to those of ISEM. Unfortunately, it also shares the limitation of being labour intensive and unsuitable for handling large numbers of samples.

Enzyme-linked assays. A revolution in serological detection of plant viruses occurred when enzyme-linked immunosorbent assay (ELISA) was introduced into plant pathology (Engvall and Perlmann, 1972; Clark and Adams, 1977). ELISA has the advantages of sensitivity, economical use of antiserum, the production of quantifiable data and the capacity to handle large numbers of samples quickly. ELISA has many variations and has been extensively reviewed (Clark and Bar-Joseph, 1984). The most common form is the 'double-antibody sandwich' method (*Figure 2A*). In this assay, virus particles in a plant sap extract are trapped by antibodies (coating antibodies) lining wells in a plastic plate. The bound particles are detected subsequently by antibodies (probe antibodies) to which enzyme molecules (usually alkaline phosphatase or horseradish peroxidase) have been cross-linked. The enzyme molecules, in turn, catalyse the conversion of a colourless substrate into a coloured product.

A common variation of ELISA is one in which the probe antibody is not linked to an enzyme. Instead, a further step is introduced which involves an enzyme-tagged moiety that recognizes the probe antibody. In one form, this moiety can be an antibody that recognizes the immunoglobulin species of the probe antibody. For example, if the probe antibodies were raised in a rabbit

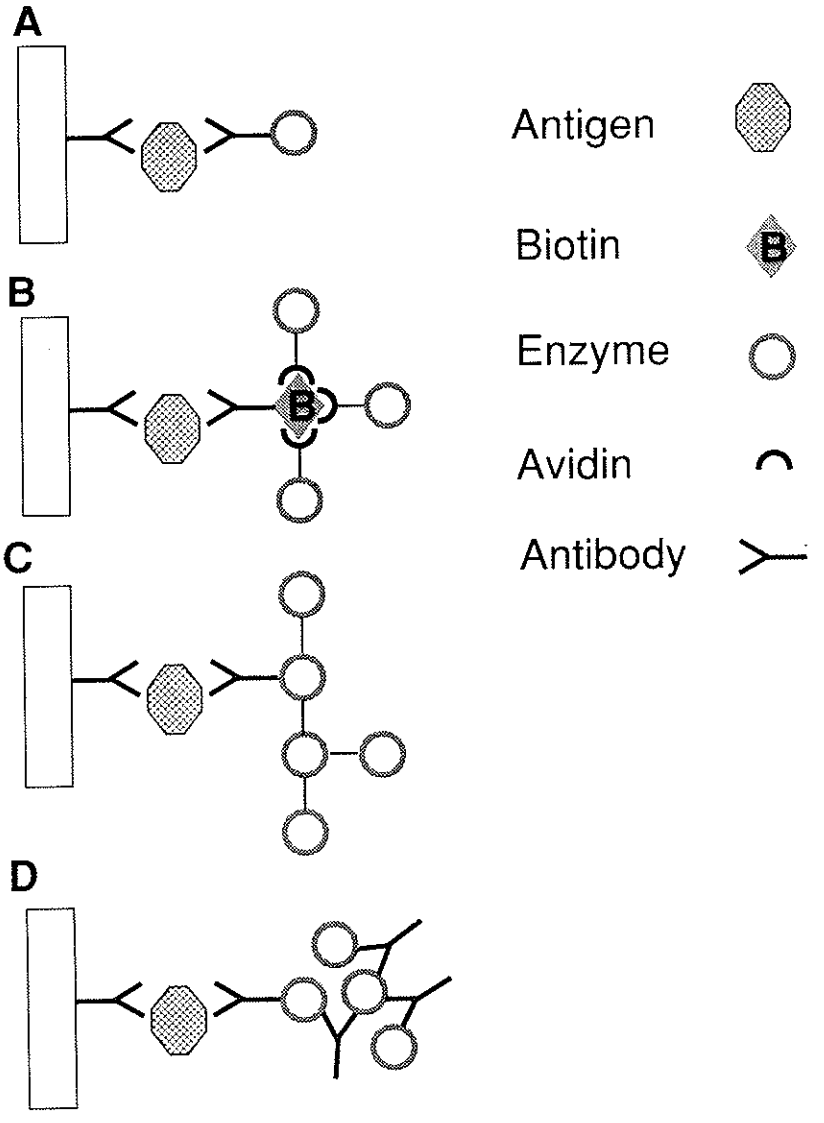

Figure 2. Signal enhancement techniques for immunoassays. The signal from a double-antibody sandwich immunoassay (A) can be enhanced by increasing the amount of enzyme bound per antigen molecule. More enzyme label can be trapped by the use of avidin-biotin complexes (B), oligomeric forms of the enzyme (C), and enzyme–anti-enzyme complexes (D).

and were mainly comprised of IgG molecules, then antibodies raised in sheep against rabbit IgG molecules can be used for this added step. Alternatively, enzyme-labelled staphylococcal protein-A can be used, since it binds specifically to the Fc portion of IgG molecules. This indirect system has three advantages compared with the double-antibody sandwich method. First, all sera produced in one animal species can be used in conjunction with a single second antibody–enzyme conjugate reacting with the Fc portion of the IgG

from the first species. Secondly, the specific antibody is not impeded in its antigen binding by having enzyme molecules conjugated to it; this generally gives the assay the capacity to react with related but heterologous antigens. Thirdly, the second antibody, or protein-A, if conjugated with more than one enzyme molecule, results in an accelerated substrate conversion rate for the assay since there is an amplification of bound signals (*Figure 2*).

With the indirect system (Clark and Bar-Joseph, 1984), the antigen must be immobilized in the wells without using the same antibody as that used for the probe. Otherwise, the enzyme-labelled immunoglobulin-specific antibodies will bind to the coating antibodies and give strong positive readings even for healthy samples. The simplest method of overcoming this problem is to use buffer conditions that promote binding of the antigen directly to the plastic, thus obviating the need for trapping antibodies. An alternative method is to trap the antigen with antibodies produced in a different animal species. Of course, this necessitates having two antisera, from different animals, produced against the same antigen. With the protein-A system (Barbara and Clark, 1982), a more elegant solution is used. Protein-A binds only to the Fc portion of an IgG molecule, whereas the $F(ab')_2$ portion contains the binding domains. Therefore, coating the wells with the $F(ab')_2$ fragments (which are simply obtained by digestion with pepsin) gives antigen-trapping molecules which are not recognized by the enzyme-labelled protein-A.

Immuno-blot technique. The immuno-blot assay (IBA) (Banttari and Goodwin, 1985) is very similar in concept to ELISA. It is based on the same principles and uses the same immunoreagents. Essentially, the plant extract is spotted onto either a nitrocellulose or cellulose membrane on to which the proteins in the extract are firmly bound. Then, under conditions that do not permit non-specific adsorption, the membrane is incubated with an enzyme–antibody conjugate, followed by a washing step, and then incubation with a substrate solution. The substrate is enzymatically converted into an insoluble coloured product that precipitates onto the membrane. When comparisons have been made between the IBA and ELISA using the same immunoreagents, the former is often more sensitive (Banttari and Goodwin, 1985; Parent *et al.*, 1985).

Improvements in serological techniques. ELISA and IBA are more sensitive, economical and convenient than any other previously used serological detection method. That these two techniques have been adopted by many practising virologists, mycologists and bacteriologists is strong testimony of their usefulness.

During the past five years a number of modifications have been made to these two techniques in order to improve them further. The attributes in which improvement has been sought are specificity, sensitivity, speed and convenience. Although these parameters are often interrelated (e.g. higher specificity or shorter incubation periods are often achieved at the cost of sensitivity), each will be considered separately.

Specificity In virology, bacteriology and mycology there is a need for some serological assays that are extremely specific and others that are broad spectrum. In quarantine, for example, the issue is not which strain of a virus, pathogenic bacterium or fungus is present, the issue is simply whether the material contains particular pathogens. Thus, a single assay that can detect all members of a virus group or all species of a fungal or bacterial genus represents a considerable saving in time and resources. In other situations, highly specific detection is required. For example, different *Rhizoctonia solani* isolates are pathogenic on different hosts, so an assay which discriminates between cereal pathogenic and cereal non-pathogenic isolates is required when testing wheat.

The antibody population in the assay almost exclusively determines the specificity of the assay. In polyclonal antiserum-based assays the specificity is governed by the antigen used for the immunization. Thus, if a broad-spectrum assay is desired, a cocktail of antigens is used for immunization. If a very specific assay is required, then homogeneous unique antigen is used. Alternatively, for a very specific serum, either the unwanted cross-reacting antibodies may be removed by adsorption with the heterologous antigen or the specific antibodies can be purified using affinity columns. However, it is often very difficult to remove the cross-reacting antibodies completely by adsorption, or to obtain antigen of sufficient uniqueness to affinity purify the desired antibodies. Monoclonal antibodies, on the other hand, are much more suited to this task. Pure antigen is not required for the immunization but, rather, hybridomas secreting antibodies of the desired specificity are selected during the screening process. There are many instances of monoclonal antibodies being used to discriminate between strains of virus, bacteria or fungi. For example, there are monoclonal antibodies that will differentiate between the veinal necrosis and wild-type strains of potato virus Y (Rose, McCarra and Mitchell, 1987), between pathovars of *Xanthomonas campestris* (Alvarez, Benedict and Mizumoto, 1985) and between *Sirococcus strobilinus* strains that colonize western hemlock (*Tsuga heterophylla*) and those that colonize either pine (*Pinus* sp.) or spruce (*Picea* sp.) (Mitchell, 1986).

An elegant approach towards production of a broad-spectrum probe for a plant virus group has been demonstrated by Shukla and his colleagues (Shukla *et al.*, 1989a). They have shown that if amino- and carboxy-terminal peptide fragments (which are on the particle surface) of potyviruses are removed by digestion with trypsin, then antiserum prepared against the remaining core particles detected most of the different isolates of potyviruses tested. This contrasts with sera produced against whole potyvirus particles which do not detect such wide serological relationships when used in ELISA. Furthermore, Shukla *et al.* (1988, 1989b) showed that the amino-terminal fragment contained linear epitopes that were unique to each potyvirus and these fragments could be used to affinity purify virus-specific antibodies from cross-reacting polyclonal sera.

A similar broad-spectrum phenomenon has been found for luteoviruses. Neither a polyclonal nor monoclonal antibody-based assay recognized all members of the group when the antigens were intact particles (Waterhouse, Gildow and Johnstone, 1988), but dissociated particles of all members are

detected by a single polyclonal serum or a specially selected monoclonal antibody (R. R. Martin and C. J. D'Arcy, unpublished data).

Sensitivity The sensitivity of an assay based on polyclonal serum is often governed by the proportion of specific to non-specific antibodies contained in the serum. A simple way of increasing sensitivity is to reduce the non-specific reaction by cross-adsorption with non-specific extracts. Monoclonal antibody-based systems, because of their homogeneity, give little or no non-specific background and, as discussed below, are more amenable to amplification techniques. Three other factors governing sensitivity are: the enzyme label used to tag the probe antibody; the enzyme substrate used; and the method of measuring substrate conversion. In the detection of plant pathogens three reporter enzymes are used most commonly: horseradish peroxidase (HRP) (EC 1.11.1.7) , alkaline phosphatase (EC 3.1.3.1) and urease (EC 3.5.1.5). Oddly, β-galactosidase (EC 3.2.1.23), which is used commonly in medical immunoassays (Tijssen, 1985), is used rarely in plant pathology. Theoretically, of these four enzymes HRP should give the most sensitive detection system whether using a colorimetric or fluorescent assay (*Table 1*). Despite its low ranking in terms of theoretical detectability, high cost and low efficiency of conjugation (*Table 1*), the lack of endogenous activity (and hence low background) in plants gives alkaline phosphatase the greatest sensitivity in practice.

Table 1. Comparison of reporter enzymes used in the detection of plant pathogens*

Enzyme	Conjugation efficiency	Relative cost	Detectability	
			Colour	Fluorescence
Horseradish peroxidase	1·0	1·0	1·0	1·0
Alkaline phosphatase	0·1	650	400	2·0
β-Galactosidase	1·3	200	40	0·04
Urease	0·1	146	–	–

* Data from Ishikawa *et al.*, 1983; Tijssen, 1985.

Theoretically, the use of fluorescent substrates, such as 4-methyl umbelliferyl phosphate (MUP), in conventional ELISA should increase the sensitivity of the assay 100-fold over that using chromogenic substrates such as *p*-nitrophenol phosphate (NPP) (Tijssen, 1985). Fluorogenic substrates may allow the detection of femtomolar (10^{-15}M) or even attomolar (10^{-18}M) levels of HRP (Puget, Michelson and Avrameas, 1977). Bioluminescence-based systems can also detect femtogram amounts of antigen (Hauber and Geiger, 1987). However, this sensitivity is rarely achieved because other factors, such as high background readings and the sensitivity of the apparatus measuring the emissions, prove to be limiting. Torrance and Jones (1982) compared the sensitivity of detection of prune dwarf, apple mosaic and potato leafroll viruses (PLRV) by ELISA using alkaline-phosphatase-labelled antibodies and both MUP and NPP as substrates. They found that using the fluorescent substrate increased the sensitivity between two- and sixteenfold. Furthermore, the

MUP-based ELISA consistently detected a greater proportion of individual viruliferous aphids (for PLRV and barley yellow dwarf virus) than NPP-based ELISA, irrespective of substrate incubation time.

Antibodies are not always tagged with enzymes for detection. Three very effective labels are: colloidal gold (Hsu, 1984) and radioactive or fluorescent molecules. The latter two tags have been used extensively in medical immunoassays but not so far in plant pathology. Radioimmunoassay (RIA) uses the same principle as IBA and ELISA except that the presence of labelled antibody is detected either in a scintillation counter or by autoradiography. Although RIA still is used widely in the medical field, there has been a general trend away from using radioisotopes for reasons of safety and convenience. Therefore it seems unlikely that RIA will be widely accepted into plant pathology.

Tagging antibodies with fluorescent molecules has also been shown to be a sensitive method of detection (Weeks *et al.*, 1986). A most useful assay, using fluorescence-labelled antibodies, which may well be adopted by plant pathologists, is the time-resolved fluoroimmunoassay (TR-FIA) (Halonen *et al.*, 1986). A major problem with using a conventional fluorescent tag as a means of detection is that many biological samples exhibit autofluorescence that masks the positive signal. However with TR-FIA, this problem is overcome by using lanthanide molecules, such as europium, as tags. When excited, these molecules have much longer decay times (100–1000 μs) than background fluorescence (<1 μs) and have the additional advantage of exhibiting a larger shift between excitation and emission wavelengths. Thus, measuring the emission after a delay period and at a higher wavelength greatly reduces the background due to autofluorescence. This technique has been used for the detection of potato viruses X, M, S, Y and PLRV (Siitari and Kurppa, 1987; Sinjarv *et al.*, 1988), and was found to be 5–100 times more sensitive than conventional double-antibody sandwich ELISA.

Amplification systems Recently, there has been marked interest in applying amplification systems to ELISA. There are two different types of signal enhancement described by this term: the systems that amplify by obtaining more enzyme molecules per antibody by using a ligand system, and those that use the action of the enzyme linked to the antibody to activate either a substrate cycle or an enzyme cascade. The former type of amplification has been achieved by coupling oligomeric forms of the enzyme to the antibody directly (*Figure 2C*) (Leary, Brigati and Ward, 1983), or through an indirect labelling method such as avidin–biotin (*Figure 2B*) (Hsu, Raine and Fanger, 1981; Kendall, Ionescu-Mayiu and Dreesman, 1983) or enzyme–anti-enzyme (*Figure 2D*) (Koertge, Butler and Dierks, 1985) complexes. However, only modest signal enhancement has been achieved by this type of method, since it also increases the background level.

The substrate cycling and enzyme cascade systems have the potential of much greater amplification than that of the ligand approach. The principles of these systems have been well described by Self (1985), Johannson *et al* (1986) and Bates (1987). In the substrate cycling system, an enzyme linked to the

probe antibody converts the primary substrate into an active form that can be cycled. A by-product of this cycle is the conversion of a secondary substrate into an easily detected product, such as a coloured molecule. The most commonly used substrate cycling system is that using diaphorase (dihydrolipoamide dehydrogenase, EC 1.8.1.4) and alkaline phosphatase (*Figure 3A*). In this system, the alkaline phosphatase conjugated onto the antibody converts the primary substrate NADP into NAD which then, in the presence of diaphorase, ethanol and alcohol dehydrogenase (EC 1.1.1.1), acts as a proton

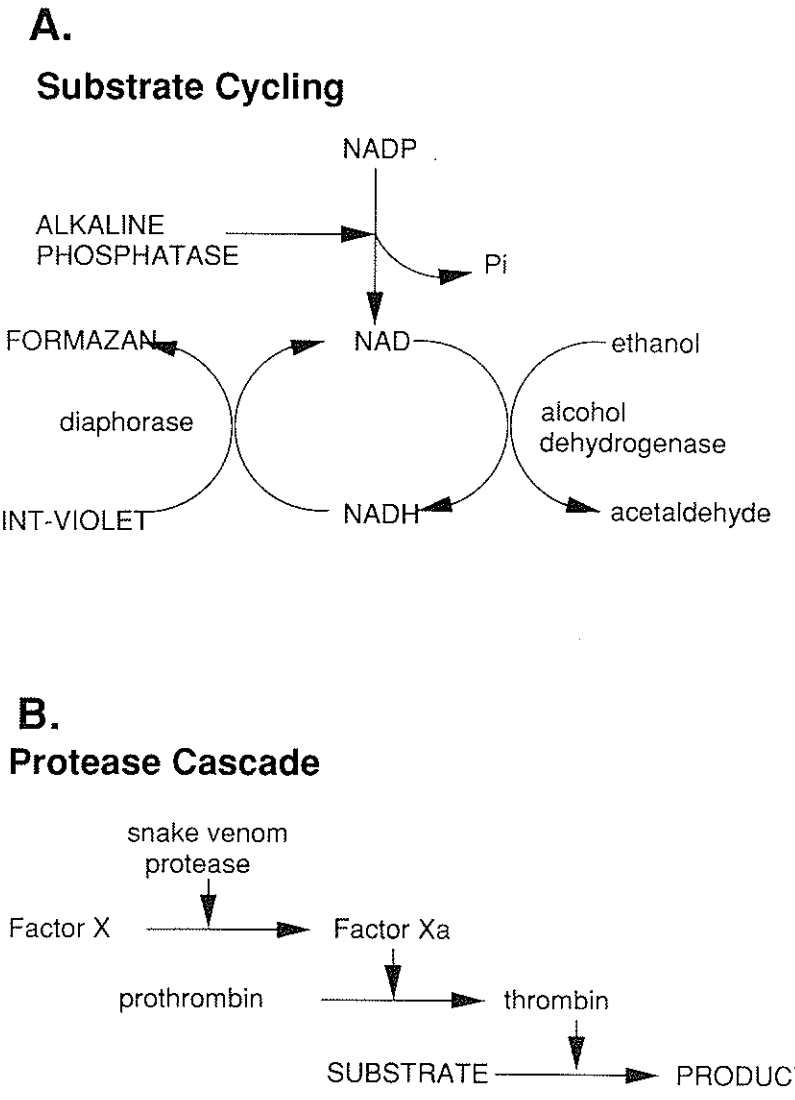

Figure 3. Enzyme amplification systems for immunoassays. The signal from an immunoassay may be enhanced by enzyme amplification which is achieved by catalytically coupling the primary enzyme [in (A) alkaline phosphatase; in (B) Factor Xa] to an enzyme amplifier cycle (A) or an enzyme cascade (B).

carrier between the oxidation of ethanol and the reduction of a tetrazolium dye (INT-violet) to produce the intensely coloured, insoluble formazan. This system requires that the enzymes and substrates are of high purity and that the alcohol dehydrogenase is completely specific for NAD rather than NADP. The assay is usually performed in two steps: the dephosphorylation of NADP followed by the addition of the reagents for the amplifier cycle which converts INT-violet to formazan. Phosphate is included in the amplifier cycle reagents, thus inhibiting further production of NAD by the alkaline phosphatase. This allows a quantification of the results, since the rate of NAD production is governed by the amount of alkaline phosphatase-conjugated antibody and the rate of formazan production is proportional to the NAD produced in this first step.

The diaphorase system has been used for the detection of barley yellow dwarf virus (Torrance, 1987) in individual aphids and was found to give about a 30-fold increase in sensitivity over conventional ELISA when sap extracts were tested. However, this amplification is considerably lower than the theoretical maximum, presumably due to the limitations of the antibodies and reagents used.

A protease cascade system (*Figure 3B*) has been demonstrated in one model system (M.S. Blake *et al.*, 1984) using the enzyme mechanisms involved in blood clotting. This system was used to analyse the avidin–biotin complex by making a bovine Factor X–biotin conjugate. The activity of the conjugate is inhibited by avidin but, when free biotin is added, Factor X can be activated by a snake venom protease to initiate the cascade. The outcome of the cascade is the conversion of a synthetic chromogenic peptide substrate to a coloured product. This has not been used in plant pathology but has the potential for amplification of ELISA or IBA of plant pathogens.

Speed and convenience In many instances the most important aspect of a phytopathological test is not the sensitivity, but the speed with which the assay can give an answer. This has led to the concept of 'plant-side' testing. A commercial company (Agri-Diagnostics, Cinnaminson, USA) has developed a system for plant-side testing of turf diseases. The system allows the identification of *Pythium* sp., *Rhizoctonia* sp. and *Sclerotinia* sp. in under one hour by using adsorbent plastic devices (*Figure 4*) that work on the same principle as IBA and ELISA (Rittenburg *et al.*, 1988).

The format of a 96-well microtitre plate, commonly used in the laboratory, can be replaced with dipstick formats in order to make the handling of samples much easier in the field. Such innovations mirror developments in medical immunoassays designed for use by the general practitioner.

An exciting development for user-friendly diagnostic assays is the visible immunodiagnostic assay. Instead of relying on enzymes or agglutination to show the presence of the antigen, visible immunodiagnostic assay uses latex microspheres conjugated onto antibodies which attach to a dipstick via a sandwich procedure. When attached to the dipstick these microspheres have an optical effect (known as Mie scattering) that causes the stick to change colour. The colour that develops on the stick is a function of the size of the microsphere. Thus it is possible to test simultaneously for more than one

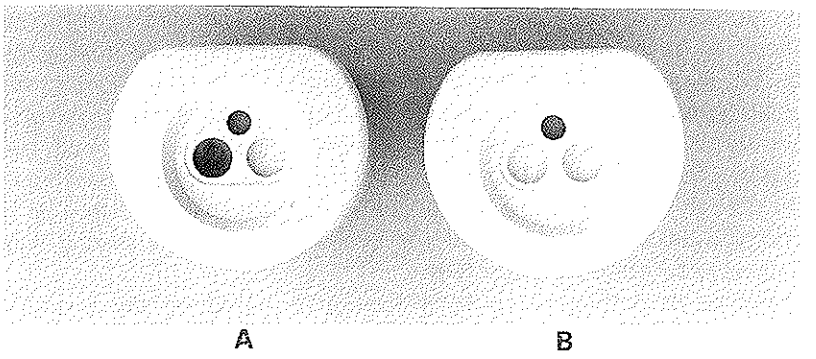

Figure 4. Rapid on-site immunoassay. A small plastic device produced by Agri-Diagnostics for rapid on-site diagnosis of plant pathogens. The devices contain pre-treated positive (top well) and negative (lower right well) controls and a well (lower left) for the test sample. Device A shows a positive test sample and device B shows a negative test sample. This figure was kindly provided by Dr Sally Miller, Agri-Diagnostics, Cinnaminson, USA.

pathogen by using different specific antibodies attached to different size microspheres. The resultant colour indicates which is present. This test can take as little as 30 minutes and is as sensitive as ELISA. Visible immunodiagnostic assay is being developed commercially by the Covalent Technology Corporation in the medical field but the company has plans to use this technology for agricultural diagnostics also (Klausner, 1987).

A well-established technique that can be used as a rapid assay is the latex test. This system is based on the agglutination of antibody-coated latex beads in the presence of antigen. The assay is simple to perform, rapid and sensitive. Potato virus Y has been tested by this method and gave unequivocal answers in two minutes (Kurppa and Vuento, 1987). In this system the antibody–latex conjugate is dried in spots onto cardboard. To perform the test these spots are simply resuspended in the sap extract and observed for granulation.

A further approach towards convenient immunoassays for agricultural pathology is the development of kits for the diagnosis of all important pathogens of a particular crop. Good examples of these are the kits produced by Boehringer Mannheim Biochemica (Mannheim), Agdia Inc. (Mishawaka, USA) and BioReba AG (Basel) for potato (*Solanum tuberosum*) testing. The kits include immunological tests and positive controls for potato viruses A, M, S, X and Y and PLRV.

Nucleic acid hybridization methods

Principles and development of nucleic acid hybridization methods. All living organisms possess nucleic acids. During evolution, mutations result in fairly random changes to the nucleotide sequences and selected changes accumulate giving rise to genetic variation. The unique genetic constitutions of individual organisms are the results of many generations of mutations and recombinations.

It is generally assumed and always confirmed by observation that closely related organisms share a greater nucleotide sequence similarity than those

which are distantly related. The principle of nucleic acid hybridization in diagnosis is based on this assumption and observation. A highly specific nucleotide sequence common to a given strain of bacteria or a given isolate of virus, but absent in other strains or isolates, may be used to test for the presence of that organism. Similarly, a highly conserved sequence present in all strains or species in a genus may be used to probe for the presence of any member of that genus. The selection of a specific sequence as a probe can be based on one of several methods but is best derived from sequence data and screening of related organisms to determine its specificity.

Detection of pathogens by hybridization is based on the formation of double-stranded nucleic acids by specific hybridization between the single-stranded target nucleic acid sequence (denatured DNA or RNA) and a complementary single-stranded nucleic acid probe. Thus, either RNA or DNA sequences may be used as probes. The kinetics of hybridization and the factors affecting the stability of the duplex are reviewed elsewhere (Britten and Davidson, 1985). If one of the strands (probe) in the duplex is labelled with a detectable marker, e.g. ^{32}P, then the formation of the duplex can be assayed after removal of unhybridized sequences.

The hybridization reaction may be performed:

1. In solution ('solution hybridization'; Young and Anderson, 1985) after which the hybrids are analysed by gel electrophoresis or by liquid scintillation counting;
2. *In situ* ('*in situ* hybridisation'; Pardue, 1985) whereby the target remains in the cell and the hybrids are visualized by microscopy and histological methods; or
3. On solid filter supports ('filter hybridization'; Anderson and Young, 1985; Mason and Williams, 1985; Williams and Mason, 1985) in which case the target nucleic acids are immobilized and the labelled nucleic acid probe is allowed to hybridize to them. The bound probe can then be detected by the appropriate detection method.

In diagnosis, the filter and *in situ* hybridization methods are used most commonly. There are many types of probes and methods of labelling them. They include cloned and uncloned nucleic acids, oligonucleotides, *in vitro* RNA transcripts, radioactive and non-radioactive probes. Their choice depends on the hybridization strategy, availability of material and equipment for preparation of probe, the concentration of target sequences to be detected, specificity and sensitivity required, and scale of operation.

Detection of pathogens by hybridization has been compared to various other diagnostic methods, including various serological methods (Sela, Reichman and Weissbach, 1984; Antoniw *et al.*, 1986; Barbara *et al.*, 1987; Calisher *et al.*, 1987; Varveri, Ravelonandro and Dunez, 1987) and found to be more sensitive. The detection limits of viruses by hybridization range between 1 and 100 pg. With subterranean clover stunt virus (SCSV) it was found that ELISA had a detection limit of 400 pg of purified virus while a ^{32}P nick-translated probe detected 1 pg purified DNA (Chu, Waterhouse and Helms, 1989). However, when infected tissues were used, ELISA was found to be more suitable because

the pathogen was detectable at a sap dilution of 1/625 while hybridization had a dilution limit of 1/125. Also, sample preparation time for ELISA is much less than for hybridization. In absolute quantity, hybridization was more sensitive in that the ELISA test required 200 µl of extract while hybrization required only 1 µl.

The potential advantages of hybridization over ELISA include:

1. All genome sequences can be cloned simultaneously and characterized readily by sequencing or gene mapping.
2. Cloned probes can be produced in unlimited supply as can monoclonal antibodies.
3. Ability to detect viroids, which contain no protein.
4. Based on sequence libraries and information on genome organization, probes with varying specificities can be obtained readily.
5. Large probes are less prone to the effects of mutation.
6. Hybridization has greater scope for increased sensitivity by amplification techniques such as enzyme-linked oligonucleotides and polymerase chain reaction as well as the amplification techniques used in ELISA.
7. Probes can be made to detect both chromosomal and extrachromosomal nucleic acids, and coding as well as non-coding regions of genomes.
8. In molecular genetic studies, whole genomes can be analysed selectively using specific probes.

Alternatively, ELISA has several advantages over hybridization:

1. Proteins are more stable than nucleic acids so that samples can be stored for longer periods of time. Thus, preliminary reaction steps can be done beforehand and stored until used.
2. ELISA is generally safer and more reliable for those already skilled in its use.
3. There is greater product diversity available.
4. Less interference by extracts occurs (hybridization may be inhibited by some plant constituents).
5. Personnel in many laboratories are not sufficiently trained or are reluctant to handle hybridization assays due to problems with sample and probe preparation.

The advantages and disadvantages of each assay method should be considered when developing a strategy for pathogen detection.

Hybridization applications include detection of various viruses and viroids (Bar-Joseph and Rosner, 1984; Hull, 1984; Diener and Owens, 1985; Lakshman *et al.*, 1986; Haber, Polston and Bird, 1987; Kingsbury, 1987; Mills, 1987; Varveri, Ravelonandro and Dunez, 1987), fastidious prokaryotes (Borkhsenius, *et al.*, 1987; Kirkpatrick *et al.*, 1987a, 1987b; Nur, Leblanc and Tully, 1987; Razin *et al.*, 1987; Roberts *et al.*, 1987; Santha *et al.*, 1987; Gobel *et al.*, 1987), as well as bacteria (Wirth and Piatt, 1982; Festl, Ludwig and Schleifer, 1986; Palva, 1986; Lazo, Roffey and Gabriel, 1987), fungi (Crowley and Oliver, 1987; Manicom *et al.*, 1987; Rollo *et al.*, 1987) and protozoa (Kukla *et al.*, 1987). It is also used routinely in medical diagnostic programmes (Hejtmancik *et al.*, 1986; Kingsbury, 1987).

Types of probes. Early probes were not cloned. They consisted of isolated nucleic acids labelled with radionucleotides either by nick translation (Rigby *et al.*, 1977), end-labelling, or cDNA synthesized by reverse transcriptase (EC 2.7.7.49) (Maniatis, Frisch and Sambrook, 1982). These types of probes are still used to screen new clone banks and for rapid preparation of unbiased strand-specific probes. However, there is a risk of contamination by host nucleic acids, and the use of uncloned probes often requires that new templates be prepared as needed. Alternatively, with cloned probes an unlimited supply of probe template can be produced.

For routine detection and to detect specific target sequences representing a small proportion of a genome (e.g. fungi or bacteria), cloned nucleic acids are used as probes. They have the advantage of uniformity and continuous supply, and are prepared by isolating the target nucleic acids, and cloning of double-stranded cDNAs into appropriate plasmids or phage vectors (Glover, 1985a, 1985b, 1987). Repetitive sequences which may cause unwanted background can be identified by restriction enzyme mapping and deleted from the probes (Arrand, 1985). Strand-specific probes may be prepared by cloning into M13 phage or other vectors that produce single-stranded molecules, or into transcription vectors. There is still a possibility of cross-hybridization between the vector sequence and the target nucleic acids unless the vector sequence is removed. With cloned probes, short sequences are generally more strain specific while longer sequences are more sensitive (Zwadyk and Cooksey, 1987). Another type of probe is *in vitro* transcribed RNA from a transcription vector system, e.g. based on phage SP6 promotor and polymerase (Butler and Chamberlin, 1982; Green, Maniatis and Melton, 1983). Multiple copies of the RNA probe are produced from a single vector sequence. These probes are free of probe reassociation problems and do not contain vector sequences. The vector, primers and transcription enzymes are available commercially. The size and specificity of the probe can be regulated by subcloning desired sequences. Background cross-hybridizations can be reduced by RNase treatment after hybridization and by taking advantage of the greater stability of RNA–RNA or RNA–DNA hybrids. A possible disadvantage is the greater care required for the handling of labile RNA.

Oligonucleotide probes are synthetic short homologous probes (Gait, 1985) that can be labelled and used to probe for specific nucleic acid sequences of a pathogen. The use of oligonucleotides as specific probes is based on knowledge of the nucleic acid or protein sequences of the target molecule. When inferred from protein sequences, a mixture of oligonucleotides may be required to cover the redundancy in the genetic code. Short oligonucleotides are more specific than longer probes derived from cloned DNA which may contain common sequences between unrelated target molecules. Both RNA and DNA oligonucleotides can be used to hybridize to either RNA or DNA targets (Buvoli *et al.*, 1987). Oligonucleotides may need to be hybridized and washed at various stringencies* to obtain the desired distinction between perfect and imperfect matches in detection and studies of sequence relatedness among

* The specificity of the probe for the target oligonucleotide can be modified by changing reaction conditions (T°C, pH and electrolyte concentration) so as to increase or decrease the stringency of the hybridization tests.

strains (Mason and Williams, 1985). They have been employed as probes to detect sequence variation in phylogeny studies (Hellman and Pettersson, 1987), mutants (Murasugi, Takemori and Hashimoto, 1987), strain differentiation (Wallace, Petz and Yam, 1986; Hellman and Pettersson, 1987) and molecular genetic studies (Horst *et al.*, 1988).

Preparation of probes. Probes are prepared in several different ways. Uncloned nucleic acid probes are derived from specific RNAs or genomic DNA. RNAs or DNAs can be end-labelled with [$\gamma-^{32}$P]dATP or transcribed into cDNA in the presence of dNTPs with one or more [$\alpha-^{32}$P]-labelled dNTPs. Cloned DNA probes are derived either from double-stranded cDNA synthesized from RNAs (Okayama and Berg, 1982; Gubler and Hoffmann, 1983) or from genomic dsDNA (Maniatis, Frisch and Sambrook, 1982). Very little starting material is necessary and the end result is a homogeneous specific probe propagated in a fast-growing bacterial system capable of high DNA yield. Many commercial kits are now available for these steps, from isolation of RNA or genomic DNA through to screening of clones (Mead *et al.*, 1988; Mierendorf, Morris and Schenborn, 1988). The desired clones in genomic libraries can be identified by screening each clone against healthy and infected tissues, screening all the clones against purified nucleic acids of the pathogens or screening a cDNA library in an expression vector with antiserum specific for the pathogen.

The SP6 polymerase system consists of a plasmid vector containing the *Salmonella* phage SP6 RNA polymerase promoter and a multiple cloning site into which the selected fragment of the DNA required as a probe can be subcloned. RNA transcripts are prepared by linearizing the plasmid vector with an appropriate restriction enzyme that cuts beyond the insert. Multiple copies of RNA representing the insert will be transcribed by the addition of SP6 RNA polymerase and rNTPs. Such transcription vectors and kit systems are commercially available [e.g. riboprobe vectors from Promega Biotech (Madison, USA) contain both SP6 and T7 promoters so that RNA can be transcribed from either strand of the insert; Krieg and Melton, 1987; Mierendorf, 1988]. Similarly, methods have been devised to synthesize enzymatically multiple copies of ssDNA probes by using immobilized templates (Hansen *et al.*, 1987).

Oligonucleotide probes may be DNA or RNA based. However, most synthetic oligonucleotides are DNA since they have more applications both as diagnostic reagents as well as in molecular biology research (Gait, 1985) and are more readily synthesized. Preparation of synthetic oligonucleotides is now routine since high-quality reagents are available commercially and most large laboratories have access to a DNA synthesizer. For those laboratories without a synthesizer the techniques of synthesis are well documented for both DNA and RNA oligonucleotides (Atkinson and Smith, 1985; Beckett and Uhlenbech, 1985; Sproat and Gait, 1985; van Boom and Wreesman, 1985) so that manual synthesis is relatively straightforward. Alternatively, there are commercial companies that will prepare oligonucleotides to order.

The length of oligonucleotides should be chosen to suit the hybridization

conditions to be used (see the section on filter hybridization). Mixtures of sequences covering all possible combinations of codon redundancy or strain variations can be made in a mixed synthesis. Computer programs are available for designing statistically optimum synthetic oligonucleotides from protein sequences (Danckaert *et al.*, 1987). However, these depend on a knowledge of codon frequencies that may not always be available, so it is probably safer to make all possible combinations.

Labelling of probes. Nucleic acid probes may be labelled with a range of radioactive or non-radioactive markers (Cunningham and Mundy, 1987; Dattagupta *et al.*, 1987; Hodgson and Fisk, 1987; Li *et al.*, 1987; Seriwatana *et al.*, 1987). Their comparative advantages and disadvantages have been reviewed extensively (Al-Hakim and Hull, 1986; Zwadyk, Cooksey and Thornsberry, 1986; Donovan *et al.*, 1987; Mifflin *et al.*, 1987; Tabares, 1987). Radioactive labels include ^{3}H, ^{35}S, ^{32}P and ^{125}I, while non-radioactive probes include various biotin-labelling systems (Al-Hakim and Hull, 1986; Dahlen, 1987; Gebeyehu *et al.*, 1987; McInnes *et al.*, 1987), fluorescein label (Zuckermann, Corey and Schultz, 1987), enzyme labels (Li *et al.*, 1987; McLaughlin *et al.*, 1987; Seriwatana *et al.*, 1987) or, more recently, labelling with steroid antigens (Boehringer Mannheim, 1988b; Schafer, Zischler and Epplen, 1988).

Radioactive dNTPs are the traditional labels used for hybridization. With hybridization to filters or in solutions, ^{32}P is the isotope of choice since its high energy results in shorter autoradiographic exposures. The isotopes used for *in situ* hybridization are generally ^{125}I or ^{35}S, since their medium energy results in low background and short exposure times compared to ^{32}P and ^{3}H, respectively.

There are several ways to incorporate radioactive label into a probe. They include nick translation, oligo-labelling, end-labelling, cDNA synthesis and transcription from a transcription vector. The method which is chosen depends on the type of template and the type of probe required.

Nick translation (Rigby *et al.*, 1977) is the traditional labelling method for dsDNA (cloned or uncloned) and many commercial kits are available that can produce a specific activity of 5×10^{8} dpm μg^{-1} DNA when using [α-^{32}P]-labelled nucleotides (Arrand, 1985). More recently, commercial kits based on multipriming of denatured ssDNA with oligonucleotide primers (random primers or hexamer primers) and extension using the Klenow fragment of DNA polymerase can produce a specific activity of 5×10^{9} dpm μg^{-1} DNA probe (Feinberg and Vogelstein, 1983, 1984; Hodgson and Fisk, 1987). Another method is to use T4 DNA polymerase to digest one end of each strand of DNA in a dsDNA template from the 3′ to 5′ direction, followed by resynthesis of dsDNA from the 5′ to 3′ direction using radioactive precursors (Morris *et al.*, 1979). This method can only be used on linear dsDNA and may be unsuitable for long DNA molecules since the single-stranded ends may produce secondary structures inhibiting the polymerase activity. Its advantage is that the probe is generally intact and can be cut with restriction enzyme if required. Defined regions of the DNA can be labelled by controlling

the exonuclease reaction. Specific activity can be as high as 10^9 dpm μg^{-1} DNA.

Linear single-stranded nucleic acids (oligonucleotides, ssRNA, ssDNA) and dsRNA can be end-labelled using the T4 polynucleotide kinase (EC 2.7.1.78) which transfers the γ-phosphate of [γ-^{32}P]ATP to a free 5′-OH group in either DNA or RNA (Richardson, 1965; Maxam and Gilbert, 1980). Both a forward and an exchange reaction are possible. Specific activities of 5×10^5 dpm pmole^{-1} termini and 8×10^5 cpm pmole^{-1} termini can be achieved with the exchange and forward reaction, respectively. Thus, end-labelling is not very efficient for large ssDNA or dsDNA molecules which can be labelled by other means (see above). For RNA, the labelling efficiency is improved by base cleavage with NaOH prior to end-labelling. The advantages of end-labelling are that the probe is strand-specific for single-stranded templates and small pieces of nucleic acids (oligonucleotides) can be labelled. Furthermore, DNA fragments from a restriction enzyme digest can be labelled before separation by gel electrophoresis, thus permitting the reliable preparation of several specific probes at once.

Terminal deoxynucleotidyl transferase (EC 2.7.7.31) can be used to attach labelled dNTPs onto 3′ ends of ss- or dsDNA, including recessed 3′ ends if cobalt ions are present in the reaction. The use of [α-^{32}P]cordycepin triphosphate (3′-deoxyadenosine 5′-triphosphate; Hu and Messing, 1982) which lacks a 3′-hydroxyl group prevents further nucleotide addition. 'Filling in' of recessed 3′ ends of dsDNA fragments such as those produced by certain restriction enzymes can also be achieved with *E. coli* DNA polymerase I (Downing *et al.*, 1979) or Klenow enzymes. This reaction can be carried out directly after digesting the DNA with restriction enzymes, and the specific activity is often higher than that given by those methods which incorporate a single label per end.

cDNA synthesis is the most efficient method for preparing high specific activity probes from single- or double-stranded nucleic acids where end-labelling is not required. For dsDNA and ssDNA, this can be carried out as described above. For ssRNA and dsRNA, reverse transcriptase (EC 2.7.7.49) is used for the synthesis of cDNA using appropriate primers (e.g. oligo-[dT] or random primers; Maniatis, Fritsch and Sambrook, 1982).

High specific activity strand-specific probes may also be prepared from DNA cloned into special vectors. These include M13 phage and RNA transcription vectors. When using M13, the hybridization probe is prepared from the single-stranded phage DNA using a M13 universal primer (Hu and Messing, 1982). However, the even series of M13 should be used so that the probe will not hybridize with sequences derived from certain plasmid vectors related to pBR322. The resulting probe has a specific activity of about 5×10^8 dpm μg^{-1} and is used without denaturation for hybridization. Labelled RNA probe is prepared from transcription vectors incorporating ^{32}P-labelled rNTP using the appropriate T7 or SP6 polymerase as outlined above.

Advances in nucleic acid chemistry have led to the development of various non-radioactive probes for nucleic acid hybridization (Kempe *et al.*, 1985; Al-Hakim and Hull, 1986; Saldanha *et al.*, 1987; Hull and Al-Hakim, 1988; Kincaid and Nightingale, 1988). The basic strategies include:

1. The incorporation of modified nucleotides that serve as tags, such as biotin-11-UTP (Langer, Waldrop and Ward, 1981; Leary, Brigati and Ward, 1983) or digoxigenin-UTP (Boehringer Mannheim, 1988a, 1988b), into probe DNA by enzymatic reactions (such as nick translations, oligo-labelling or end-labelling with *E. coli* DNA polymerase I) can be detected by streptavidin or anti-digoxigenin enzyme assays, respectively.
2. Direct modification of probes by one of several methods. For example, use of a photoreactive biotin derivative that binds to ss- or dsDNA or RNA when irradiated with visible light (Forster *et al.*, 1985; McInnes *et al.*, 1987). Another one-step biotinylation method uses biotin-hydrazide to label ssDNA and RNA; it does not require special equipment and has a detection sensitivity of 1 pg (Reisfeld *et al.*, 1987). These biotin-labelled DNAs are used as probes in hybridization reactions. The bound probe is detected by incubation with avidin or streptavidin which have been coupled chemically to an enzyme capable of catalysing a reaction that can be detected colorimetrically, fluorometrically or photometrically. Streptavidin conjugates appear to be more stable than avidin conjugates (Shi, Itzkowitz and Kim, 1988). As little as 1–5 pg of target sequence can be detected.
3. Covalent linking of an enzyme to single-stranded nucleic acids (e.g. oligonucleotides) which then can be used directly as hybridization probes (Renz and Kurz, 1984; Li *et al.*, 1987; McLaughlin *et al.*, 1987; Seriwatana *et al.*, 1987). Again, the final stage of the procedure is to develop a colorimetric or other appropriate reaction product. Approximately 1–5 pg of target sequence can be detected (Renz and Kurz, 1984). However, the enzyme must survive the hybridization conditions.
4. Another immunological approach to hybridization is to use antibodies to chemically modified DNA probes (Gratzner, 1982; Tchen *et al.*, 1984). When such a probe is hybridized to a target DNA, the hybrids can be detected serologically. A probe based on this technique is available commercially (Chemiprobe, Orgenics Ltd., Yavne, Israel). It is stable, with a sensitivity similar to that of ^{32}P-labelled oligo-DNA probes (Chu, unpublished observations).
5. A novel approach to non-radioactive probes is a bioassay using a probe vector which transforms *E. coli* only when hybridized to target DNA. This assay is quantitative over a range of 0·1–100 pg of target DNA. When different probe vectors, each containing a different marker are used, several target DNAs can be detected simultaneously (Hartley *et al.*, 1986).

Non-radioactive probes have the advantages of stability, long shelf-life and elimination of the hazards associated with handling radioisotopes. Also, detection can be rapid since autoradiography (which requires long exposures when using ^{3}H as the label) is not involved. This is a significant advantage for *in situ* hybridization. The amplification methods described in the section on serological assays can be used to amplify non-radioactive signals.

Recently, non-radioactive probes have been used for detecting viroids (McInnes, Habili and Symons, 1989), viruses (Donovan *et al.*, 1987; Habili,

McInnes and Symons, 1987; Saldanha *et al.*, 1987; Tomlinson *et al.*, 1988) and bacteria (Ezaki *et al.*, 1988), as well as for the routine detection of pathogens by *in situ* hybridization (Burns *et al.*, 1987).

The main disadvantage of non-radioactive methods is that they are often less sensitive than ^{32}P-labelled probes (Zwadyk, Cooksey and Thornsberry, 1986). This is primarily due to background problems encountered when plant extracts are used. Also, colorimetric products produced on filters are more difficult to record and quantify than signals from radioactive probes, and the signal may not be removable for reuse of the filter.

Filter hybridization techniques. Most hybridization assays used for the detection of plant pathogens are performed on filters. The technique, based on the original experiments of Gillespie and Spiegelman (1965), involves immobilizing denatured DNA or RNA on an inert support such as nitrocellulose, so that self-annealing is prevented but bound sequences are available for hybridization with complementary nucleic acid probes. To facilitate analysis, the probe is generally labelled. Hybridization is followed by extensive washing of the filter to remove unreacted probe. The hybrids are then detected by autoradiography (^{32}P-probes) or various enzyme–immunochemical methods (Moeremans *et al.*, 1984; Moeremans, Daniels and De Mey, 1985; Hull and Al-Hakim, 1988). Methods of filter hybridization that are used in detection of plant pathogens include: colony and dot-blot hybridization, Southern and Northern blots (Bio-Rad Bulletin 1149).

Colony and dot-blot hybridization give an estimation of the relative concentration of a specific target nucleic acid within a sample. They are most useful when the sample numbers are large. Replicate filters can be prepared easily and all the samples can be assayed and developed simultaneously (Bar-Joseph and Rosner, 1984). Dot-blot hybridizations are used in routine detection of plant pathogens. Generally they cannot distinguish between the different types and sizes of nucleic acids hybridizing to the probes, but can be used qualitatively since this technique is capable of discriminating between closely related but different target sequences (Beltz *et al.*, 1983). Dot-blot hybridization can also be used quantitatively with appropriate calibration (Kafatos, Jones and Efstratiadis, 1979). Compared to solution hybridization, the rate of hybridization is slow and it is therefore less sensitive (Anderson and Young, 1985; Amasino, 1986).

The basic procedure for Southern blotting is well documented (Southern, 1975; Sealy and Southern, 1982) and has been used widely to study individual DNA species separated on agarose or polyacrylamide gels, e.g. RFLP of bacterial or fungal strains. The separated DNA is transferred by capillary action (nitrocellulose and nylons) or electrophoretically (using only certain nylons requiring low ionic strength for binding, such as Zeta-Probe) to a filter. The immobilized DNA is then detected by use of a hybridization probe.

A combination of hybridization, Southern blot and restriction mapping techniques have provided the basis for the use of molecular markers that will allow extensive use of genetic analysis for the diagnosis of plant pathogens.

Among such molecular markers are restriction fragment length polymorphisms (RFLPs) (Botstein *et al.*, 1980) and oligonucleotide polymorphisms (Kazazian, 1986; Soller and Beckmann, 1988). RFLPs arise from variation of DNA sequences between the genomes of individuals or populations. They are commonly detected using labelled cloned DNA probes on Southern blots of the genomic DNA cleaved with appropriate restriction enzymes. In its simplest form, RFLP analysis includes restriction enzyme maps of less complex genomes, e.g. viruses and plasmids. The ideal RFLP loci are co-inherited with traits being analysed and can be used as markers even though almost nothing is known about the traits. RFLP markers have several advantages over physiological (e.g. virulence), biochemical (e.g. isozymes) or morphological markers. Any tissue can be used for RFLP analysis and the technique may be useful as an initial step in identifying strain-specific probes. RFLP analysis may provide a rapid alternative to the screening of numerous clones in complex genomic libraries of prokaryotic and eukaryotic cells.

Techniques for rapid and efficient detection of RFLPs are well established (Botstein *et al.*, 1980; Beckmann and Soller, 1983; Beckmann and Soller, 1986; Boehm, Werle and Drahovsky, 1987; Landry and Michelmore, 1987). Typically, the genomic DNAs are digested with a type II restriction endonuclease. The DNA fragments that are generated are then Southern blotted, i.e. separated by gel electrophoresis, hybridized to a labelled DNA probe and visualized by appropriate methods. In some instances, the RFLPs may be observed by direct staining of the gel without using Southern blots (Klich and Mullaney, 1987). The observed number of bands is dependent upon the number of loci in the genome which are homologous to the probe and the number of restriction sites within these DNA sequences. Any specific differences in the DNA sequence between isolates due to substitutions, additions, deletions, inversions or translocations may alter the size and number of the fragments obtained. Several restriction enzymes can be employed to identify RFLPs with each probe tested. Thus, numerous RFLP maps can be generated for each isolate of a pathogen so that distinctive RFLP maps can be obtained. When multiple loci are detected, identification of alleles are more difficult. However, the same blot can be probed many times with different probes to produce a suitable RFLP map. Such maps can be important in determining the population genetics of the pathogen. Such information can be useful for both epidemiological and taxonomic studies.

Recently, RFLP analysis has been used for identification and taxonomy of strains of pathogens such as fungi (Klich and Mullaney, 1987; Magee, D'Souza and Magee, 1987; Manicom *et al.*, 1987; Michelmore and Hulbert, 1987; Hulbert and Michelmore, 1988), nematodes (Curran and Webster, 1987) and phytopathogenic bacteria (Lazo, Roffey and Gabriel, 1987).

Oligonucleotide polymorphism analyses are a cheaper alternative to RFLP mapping in detecting variations within and between populations (Soller and Beckmann, 1988). Synthetic oligonucleotide probes are used to hybridize to and detect specific allelic variants of a particular gene.

Northern, or RNA, blotting (Alwine, Kemp and Stark, 1977; Thomas, 1980, 1983; Williams and Mason, 1985) is used to characterize the size and number of

target RNA species in a nucleic acid population. It can be used for differentiation between serologically related strains of pathogens. The method, like Southern transfer, involves electrophoretic separation of the RNA species in an agarose or polyacrylamide gel, transfer to a filter by capillary action or electrophoretic transfer (polyacrylamide gels) and subsequent detection of specific RNA sequences by hybridization with a labelled probe (Wahl, Stern and Stark, 1979). The RNA is denatured prior to transfer to filters, either by running in a denaturing gel or by treating the gel with weak alkali after electrophoresis. Glyoxal and dimethyl sulphoxide are recommended for denaturing RNA (McMaster and Carmichael, 1977; Thomas, 1983).

Many factors affect the hybridization reaction. The main ones are probe concentration, base composition, type of probes, temperature, ionic strength, pH, viscosity, levels of formamide and dextran sulphate in the hybridization solution, and degree of mismatching (Anderson and Young, 1985).

There are also several types of filter materials. Nitrocellulose filters bind DNA and RNA very efficiently, except for fragments less than 500 nucleotides long. Nitrocellulose is also fragile. Nylon-based filters are easier to handle and can be rehybridized several times (Gatti, Concanon and Salser, 1984). Filters with a pore size of 0·45 μm are used for large DNA molecules, while 0·1–0·22 μm pore sizes are more efficient for RNA and small DNA (<500 nucleotides) molecules. New methods have been developed to bind covalently nucleic acids to filters and so prevent the filter-bound nucleic acids from being washed off during hybridization. These include UV-induced binding (Church and Gilbert, 1984; Khandjian, 1987) and alkali treatment (Reed and Mann, 1985).

Nitrocellulose filters require high ionic strength for quantitative binding of RNA and DNA (Southern, 1975) while requirements for nylon vary according to different manufacturers. Chemically treated cellulose and nylon papers bind nucleic acids covalently but their binding capacity is much lower than nitrocellulose, typically 1/40–1/80 (Bio-Rad Bulletin 1110). All filters require that the nucleic acids (including single-stranded nucleic acids) be denatured for binding. They can be denatured before or after binding to the filter. Alkali treatment of DNA bound to Zeta-Probe nylon simultaneously denatures and covalently binds DNA (Reed and Mann, 1985) and is therefore convenient for genomic and plasmid dsDNA blots. RNA can be denatured with glyoxal (McMaster and Carmichael, 1977) prior to application to filters (Thomas, 1983). A minimum of 10 μg DNA per dot is required for genomic DNA blots to detect a single copy sequence in eukaryotic DNA. For multiple copy sequences proportionally less nucleic acid need be spotted. Usually, it is necessary to sonicate or digest the genomic DNA to help bind the DNA to the filter more efficiently. When single-stranded nucleic acids are applied correctly, both nitrocellulose and nylon seem to give a similar sensitivity of detection and are used more widely than treated papers.

All the pre-hybridization and hybridization steps used are well established and documented (Anderson and Young, 1985; Mason and Williams, 1985). Several points to note are:

1. The specific activity of the probe needs to be only 10^7 dpm μg^{-1} to give a

strong signal after overnight exposure. If a higher specific activity probe is used, exposure time can be reduced.

2. If required, hybridization time can be reduced to a few hours.
3. If the probe is not expected to form a perfect match with the filter-bound nucleic acid, then the stringency of washing and hybridization should be reduced (Mason and Williams, 1985).
4. 10 μg ml^{-1} poly (A) or 10 μg ml^{-1} poly(C) should be added to the pre-hybridization and hybridization buffers to prevent spurious hybridization if the probe contains segments of poly (dA) (dT) or poly (dG) (dC) homopolymers.
5. If the proportion of specific probe or specific target sequence is low (e.g. when using labelled total mRNA or cDNA made against total mRNA population), the concentration of probe and nucleic acid should be as high as possible (up to 1 μg ml^{-1} for probe and 10 μg μl^{-1} for nucleic acid spotted on the filter) and the hybridization time should be increased to 24–48 hours.
6. When oligonucleotides are used as probes, the hybridization should ideally be carried out at a temperature at which perfect hybrids are stable, but mismatched hybrids are unstable. This is achieved by hybridizing at about 5°C below the temperature (*Td*) at which a perfectly matched hybrid will be half dissociated. The *Td* for oligonucleotides can be estimated by the following relationship (Suggs *et al.*, 1981):

$$Td = 4°C \text{ per GC base pair} + 2°C \text{ per AT base pair.}$$

   For a mixture of oligonucleotides, a temperature of 5°C below the lowest *Td* should be used.
7. The usual precautions in handling RNA (Clemens, 1984) should be observed when RNA–RNA hybridization is involved. Also, the hybridization should be performed for the minimum possible time, in a slightly acidic buffer containing formamide, and at the lowest temperature allowing efficient hybridization. When glyoxal is used to denature RNA, the glyoxal should be deionized and stored in small aliquots so that a fresh aliquot is used each time. These precautions will minimize hydrolysis of the RNA.
8. When using a new diagnostic probe for the first time, it is necessary to try a range of hybridization conditions to optimize the detection assay, such as hybridization temperature and concentration of probe, etc.
9. Certain precautions should be taken when using biotin-labelled nucleic acids. The probe should be denatured using high temperature rather than alkali, because the bond between the biotin and the nucleic acid is alkali labile. Subsequently, the hybridization should be carried out in formamide, rather than aqueous solution at high temperature. The thermal stability of biotin-labelled hybrid is slightly lower than radioactive hybrid so that the concentration of formamide is lowered from 50% to 45%. Some polyethylene bags are not suitable as they lead to high backgrounds. It has also been observed that efficient hybridization with biotinylated DNA probes requires higher probe concentrations than those normally employed for ^{32}P probes (e.g. 50–100 ng ml^{-1}; Gebeyehu *et al.*,

1987), due to slower reassociation rates. Furthermore, use of proteinase K may be necessary to remove completely the streptavidin-biotinylated probe for reprobing.

In situ *hybridization.* The principles and practical aspects of *in situ* hybridization have been reviewed recently (Haase *et al.*, 1985; Pardue, 1985; Hofler, 1987; Infantolino and Pinarello, 1987; Penschow *et al.*, 1987; Valentino, Eberwine and Barchas, 1987). This technique provides for the specific hybridization of a nucleic acid probe to nucleic acids within cytological preparations, so that a high degree of spatial localization and quantification of the target sequences can be determined. The precise localization of target sequences may be observed at the organelle, cellular or tissue level. It is also possible to detect nucleic acids of pathogens that are present in only a small number of cells or at low concentrations in cells (Coats *et al.*, 1987). Such nucleic acids might never be detected in nucleic acids extracted from a whole tissue due to dilution by other nucleic acid species from uninfected cells that do not contain the nucleic acid of interest. Thus, in cases such as slow diseases, latent infections and tissue- or organ-restricted diseases, *in situ* hybridization is the most sensitive technique because it may detect a few infected cells among many uninfected cells. The applications of *in situ* hybridization in diagnosis of plant diseases include the identification of latent infections and detection and localization of pathogens in tissues and plant cells which are difficult to examine by standard histological or microscopic methods such as those used to reveal mycoplasmas, viruses and seed-borne pathogens.

Most *in situ* hybridizations are carried out with preparations that are analysed by the light microscope, although preparations for analysis by electron microscopy can also be used (Hutchinson *et al.*, 1982; Langer-Safer, Levine and Ward, 1982). The probes used are similar to those employed in filter hybridization assays, except that non-radioactive probes or ^{3}H and ^{35}S, which are lower-energy isotopes than ^{32}P, are used (Lewis *et al.*, 1987; Pachmann, 1987). As discussed previously, non-radioactive probes are increasingly preferred for *in situ* hybridization procedures, due to their rapid signal development (Pinkel *et al.*, 1986; Hopman, Wiegant and van Duijn, 1987; Lewis *et al.*, 1987; Terpstra *et al.*, 1987).

Improvements in hybridization techniques. During the past few years there have been many improvements and developments in hybridization and associated techniques. Some of these changes are listed below.

Improved methods of producing cloned probes Such as:

1. Electroporation (Dower, Miller and Ragsdale, 1988; Fiedler and Wirth, 1988);
2. Ligase-mediated detection, in which two adjacent probes are ligated after hybridization (Landegren *et al.*, 1988);
3. Use of multiple smaller non-overlapping specific probes to enhance simultaneously sensitivity and specificity (Parkkinen, 1988);
4. New approaches to differential hybridization to identify rare mRNA clones from cDNA libraries using single-stranded cDNA (Boll *et al.*, 1986);

5. Use of UV-irradiation to covalently bind DNA to solid supports (Stollar and Rashtchian, 1987) and immobilization of DNA to Sephacryl 500 dextran supports, which improve hybridization efficiencies to 80% compared to 20% with filter hybridization (Goldkorn and Prockop, 1986; Gingeras, Kwoh and Davis, 1987; Thompson and Gillespie, 1987; Wolf *et al.*, 1987);
6. Hybridization strategies that reduce background from samples or enable the use of solution hybridization, which can reduce hybridization times to a few minutes (Gingeras, Kwoh and Davies, 1987; Karjalainen, Rouhainen and Soderlund, 1987; Nicholls, Langdale and Malcolm, 1987; Wilkins and Snell, 1987; Palva, Nyberg and Palva, 1988; Urdea *et al.*, 1988);
7. Development of charged nylon supports with increased binding capacity and the ability to bind fragments as small as 10 bases (Gross, Huang and Gerrard, 1985); these are also suitable for electrophoretic transfer of DNA and RNA (Reed and Mann, 1985);
8. Development of microassays for small numbers of plant cells so that specific nucleic acids in 10 protoplasts (0·15 pg) can be detected (Crossway and Houck, 1985; Williams, 1987);
9. Development of automated DNA electrophoresis, hybridization and electronic detection of signal (Zapolski *et al.*, 1987).

In addition to these improvements, there have been great advances in the development of novel non-radioactive probes, such as those based on incorporation of digoxigenin-dUTP (Boehringer Mannheim, 1988b), bioluminescence (Hauber and Geiger, 1988) and chemiluminescence (Thorpe and Kricka, 1987; Urdea *et al.*, 1988); new hybridization amplification methods; and ELISA-based solution hybridization techniques. These developments are discussed in more detail below.

Non-radioactive digoxigenin-labelled nucleic acid probes are prepared either by covalently attaching digoxigeninamidocaproic acid-*N*-hydroxysuccinimidoester to an oligonucleotide probe (Schäfer, Zischler and Epplen, 1988), or by oligolabelling ssDNA templates using digoxigenin-dUTP as the label precursor (Boehringer Mannheim, 1988b). Using such an oligonucleotide probe, a more intense signal was obtained by lowering the hybridization temperature to 3–5°C below that required for ^{32}P-labelled oligonucleotides. The hybridized probes were detected colorimetrically using an alkaline phosphatase anti-digoxigenin antibody conjugate and 5-bromo-4-chloro-indoxyl phosphate (BCIP) and nitro blue tetrazolium (NBT) substrate. In Southern blots, strong bands were observed after 2–3 min and weaker bands after several hours of development. The coloured precipitate can be stripped using dimethylformamide for rehybridization of the membrane. In dot blots using oligo-labelled cDNA probes, 0·1 pg of homologous DNA was detected after 12 h of development, while 1 pg was detected in 1 h. Single-copy genes can be detected using 1 μg of mammalian DNA. The assay could be completed in 24 h but the development time can also be extended for up to 3 days without substantial background problems.

Bioluminescence reactions are simple, rapid, highly sensitive and quantitative (Kricka, 1988). Bioluminescence can be used for both immunoassays and

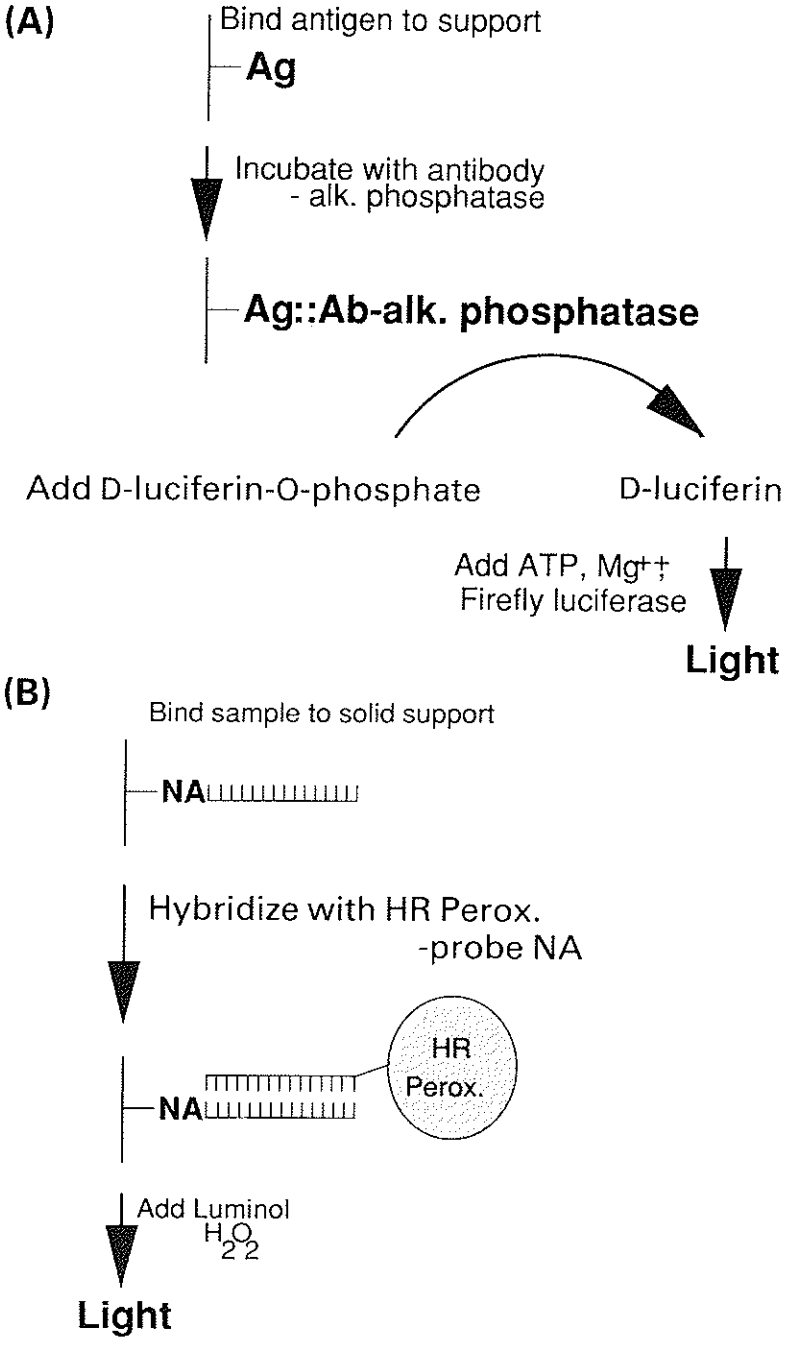

Figure 5. Bioluminescence and chemiluminescence detection systems. The bioluminescence assay (A) is that described by Hauber and Geiger (1988), shown here for the detection of antigen. An adaptation of this approach, using different reagents, is the chemiluminescence approach (B) for the detection of a nucleic acid sequence (Thorpe and Kricka, 1987; Urdea *et al.*, 1988). Abbreviations: Ag, antigen; Ab, antibody; alk. phosphatase, alkaline phosphatase; NA, nucleic acid; HR Perox, horseradish peroxidase.

hybridizations (Baldwin, Holzman and Holzman, 1986; Leong, Milstein and Pannell, 1986; Tanaka and Ishikawa, 1986; Terouanne *et al.*, 1986, 1987; Brochu *et al.*, 1987; Geiger and Miska, 1987; Hauber and Geiger, 1988; Kricka, 1988) with great sensitivity (*Figure 5A*). The signal can be measured with a liquid scintillation counter (Nguyen, Morange and Bensaude, 1988) or recorded on photographic film. A kit marketed by Amersham uses a modified horseradish peroxidase which can be conjugated to the probe nucleic acid in 10 min. After hybridization, washing the filter and adding the substrate the blot can be developed in just a few minutes (*Figure 5B*). This type of luminescence is called chemiluminescence since it does not use luciferase.

Another possible hybridization assay based on luciferase is one that involves using two adjacent DNA probes, one labelled with luciferase and the other labelled with a fluorophore. When both probes are bound to a target DNA, sufficiently near to each other, fluorescence emission can be obtained and measured (Heller *et al.*, 1983).

Finally, successful cloning and expression of luciferase genes (Kricka, 1988) could provide a way for using tagged strain-specific phage and plasmids for the sensitive identification of specific strains of prokaryotic plant pathogens (Shaw and Kado, 1986; Ulitzur and Kuhn, 1987).

Amplified hybridization techniques Amplification of specific nucleic acids can be used for hybridization tests for low abundance sequences, e.g. in the detection of plant pathogens present in low concentrations, by amplification of either the target or probe DNA.

Target nucleic acids can be amplified by various enrichment methods, including either partial purification or isolation and subsequent concentration of the nucleic acids, or multiplication of the target. In purification, the strategy is to remove as much of the contaminating host nucleic acids as possible and, when comparing strains of a pathogen, to remove as much of the nucleic acid common between the strains as possible. Various methods are available for isolation or enrichment of specific pathogens in plant tissues prior to nucleic acid hybridization. The enrichment of target mRNAs from genes expressed more in one strain of pathogen than the other can be made by allowing sequences common to both strains to anneal either in solution or on a solid matrix. The reannealed sequences can be separated from unannealed sequences by hydroxyapatite chromatography or by successive washing of the solid matrix (Arrand, 1985). Such enriched sequences can be used for cloning or as targets for hybridization. Similarly, if sequence information is available, specific target sequences can be enriched by hybridization to immobilized complementary DNA.

A more recent method of specific enrichment involves the multiplication of target DNA. It involves hybridization of synthetic complementary oligonucleotide primers to the target sequences and synthesis of multiple copies of complementary DNA using a heat-stable DNA polymerase. This can be done in a crude extract without prior purification of the nucleic acids. This method is known as polymerase chain reaction (PCR) (Saiki *et al.*, 1985, 1988; Scharf, Horn and Erlich, 1986). The method has been employed to enrich dsDNA sequences to facilitate direct sequencing (McMahon, Davis and Wogan, 1987;

Wong *et al.*, 1987); analysis of genetic disorders and chromosomal rearrangements (Kogan, Doherty and Gitschier, 1987; Lee *et al.*, 1987); detection and creation of single base changes or deletions in whole genes (Saiki *et al.*, 1986; Bos *et al.*, 1987; Erlich, Gelfand and Saiki, 1988; Rochlitz *et al.*, 1988; Weier and Gray, 1988), and detection of viral pathogens (Kwok *et al.*, 1987). Typically, the target dsDNA is heat denatured, a pair of synthetic oligonucleotide primers are then hybridized to both strands of the target DNA, one to the 5′ end of the sense strand and one to the 5′ end of the antisense strand by an annealing step. The enzyme then synthesizes new DNA on the templates to produce theoretically twice the number of target DNAs. The sample is then heated to denature the DNA, followed by annealing with excess primer. If this cycle of denaturation, annealing and extension is repeated many times, there is an exponential increase in the number of target DNA molecules. The method is simple, versatile, reproduces DNA sequences between the primers with high fidelity and an efficiency of up to 85% per cycle (Weier and Gray, 1988) and can be automated. The PCR product can be used not only as a target for hybridization but can also be used directly for sequencing (Simpson, Crosby and Skopek, 1988) to determine strain variations, to facilitate cloning (Simpson, Crosby and Skopek, 1988) or as a specific probe. *Taq* DNA polymerase is stable to heating at 95°C, so it will survive the denaturation step without addition of fresh enzyme during each cycle. Its optimal operating temperature is between 60°C and 85°C (Chien, Edgar and Trela, 1976), so more stringent selection of target DNA can be obtained while achieving maximum yield and length of products (Saiki *et al.*, 1988). The enzyme is available commercially in highly purified form (Perkin Elmer Cetus, Norwalk, USA) and can yield amplification of DNA sequences of up to 2 kbp by 10^5–10^7-fold (Saiki *et al.*, 1985, 1988; Rochlitz *et al.*, 1988).

One possible problem in PCR is synthesis of non-target sequences due to partial homology to the primer. Therefore the annealing temperature should be set to minimize non-specific annealing. Initial cycles can be more stringent and less efficient while the later cycles can be less stringent and more efficient after the desired sequence has increased significantly. It should be possible to select primers that amplify strain-, species- or genus-specific target sequences.

PCR can be used to amplify pathogen DNA for strain identification and development of strain-specific probes, production of uncloned probes from mRNAs and amplification of cloned probes *in vitro* to eliminate vector sequences (Lo, Mehal and Fleming, 1988; Biernat, Gobel and Koster, 1989). The simplicity and sensitivity of PCR gives it great potential for routine and large-scale detection of pathogens in difficult areas such as seed certification.

In addition to amplifying target sequences, it is also possible to amplify probe signals. To a certain extent these signals can be improved by increasing the amount and specific activity of the probe itself or by multiplying the signal-generating moieties that detect the hybridized probe. As indicated above, the specific activity and concentration of the probe varies with the method of labelling and type of probe. Similarly, multiple enzyme molecules can be attached to each DNA probe instead of one (Fahrlander and Klausner, 1988).

The second means of amplifying the probe signal involves increasing the efficiency of detecting the hybrids. In detecting ^{32}P-labelled radioactive hybrids, the use of an intensifying screen is a means of amplifying the detection. When using non-radioactive probes such as biotin, digoxigenin and enzyme labelling, the various amplification methods used in immunological detection techniques described above can be employed. Using alkaline phosphatase conjugated to the oligonucleotide secondary probe and detection with NBT/BCIP substrate system, as little as 0·8 pg of hepatitis B virus DNA in a background of 100 ng of human genomic DNA was readily and reproducibly detected in a total assay time of 5 h (Fahrlander and Klausner, 1988). This approach should be applicable to plant pathology.

ELISA-based nucleic acid hybridization Large-scale application of hybridization for pathogen detection could be greatly enhanced by the adoption of ELISA technology. The first steps in this direction have been achieved by the use of non-radioactive, enzyme–immunological detection methods to visualize hybridization. The use of antigen/antibody (digoxigenin) or affinity bridges (biotin–avidin) to bind probes non-covalently onto favourable solid surfaces, such as latex beads, polystyrene surfaces or Sepharose beads, that are amenable to ELISA techniques will further enhance the development of ELISA-based hybridization (*Figure 6*).

The use of solution-based sandwich hybridization strategies using two probes can also be developed. This will involve a specific capture probe, which can be immobilized on an ELISA plate or other solid support after hybridization, and a second reporter probe whose presence can be measured enzymatically after rapid removal of unhybridized reporter probes by centrifugation, filtration, magnetism or washing (Urdea *et al.*, 1988).

The recent development of antibodies specific for RNA:DNA hybrids allows for specific capture of RNA:DNA hybrids from solution (Rashtchian *et al.*, 1987; Stollar and Rashtchian, 1987). Such assays can be completed in 2 hours and detect target nucleic acids at picogram levels.

Selective media

Many detection methods used for the identification of plant pathogens, especially fungi and bacteria, cannot identify the pathogens to the species or strain level. The culture of pathogens in artificial media is useful when such identification is required (Takayama, Kawai and Suetsugu, 1985). Similarly, many detection methods, such as serological and nucleic acid hybridization methods, cannot discriminate between viable or dead cells of these pathogens. Culture and subsequent pathogenesis testing are therefore required for confirmation of diagnosis, e.g. of soft rot, *Erwinia* sp. (Kelman and Maher, 1987). Thus, the development and use of better selective media is still required in the detection and identification of plant pathogens.

Most bacteria and fungi can be grown in culture under appropriate conditions (Stolp and Starr, 1981; Fahy and Hayward, 1983; Hayward, 1983; Dhingra and Sinclair, 1985; Davis *et al.*, 1987). They have unique metabolic

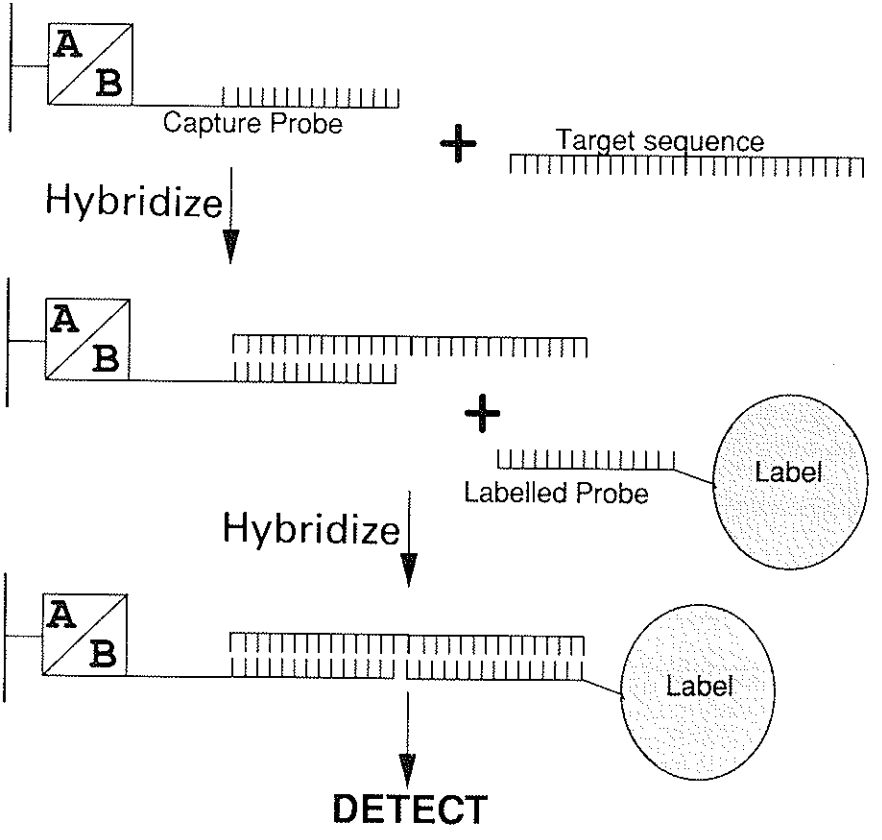

Figure 6. Outline of the two-stage sandwich hybridization scheme for detection of a target nucleic acid. The assay involves detection of the label by one of a number of methods (see text). Abbreviations: A, avidin; B, biotin.

pathways and such nutritional diversity is exploited in the development of new selective media (Brisbane and Kerr, 1983; Conway, 1985; Perombelon, 1987; Schaad, 1987). The availability of standardized methods of characterizing the biochemical and physiological properties of plant pathogens would greatly facilitate the development of new selective media. This is especially important for MLOs, spiroplasmas and other fastidious prokaryotes (Chen *et al.*, 1982; Lee and Davis, 1984, 1986). Similarly, a knowledge of the distribution of viable pathogens in infected plants will also enhance the reliability of isolation, especially of those prokaryotes limited to the vascular tissues, e.g. the phloem-limited bacteria (Kostka and McAleese, 1987; Raju and Wells, 1987).

The advantages of selective media or cultural methods in pathogen detection are that:

1. Isolation and initial culture can be made in the field so that any problem of storage and maintaining the quality of samples is avoided.
2. Symptoms can be correlated with cultures quickly.
3. Isolation can be used to confirm other diagnostic procedures.
4. Further studies, or identification, of the pathogen are facilitated by the availability of pure cultures.

Recently, selective isolation methods utilizing specific antibodies have been developed to assist in the culture of specific bacteria on non-selective or semi-selective media (Stead *et al.*, 1987; van Vuurde, Ruissen and Vruggink, 1987). The approach involves the selective isolation and concentration of bacteria by immunoaffinity chromatography (Ruissen *et al.*, 1987). After loading samples and incubating at room temperature for 20 min, the non-bound microbes are washed off the column with buffer and then the bound bacteria are eluted with chaotropic salt solutions. The eluted bacteria may be plated directly, or concentrated on millipore filters and then placed onto nutrient agar. Any cross-reacting bacteria can be identified from the culture. This method has been found to be more reliable and sensitive than immunofluorescence or ELISA (Stead *et al.*, 1987).

NON-SPECIFIC METHODS

Light microscopy

Histological investigations of a range of virus-infected plant cells have shown the presence of crystals and/or macro-structures in the cells (*see* Francki, Milne and Hatta, 1985; Edwardson and Christie, 1986). These crystals appear to be composed of regularly arranged virus particles and the macro-structures, known as X-bodies or inclusion bodies, often have a characteristic morphology and cellular location (Bald, 1966; Christie and Edwardson, 1977; Edwardson and Christie, 1978; Russo, Di Franco and Martelli, 1987). The detection and identification of some plant viruses can be achieved readily by the observation of particular inclusion bodies that they induce in the cells they infect (Ko, 1988). For example, the geminivirus group induces large blue-violet nuclear inclusion bodies as well as fibrillar bodies in phloem cells in azure-A-stained

tissues (Christie *et al.*, 1986). For viruses such as these, light microscopic examination of stained sections is a rapid and relatively sensitive means of detection. However, for most viruses, light microscopy is of limited value (Rubio-Huertos *et al.*, 1985).

Classification and identification of many unicellular plant pathogens and fungi are based on the observation of morphological structures of the organism with the light microscope (Dhingra and Sinclair, 1985). The characteristic features of such pathogens include the edge, size, shape and colour of the colonies, the number and position of the flagella and the type of sexual and asexual spores.

Electron microscopy

Electron microscopy (EM) has been used predominantly to detect and study viruses (Francki, Milne and Hatta, 1985) rather than bacteria and fungi. Many laboratories are equipped with an electron microscope used to assess the presence, morphology and location of pathogens. In many cases the use of electron microscopy is the most rapid method of identifying an unknown virus (Francki, Milne and Hatta, 1985; Nilsson and Tomenius, 1987; Buchen-Osmond *et al.*, 1988). Since most biological molecules are not totally opaque to the electron beam, methods have been developed to enhance contrast. These include the introduction of metal shadowing (Williams and Wyckoff, 1944) and negative staining with heavy metallic compounds (Hall, 1955; Brenner and Horne, 1959). The latter method is rapid and simple, yet usually more informative than metal shadowing and is usually the method of choice today (Horne and Wildy, 1961, 1979). The stains commonly employed include phosphotungstic acid (Brenner and Horne, 1959), uranyl acetate (Huxley and Zubay, 1960), ammonium molybdate (Horne, 1967) and uranyl formate (Finch, 1964). Those viruses whose stability depends on electrovalent bonding between protein and RNA appear to be disrupted by staining with phosphotungstic acid at neutral pH (Francki, Milne and Hatta, 1985). Similarly, phosphotungstic acid can produce artefacts with enveloped viruses, e.g. rhabdoviruses (Francki, 1973). Uranyl acetate (pH 4·2) is a good general negative stain for plant viruses, although it also is not suitable for rhabdoviruses and may stain nucleic acids, causing positive staining (Francki, Milne and Hatta, 1985). Uranyl formate reveals the fine structure of rod-shaped viruses more effectively (Finch, 1964). Ammonium molybdate seems to be a less disruptive general stain. EM techniques used in diagnosis include immunosorbent electron microscopy and *in situ* hybridization. These are outlined above in sections on serological methods and nucleic acid hybridization methods, respectively. Other techniques used in EM are the leaf-dip method, thin-section EM and scanning EM.

Leaf-dip method. It is possible to detect and identify the particles of many viruses based on their particle morphology simply and rapidly by extracting and examining the juice obtained from infected plants (Hitchborn and Hills, 1965). This is the method of choice when presented with a plant sample with suspected

viral infection. Since biological objects are readily destroyed when exposed to the electron beam, a method of ensuring minimum exposure to the region of interest, until ready for photographing, has been devised (Williams and Fisher, 1970) and refined recently (Wrigley, Brown and Chillingworth, 1983). This is important for the identification of virus-like particles (VLP) amongst the cell debris.

Thin-section electron microscopy. As a non-specific diagnostic technique, thin-section electron microscopy is used when pathogens are not detectable by the leaf-dip method. It is generally restricted to the detection of unknown viruses and MLOs.

The first use of EM to detect viruses in thin sections was reported by Black and his colleagues for tobacco mosaic virus (TMV) (Black, Morgan and Wyckoff, 1950). After that time, new techniques for fixation, embedding, section cutting and staining were developed for thin-section electron microscopy. Plant tissues had been difficult to use due to the hardness and impermeability of the cell walls to fixatives and embedding resins, while the acids in vacuoles interfered with specimen preparation. Major progress included the introduction of the diamond knife (Fernandez-Moran, 1953), use of glutaraldehyde (Sabatini, Bensch and Barrnett, 1963), application of epoxy embedding resins (Glauert, Rogers and Glauert, 1956) and the use of immunogold staining (Ferrari *et al.*, 1988). The difficulty of distinguishing ribosomes from small polyhedral virus particles in thin sections has been overcome by several methods (Milne, 1967; Honda and Matsui, 1974; Hatta and Francki, 1979). Today, micrographs of thin sections of plant tissues infected by all known groups of plant viruses have been published (Francki, Milne and Hatta, 1985).

Scanning electron microscopy (SEM). SEM has been employed mainly to detect the presence of fastidious prokaryotes in plants infected with the yellow types of diseases (Bove, 1984). These organisms occur in the phloem and xylem elements which are usually devoid of interfering cytoplasmic material, thus making their detection by SEM possible. The two-dimensional structure of these micro-organisms, as observed by transmission EM, can resemble some cell organelles, but their three-dimensional structure, observed by SEM, are unique. SEM has also been useful for rapid diagnosis of bacterial wilt of bentgrass (*Agrostis* sp.) (Roberts, Baker and Vargas, 1983).

Other histological and microscopic methods

Although serological techniques are being implemented for many plant pathogens, histochemical and related microscopic techniques are still used to diagnose unknown diseases and those suspected to be caused by certain pathogens such as MLOs and phloem-limited bacteria or fungi (Dhingra and Sinclair, 1985; Raju and Wells, 1987). These methods are rapid and non-specific, but relatively sensitive and are useful for preliminary diagnosis or where good quality antiserum is not yet available. Histological stains for

MLOs include the DNA-specific stain 4, 6-diamidino-2-phenylindole (DAPI) (Hiruki, Giannotti and Dijkstra, 1974), the callose-specific stain aniline blue, or Dienes' stain (Deeley, Stevens and Fox, 1979). DAPI staining is rapid (20 min) and can detect MLOs as early as 3 weeks post-infection, much earlier than the development of visible symptoms (Hiruki and Da Rocha, 1986).

Another useful method for detection of suspected MLOs is the phase-contrast microscope. It is more suitable than the conventional bright-field light microscope because, under phase-contrast microscopy, phloem-limited bacteria exhibit an undulating motility in 30% glycerol that ceases upon addition of penicillin.

Analysis of dsRNA

This technique has been developed mainly for the detection of unclassified RNA plant viruses (Bar-Joseph *et al.*, 1983; Dodds, Morris and Jordan, 1984; Jordan, 1986). It may also be used to detect viral diseases before symptoms appear (Lejour and Kummert, 1986). This method is based on the fact that RNA plant viruses either have dsRNA as their genome (e.g. phytoreoviruses) or use dsRNA as an intermediate for replication (ssRNA viruses). In some viruses, dsRNA accumulates during replication (e.g. velvet tobacco mottle-virus, see Chu, Francki and Randles, 1983) while in others, the level of dsRNA varies with time after infection and may not be readily detectable. The yield of dsRNA also varies with different viruses (Valverde, Dodds and Heick, 1986).

dsRNA may be isolated and labelled (Rosner *et al.*, 1983) or cloned as cDNA for development of nucleic acid probes, thus bypassing virus purification steps (Jelkmann, Martin and Maiss, 1989). This is an advantage if the virus is uncharacterized or difficult to purify (e.g. Chu, Helms and Martin, 1989). A further advantage is that the dsRNA profile can aid in the classification of an unknown virus. It has been advocated that strains of viruses can be differentiated according to their characteristic double-stranded forms of subgenomic mRNAs (Valverde, Dodds and Heick, 1986). Similarly, dsRNA analysis may reveal the presence of infection complexes such as satellite RNA, multiple infections and cryptic virus (Lejour and Kummert, 1986). A problem with this approach is that dsRNA can be found in healthy control plants (Kurppa and Martin 1986; Valverde, Dodds and Heick, 1986; Spiegel, 1987). Such non-pathogenesis-related dsRNAs include those from fungi (Morris and Dodds, 1979; Dodds, Morris and Jordan, 1984) or plant hosts (Wakarchuk and Hamilton, 1985). These non-viral dsRNAs can be distinguished from viral dsRNA if adequate controls are used, and it should be emphasized that the presence of dsRNA be correlated with other detection methods wherever possible, e.g. symptoms and partial purification of VLPs. Other possible problems associated with dsRNA analysis are its lower sensitivity compared to EM or bioassay and that the method requires laborious nucleic acid purification. This is of special significance in fruit trees and other perennial species which contain high levels of polysaccharides and phenolics. A modification of the extraction procedure is usually required and retest of negatives is recommended.

A new approach is the use of anti-dsRNA antiserum to detect dsRNA in crude preparations (Gabriel, 1986). However, this seems to have even lower sensitivity than normal silver staining for detection of dsRNAs.

Analysis of protein and nucleic acid components of pathogens

As in the analysis of dsRNA, the identification of protein and nucleic acid components by gel electrophoresis can be useful for the specific detection and characterization of unknown pathogens, especially viruses and viroids, in crude or partially purified preparations. However, these analyses must be carried out in association with other tests that show the presence of a pathogen, such as transmission tests. This approach has been used to identify ssDNA associated with foliar decay disease of coconut (*Cocos nucifera*) (Randles *et al.*, 1986; Hanold, Langridge and Randles, 1988). Similarly, analysis of the protein and nucleic acid components of partially purified virus preparations from plants infected with subterranean clover stunt virus (SCSV) revealed that SCSV is associated with a new type of ssDNA virus (Chu and Helms, 1988). This is the most useful method for detection of viroids which cannot be detected by EM or serological methods and for which nucleic acid hybridization probes are not available (Schumacher *et al.*, 1986). Rapid diagnosis of Chinese yam necrotic mosaic virus infection, using leaf homogenates, can be completed in 7 hours (Shirako and Ehara, 1986).

The diagnostic value of this method has been improved recently by minipurification methods for many viruses (Lane, 1986); sensitive staining methods, such as silver stains, for nucleic acids (Berry and Samuel, 1982) and proteins (Merril *et al.*, 1981); and enhanced glycoprotein staining methods in polyacrylamide gels (Munoz *et al.*, 1988). A new direct blotting technique for proteins allows completion of a Western blot analysis from electrophoresis to results in 4 hours with a sensitivity of 200 ng per band (Beck, 1988).

Bioassay and in vitro *culture*

Transmission and pathogenicity tests. Many uncharacterized diseases are still diagnosed by transmission to indicator hosts and/or vector transmission properties. Transmission and pathogenicity tests are based on a set of diagnostic hosts susceptible or non-susceptible to a plant pathogen, with the susceptible hosts producing characteristic symptoms (Buchen-Osmond *et al.*, 1988). It is used mainly to detect unknown pathogens by their reactions with a range of specific sensitive indicator hosts, e.g. graft inoculation of citrus budwood to specific indicator hosts. These tests are routinely used to identify cultures of bacteria and fungi and other plant pathogens (Kelman and Maher, 1987; Kostka and McAleese, 1987; Sletten, 1987; Garnsey, 1988; Spiegel, 1988). The tests are labour intensive, time consuming and not always highly efficient, but are sometimes still the simplest and often one of the most sensitive techniques. They remain the benchmark to which other techniques are compared for sensitivity and reliability.

Improvements in graft and host-transmission tests can be achieved by

identifying a standardized set of virus-indexed host species for each pathogen (van Dijk, van der Meer and Piram, 1987; Foster, 1988; Spiegel, 1988), or the development of new hosts that will produce characteristic symptoms in a shorter time, e.g. assays for unknown sweet potato (*Ipomoea* sp.) viruses have been improved by graft assay to *Ipomoea setosa*, a new indicator host (Moyer, 1988). The source of variation should also be identified and reduced. One means of doing this is through standardization of indicator host species and cultivars (Buchen-Osmond *et al.*, 1988). Another improvement will be through the use of mechanical aids to improve the speed, reliability and sensitivity of transmission tests. For example, use of the Pollahne leaf press and compressed air guns to control wounding inoculation onto indicator plants. Using this approach, sensitivity can be increased 500-fold (Laidlaw, 1986).

In vitro *culture.* Traditionally, transmission tests are performed in glasshouses using indicator plants grown from commercially available seeds. Occasionally, a problem of virus contamination in indicator hosts is observed, e.g. seed-borne and/or cryptic viruses in white clover (*Trifolium repens*), bean (*Vicia* sp.) and pea (*Pisum* sp.) seeds (Chu, unpublished observation) and soil-borne virus in strawberry (*Fragaria* sp.) indicator plants (Tanne, 1984). A possible solution is to use *in vitro* cultured pathogen-free plant stocks or tissue culture (Withers, 1988). *In vitro* indexing has several advantages: the test plants are cloned from pathogen-free material; the pathogen is contained; there is an unlimited source of test plants; and *in vitro* cultures of reference pathogens can be maintained. The application of *in vitro* cultured plant material is especially important for vegetatively propagated indicator plants, where introduction of foreign diseases could be passed on until detected [e.g. citrus exocortis viroid testing on citron (*Citrus medica*) and *Xanthomonas ampelina* on vines (*Vitis vinifera*)]. Seedlings can also be inoculated *in vitro* so that seed-borne pathogens are contained. The problems associated with *in vitro* indexing are that sensitive methods are sometimes required to detect pathogens developing *in vitro* and that the titre in culture can be lower than under natural conditions. Also, symptoms may be modified. However, *in vitro* indexing can be simple and give results rapidly. For example, larch (*Larix* sp.) plantlets susceptible to *Gremmeniella abietina* produce diagnostic symptoms in 4 weeks under *in vitro* inoculation compared to 1·5 years when inoculated in the field (Abdul Rahman *et al.*, 1986). Tissue culture bioassay is usually carried out under controlled environmental conditions, thus removing the variation of pathogen concentration due to seasonal variation, and assays can be performed throughout the year. Indexing on tissue-cultured seedlings include tests for citrus exocortis viroid (Navarro, 1981), citrus tristeza (Withers, 1988), citrus canker (Lopez and Navarro, 1981), crown rot of apple and peach (Jeffers and Aldwinckle, 1986), apple scab (*Venturia inaequalis*, Yepes and Aldwinkle, 1986) and *Xanthomonas ampelina* (Lopez, Arregui and Navarro, 1983).

Culture and isolation. Unlike viruses and viroids, many fungi and bacteria can be cultured on nutrient media with ease. For these pathogens, isolation is used routinely for detection, and for some it is the only reliable detection method,

e.g. Panama disease pathogen. Culture methods are sometimes used as standards to which new detection methods are compared, for determination of the viability of pathogens and to identify unknown pathogens.

Biochemical and physiological tests

After isolation, the identification of some unknown pathogens may be achieved by consideration of morphological properties, followed by specific serological or nucleic acid hybridization assays. In some cases involving bacteria and fungi, this is not possible because their morphologies are very similar, or because serological techniques are either not capable of differentiating between pathovars or are not sufficiently reliable. In these cases, other properties such as chemical composition, nutritional requirements, enzymatic reactions, pathogenicity and susceptibility to viruses and antibodies can be used. Many of these tests are rapid and sensitive but require pure cultures of the pathogens. Chemical tests include Gram-staining, and characterization of cell wall peptidoglycans and polysaccharides, cellular fatty acids, proteins and DNA base composition (Joshi and Vidaver, 1984; Miller and Sasser, 1984; Laroche, 1987). Recent advances in high pressure liquid chromatography technology can be used to analyse these biochemicals rapidly (Horie *et al.*, 1988; Webb *et al.*, 1988). Enzymatic reactions include maceration and pectolytic enzyme assays (Lukezic and Levine, 1987) and assays for virus-specific enzymes (Gronowitz and Kallander, 1983). Using a combination of these tests, specific identification of bacterial strains can sometimes be achieved. Alternatively, some strains of bacteria may be identified by the presence of specific plasmids (Lazo and Gabriel, 1987), bacteriophages (Persley, 1983; Cuppels, 1984; Davis *et al.*, 1987; Fujimoto and Vidaver, 1987) or presence or sensitivity to bacteriocins and antibiotics (Vidaver, 1983; Perombelon and Hyman, 1986).

AUTOMATION, COMPUTER TECHNOLOGY AND PORTABILITY

There have been many developments in the production of equipment for use in the analysis and diagnosis of pathogens. They include ELISA readers, bar coders, image analysers, new generations of microtomes and microscopes and various specialized machines. These range from semi-automatic to fully automatic with computerized analysis and interpretation of results. They are designed for laboratories which perform large-scale detection of specific pathogens, especially in the medical field. However, they are now used increasingly in more specific, agriculturally oriented diagnostic laboratories and in research laboratories. They have the advantage of reproducibility and rapid analysis of data. Together with these machines, new techniques to facilitate and streamline large-scale diagnosis have emerged, e.g. automatic bar-coding of samples, image analysis for digital quantitation of nucleic acid hybridization signals (Sullivan *et al.*, 1987; Rulli, 1988), pre-colour-coded tubes, microwave technology for specimen preparation in microscopy (Coats *et al.*, 1987) and fully programable machines for UV cross-linking of nucleic acids

to membranes, which reduces DNA-binding time from 2 hours to 30 sec (Stratagene, La Jolla, USA). Similarly, PCR machines are now available for automation of this new technique in molecular biology.

While these developments allow for the rapid processing of large numbers of samples, they have the disadvantage of increased cost to basic laboratories, especially in developing countries. For this reason, and to ensure quality control and a uniform testing environment, it is likely that plant pathogen testing centres will become more common. Plant extracts could be loaded into microtitre plates for ELISA or onto nucleic acid binding membranes for nucleic acid hybridization, then washed and sent to a centralized testing facility where the assays would be performed. This has the advantage that positive and negative controls need to be maintained at fewer locations. Such a facility has been operating in the USA for several years (Agdia, Inc., Mishawaka, USA).

Future developments

There are many detection techniques available for well-characterized pathogens. Various labelling methods combined with variations of immunological and hybridization procedures have been devised to increase sensitivity and improve specificity of assays. The method of choice depends on the individual pathogen–host combination, sensitivity levels required and the scale of operation. Each assay chosen should be thoroughly tested in each laboratory before being used on a large scale, since climatic variation, sampling time, physiological status of test plants and other factors may influence the sensitivity achieved. When selecting a technique to be used for an assay, sensitivity, specificity and predictive accuracy of the assay should be considered.

Several detection methods may be equally suitable for a given application. Then other criteria such as cost, available resources, facilities and the experience of the user may be the deciding factors. Often, a combination of traditional and molecular detection methods may have to be used. For some applications, such as plant quarantine and certification programmes, biological detection methods may have to be used until molecular methods become acceptable to the regulation bodies that control assay requirements. As molecular methods are adapted for plant pathogen detection, the recent advances should be included as part of the repertoire of assays to be considered.

Given the recent advances in virus detection one might think that most problems have been solved. However, there are still many difficulties with the detection of plant pathogens that must be addressed. Many of the detection methods have been applied to model systems, and this is especially true for bacterial and fungal pathogens. Suitable reagents still need to be developed for many pathogens that are of economic importance. Nucleic acid probes or antisera with various specificities (strain, species and genera) still need to be developed for most plant pathogens. In addition to developing new diagnostic reagents there are still many pathogens that need to be isolated before diagnostic reagents can be developed.

More sensitive and reliable detection methods are still required, or need to

be adapted, for pathogen detection in applications such as plant quarantine, latent infections, certification programmes and detection in vectors. Improvements are also needed in the interpretation of results and the early diagnosis of plant pathogens. Based on the recent advances in the development of novel, non-radioactive and more sensitive detection methods, it is possible to extrapolate and speculate on future developments in these areas. Further advances will depend on continued development and application of new technologies, including material science, electronics, molecular biology and immunology—for example, selection of high-affinity monoclonal antibodies (Yan, Kitano and Ise, 1988; Yokoyama, 1988).

In future there may be an increase in the development of specialized diagnostic centres. They will be computerized, automated and equipped with expert systems for identification of pathogens and interpretation of data (Delhotal, 1987; Buchen-Osmond *et al.*, 1988). These laboratories may conduct research on the development of probes for individual diseases, crop-specific diseases (Peterschmitt, Chatenet and Baudin, 1987) and broad-spectrum pathogenesis-related assays, e.g. proteins and mRNAs specific to infection (Le Minor, Chalon and Veron, 1972; Cornelissen *et al.*, 1986), dsRNA for RNA viruses, general tests for bacteria and fungi based on cell wall constituents, and methods for simultaneous detection of more than one pathogen (Poor, Santa and Sittampalan, 1988). These laboratories would use the most sensitive methods, probably using monoclonal antibodies and non-radioactive nucleic acid hybridization assays, for seed certification and early diagnosis and for special crop–disease situations. Such laboratories could be the source of reference for standard diagnostic assays and RFLP maps for strain differentiation.

In the field of quarantine, strategic research is needed to determine the aetiology of important but still unidentified diseases in many priority crops, e.g. sweet potatoes, in order to develop suitable diagnostic methods.

Studies should be conducted to determine the range of concentration of a pathogen normally present in diseased plants for each pathogen–host combination so that the level of sensitivity of the assay required for reliable detection of the pathogen can be established. This will allow an objective choice of assays that will detect at this level, or research can be concentrated in the development of assays with levels of sensitivity that will satisfactorily identify the pathogen.

New transmission methods and hosts may be required for new unidentified pathogens. Thus improvements in the detection of new viruses will include identification of sensitive broad-spectrum hosts, e.g. *Nicotiana* species. Many present methods rely on non-standardized indicator species. Detection of diseases that are difficult to transmit (e.g. many fruit tree viruses) can be improved by selection of new indicator hosts to decrease the amount of time required before symptoms develop and to increase the sensitivity of the assay. Another approach is to develop and use group-specific assays to identify unknown pathogens, e.g. for ISEM or immunosorbent immunofluorescence (van Vuurde, Ruissen and Vruggink, 1987) one could use a mixture of group-specific antisera to trap the unknown pathogen. After visualization and

determination of the group affiliation based on other properties of the pathogen, one can then systematically decorate the same specimen with more specific antibodies.

A major future thrust in the application of nucleic acid hybridization for routine use is to adapt ELISA technology. The first steps in this direction have been achieved by the use of non-radioactive enzyme-immunological detection methods to visualize the hybrid molecules. Further achievements should be imminent due to recent successes in the use of solution-based sandwich hybridization strategies (Urdea *et al.*, 1988). A possible ELISA–hybridization strategy involving sandwich hybridization is to prepare two probes specific to different parts of the target molecules. One probe (capture probe) is labelled with digoxigenin while the other is labelled with biotin. After hybridization of the probes to the target, the hybrids can be captured onto solid supports (ELISA wells) coated with antibodies to digoxigenin. After washing away unhybridized reporter probes, the bound reporter probes in the hybrids can be detected by a biotin–streptavidin signal detection system.

Conclusions

The sensitivity and specificity of plant pathogen detection has been enhanced greatly with the advent of ELISA and nucleic acid hybridization assays. In many instances, these are now the methods of choice for detection of plant pathogens because they are sensitive, specific, fast and readily adapted to large-scale testing. However, one must be aware of the shortfalls of these methods and they must be used in conjunction with some basic knowledge of the host and pathogen. A positive ELISA or nucleic acid hybridization result in an assay for a plant pathogen does not mean that viable pathogen is present. Also, in vector studies, presence of a pathogen in or on a vector is meaningless if the vector is not capable of transmitting the pathogen (i.e. any aphid feeding on a plant infected with barley yellow dwarf virus will test positive for the virus, but if the aphid is not a vector for this virus, then it does not contribute to the ecology and epidemiology of barley yellow dwarf virus). There continues to be a need for biological testing of plant pathogens to support the data obtained with these newer methods. There is still a need to develop these sensitive methods for the detection of many economically important plant pathogens. If these non-biological detection methods are to be accepted by quarantine and certification regulatory bodies, they must be shown to detect all strains of a pathogen and be as sensitive as the methods used currently.

Improvements in speed, sensitivity and specificity will be adapted for the detection of plant pathogens in the near future. The introduction of various amplification systems for ELISA, better non-radioactive probes and the use of PCR to amplify signals for nucleic acid hybridization as well as novel techniques still in the early stages of development will improve the detection of plant pathogens. Most of these methods are developed for diagnostics in the medical field where cost per test is often not a major factor. To be used widely for the detection of plant pathogens the cost of these newer detection technologies must be comparable to that of the standard double-antibody sandwich ELISA.

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