Session 1

The Scientific Basis of Diagnostic Techniques

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DIAGNOSTICS : OPPORTUNITIES AND NEEDS

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ABSTRACT

Agriculture provides approximately 1.4% of GDP in UK. Approximately 4.5 million hectares of crops are grown, cereals, especially wheat, and oilseed rape occupying over 77% of that total area. In cereals the prime causes of yield loss are the stem base and various leaf spotting diseases; a broadly similar scenario exists with oilseed rape. In potato the primary disease is late blight but a range of other diseases cause blemishing and loss of value. Virtually no attention has been paid at the practical level to root diseases, due primarily to difficulties in identification of their presence/absence or extent in growing crops. Nematodes are a further problem, particularly the different species and strains of potato cyst nematode. Ideally Agriculture has need of a single diagnostic system for each plant species or botanical family that would positively identify which pests or pathogens were present at a "pre-observable level"; this would obviate the prophylactic use of agrochemicals and improve targeting and efficiency of those that were needed, following positive diagnosis. For use in the field such diagnostics need to be cheap, easy to use and to provide clear uncomplicated results, with total reliability.

INTRODUCTION

Agriculture in UK and other member states of the European Union is in a state of transition from tonnage production-based aids to area-based aids and, in the longer term there will probably be a decoupling of aid from production in some radical reform of the Common Agricultural Policy. In parallel with this the agreements from the Uruguay Round of General Agreement on Tariffs and Trade (GATT) and likely developments in the next round of GATT will lead to very substantial reductions in tariffication and that in turn to more free trade. Except in times of shortage caused by unpredictable weather, and pest or disease factors, there will be a downward pressure on prices. Estimates suggest that wheat prices may reach £85/tonne in the first few years of the next millennium. This will have the effect of forcing producers to cut production costs.

At the same time there are and, increasingly so, will be, considerable pressures to improve quality of produce, especially human foodstuffs. The development of 'one-stop' shopping for household foods and an increase in the proportion of foods purchased as partially-prepared or wholly-prepared meals or meal components has concentrated the power to control quality specifications and producer prices into the hands of a very small number of purchasers of primary agricultural and horticultural products.

Furthermore, there is a continuing impetus from consumers and the environment to reduce the levels of agrochemicals of all types that are applied to plants or their products.

These foregoing issues emphasise the need for a highly accurate and timely application of only those crop protection products that are economically essential to crops and which will produce highest quality produce; overpriced or blemished produce has no future in an increasingly competitive marketplace.

Furthermore, in leisure products - houseplants and garden plants - there is a similar need to ensure highest quality pest and disease-free plants to consumer; any weakness on the part of UK to do this will be heavily exploited by Dutch competition in particular.

UK PRODUCTION IN AGRICULTURE AND HORTICULTURE

The contribution from UK agriculture alone to Gross Domestic Product (GDP) from domestic production was almost £7.8 billion in 1994 (MAFF *et al*, 1995), that being 1.4% of total UK GDP.

In the calendar year 1994 UK expenditure on household goods was forecast to be £73.2 billion.

Total land in agriculture and horticulture, excluding common rough grazings was 17.25 million ha of which mainstream crops accounted for approximately 4.5 million ha of area. The horticultural area within that was 240,000 ha with an output value of £1.9 billion in the same period.

Details of individual crops or crop groupings for horticulture are shown in Table 1.

Table 1

Areas of crops in UK		At June of each year		
		Average of 1983-85	1990	1994
Crop areas ('000 hectares)				
Total		5,136	5,013	4,469
This comprises:				
Total cereals		4,005	3,657	3,042
of which:	wheat	1,845	2,013	1,811
	barley	2,029	1,516	1,106
	oats	115	106	109
	rye and mixed corn	14	12	10
	triticale	..	9	6
Other arable crops (excluding potatoes)		719	971	1,073
of which:	oilseed rape	262	390	404
	sugar beet not for stockfeeding	201	194	195
	hops	5	4	3
	peas for harvesting			
	dry and field beans	96	216	228
	linseed (b)	-	34	58
	other crops	155	133	184
Potatoes		195	177	164
Horticulture		207	208	189
of which:	vegetables growing in the open	137	142	127
	orchard fruit	40	34	32
	soft fruit	17	15	13
	ornamentals	12	14	14
	glasshouse crops	2	2	2

The data in this table cover all holdings (including minor holdings) in England and Northern Ireland but exclude minor holdings in Scotland.

Clearly from the foregoing data the market potential for agricultural and horticultural produce is massive and although dynamic and therefore subject to seasonal and market fluctuations, continues to have considerable potential.

PATHOGENS CONSTRAINING PRODUCTION AND QUALITY

Cereals

(a) Wheat

In a survey of stem base diseases and Fusarium ear diseases in winter wheat crops in Great Britain during 1989 and 1990 (Polley & Turner, 1995) it was confirmed that disease presence was widespread. See Tables 2 and 3.

Table 2

Frequencies of winter wheat stems affected by stem base diseases at growth stage 31 classified by symptom category

	Symptom category	Percentage of stems affected 1989	1990
1.	Eyespot	5.9	9.0
2.	Eyespot + *Fusarium*	13.4	6.8
3.	*Fusarium* 1 (leaf sheath - date-brown)	-	10.3
4.	*Fusarium* 2 (leaf sheath - charcoal-grey)	10.5	0.3
5.	*Fusarium* 3 (leaf sheath/blade - charcoal-grey)	2.9	0.8
6.	*Fusarium* 4 (leaf sheath margins - date/brown)	6.2	2.8
7.	Vascular discolouration	14.4	7.6
8.	Sharp eyespot	5.2	7.4
9.	Sharp eyespot + *Fusarium*	4.4	2.6
10.	Small discrete lesions	4.2	0.3
11.	Symptomless	32.8	50.2
	Total number of stems assessed	7275	3850

Source: Polley & Turner, 1995

Table 3

Incidence and severity of stem base diseases and the incidence of *Fusarium* ear diseases at growth stage 73-75

Disease	Percentage of stems or ears affected 1989 Slight	1989 Moderate	1989 Severe	1989 Total	1990 Slight	1990 Moderate	1990 Severe	1990 Total
Stem base diseases								
Eyespot	13.6	9.2	0.4	23.2	19.8	10.9	0.6	31.3
Sharp eyespot	7.2	6.6	0.1	13.9	7.1	4.9	0.1	12.1
Fusarium (internodal)	24.0	13.5	3.1	40.6	22.3	6.4	0.2	28.9
Fusarium (nodal)	-	-	-	18.7	25.2	5.7	0.2	31.1
Ear diseases								
Fusarium ear blight	-	-	-	0.4	-	-	-	0.5
F. poae glume spot	-	-	-	6.2	-	-	-	12.7

Source: Polley & Turner, 1995

However incidence varied from season to season and locality to locality. Moreover, whilst the identification methodologies employed in this research separated diseases out relatively easily and discreetly, such techniques could not readily be employed in the field situation. Clearly definitive laboratory diagnosis is absolute but in terms of cost and time, let alone the logistics of transporting and handling large quantities of plant material, field diagnosis would require more simple and quick methodologies. Financial value of loss caused by these diseases is difficult to quantify but Priestley and Bayles (1988) suggested values between £7 and £9 million per annum for the eyespot alone. Bateman and others (1995) went on to consider sensitivity of W or R-types of *Pseudocercosporella herpotrichoides* to fungicides (eg carbendazim); in practical agriculture the differences in sensitivity of these two types to various fungicides is of major significance, as indeed is the fact that they have different rates of infection of wheat and perhaps different effects upon yield (Goulds & Fitt, 1991).

Polley (personal communication) confirmed the position on foliar disease of wheat in UK in the period 1989-1995. Data are shown at Table 4.

Data recorded on leaf 2 showed the highest disease incidence to be of *Septoria tritici*, with mildew and brown rust being second and third in order of significance respectively.

In 1995 value of foliar disease losses attributable to foliar disease in wheat amounted to £10.4 million with wheat at £110/t, or approximately £12.0 million with wheat at £130/t. These losses are in excess of 33% of total losses in wheat attributable to pathogens.

Table 4

Foliar disease severity on leaf 2

Disease	% disease on leaf 2						
	1989	1990	1991	1992	1993	1994	1995
Septoria tritici	0.7	0.6	4.3	1.2	4.2	1.2	0.8
Septoria nodorum	0.01	0.02	0.1	0.1	0.5	0.3	0.02
Mildew	0.4	0.5	1.1	0.5	0.8	0.4	0.15
Yellow rust	0.9	0.2	0.02	0.0	0.01	0.01	0.0
Brown rust	0.2	0.6	0.1	0.1	0.8	0.1	0.1

Source: Polley (personal communication)

(b) Barley

In a major survey of diseases of spring barley incidence of seven pathogens was reported (Polley *et al*, 1993). Severity of each pathogen varied between sites, seasons and the cultivar examined. Pathogens were, in descending order of incidence:

Mildew *(Erysiphe graminis)*
Rhynchosporium secalis

Brown rust *(Puccinia hordei)*
Yellow rust (*P. striiformis*)
Halo spot *(Selenophoma donacis)*
Septoria spp.
Net blotch *(Pyrenophora teres)*

Estimated mean yield loss per annum from the three major diseases (presuming individual losses to be additive) was 3.2% in England and Wales although the range for individual diseases ranged from 10.8% to 4.2% for mildew, 1.4 to <0.1% for *Rhynchosporium* and 7.7% to 0.3% for brown rust. Obviously diseases like mildew can be expected in every season whereas brown and yellow rust incidence would be occasional, dependent upon weather. The remaining "spot/blotch" diseases are less easy to predict or identify in the field, especially during periods of physiological stress.

Similar studies in 1981-1991 were reported on winter barley (Polley *et al*, 1993). Mildew, net blotch, *Rhynchosporium*, brown rust and halo spot were identified on foliage and sharp eyespot and eyespot on the stem bases. Yield loss estimates are of broad indicative value only because of seasonal and site variations but exceeded 5%.

Oilseed Rape

The predominant acreage of rapeseed is of low erucic/low glucosinolate types and is autumn established. Hardwick *et al* (1991) considered light leaf spot *(Pyrenopeziza brassicae*, *Alternaria* (particularly *A. brassicae*) and stem canker, *Leptosphaeria maculans* to be the predominant disease threats to UK rapeseed production. In a more recent review Hardwick *et al* (1995) reported on disease incidence in rapeseed between 1986 and 1990 and went on to evaluate benefits accruing from use of fungicides; these latter amounted to -£15 to +£58 per ha. In the reported work the great significance of further research on predicting severe epidemics of diseases and rapid diagnostic methods and thereafter early quantification of disease was highlighted. Additionally emphasis was laid upon development of molecular diagnostics to aid in identifying early and latent disease infections.

Sansford and others (1995) confirmed a range of reduction in yield of 0.009-0.98 t/ha for each 1% increase in light leaf spot infection, depending on when the infection occurred on the plant. They also indicated a loss of 0.032 t/ha for each 1% infection with *Sclerotinia sclerotiorum*. With stem canker (*L. maculans*) the timing of infection as well as level of infection were important in determining yield loss.

Potato

The potato crop suffers from a range of pathogens and from potato cyst nematode (PCN). Of the pathogens undoubtedly late blight (*Phytophthora infestans*) of potato is the major problem both in UK and worldwide. This is closely followed by the pest, potato cyst nematode (PCN).

Yield reductions from late blight have been assessed in a range of ADAS experiments and amount to 15-29 t/ha (Bradshaw - Personal communication), depending upon season. Additionally blight infections in potato tubers lead to quality losses through blemishes and,more importantly, to ingress of secondary bacterial soft rots. These latter destroy individual tubers in their entirety and obviously have major deleterious effects upon quality of the remainder. Whilst primarily occurring as asexual types in UK the A2 mating/sexual strain has been identified and, also, some strains of late blight are resistant to the phenylamide group of fungicides. Currently most potato crops are treated as routine with prophylactic treatments against blight. Nonetheless it would be prudent from the industry and primary buyer viewpoints to reduce fungicide usage. However, to accommodate that change clear indications of the presence of blight spores and their type at the very initiation of an infection, allied with reliable prediction of conditions conducive to blight spread are pre-requisites.

PCN poses different problems : both *Globodera pallida* and *G. rostochiensis* occur in UK. Ideally control of each will revolve around integration of cultivar resistance to and tolerance of PCN with rotation and nematicides. This would be most easily facilitated by rapid and cheap identification techniques for the species and for its distribution in the field. It is accepted however that these aspirations may take some time to be achieved.

In addition to the two primary areas of current application of diagnostics in the potato, namely, late blight and potato cyst nematode, there are a number of tuber blemishing diseases which are of increasing significance even though they do not have major direct effects upon yield. It is important to recognise that by the year 2000 AD approximately 75% of UK potato production, equivalent to 4.5 to 5 million tonnes of tubers per annum, will be sold through major supermarkets. In this situation, where tubers are pre-washed and sold in transparent packaging or clear plastic bags the significance of appearance is paramount : potatoes of poor appearance will not be selected by the consumer.

Hence diseases like silver scurf *(Helminthosporium solani)* and black dot (*Colletotrichum coccodes)* need to be monitored and controlled. Similarly the scabbing diseases *(Streptomyces scabies* and *Spongospora subterranea)* and in future, because of likely changes in storage practice, gangrene *(Phoma exigua)* and skin spot *Polyscytalum pustulans)* will need identification and prevention or treatment. Diagnostics could help.

The "Unknown" Diseases

Whilst foliar, stem base, tuber or fruit diseases are relatively easy to notice and identify, the position of the various pests or pathogens affecting roots has not been fully elucidated and in terms of application of knowledge to control such problems at the practical agricultural level, much needs to be done. Undoubtedly root pruning diseases like *Rhizoctonia* are already known to have adverse effects on yield and quality of, for example, the potato crop. However in terms of nutrient exchange between the root system and the soil and in terms of effects upon moisture uptake and subsequent effects upon physiological efficiency, the practical agriculturist has much to learn but, regrettably, even when learned has few acceptable tools for diagnosis of the problem or its treatment.

It is a challenge to diagnostics science and to those involved in producing control agents for pathogens involved in restricting efficiency of root systems to resolve these problems.

Similarly, the use of diagnostics to prove the suitability of inputs (especially water) to irrigate agriculture and particularly horticulture provides further areas for investigation.

WHAT DOES AGRICULTURE WANT FROM DIAGNOSTICS?

The role of Agriculture in so far as crops are concerned is to manipulate various plant species to produce maximum economic yield with minimal unit cost of production by assembling together all the knowledge and expertise that would give that particular plant species unrestricted opportunity. Regrettably restrictions caused by pests and pathogens are often not discernible in the field or greenhouse situation until such times as they are clearly creating losses in yield and/or quality. Often those losses are irretrievable. Hence diagnostics must provide earliest possible warning of the presence of an organism capable of producing economic yield loss and a precise identification of the organism itself. Thereafter plant protection technologies, perhaps in future allied to artificial intelligence, will offer strategies for control and identifying economic responses.

In practice diagnostics must be cheap, easy to use and foolproof in the practical field situation. Nevertheless it is accepted that research may have different criteria upon which to judge needs from diagnostics.

APPLICATION OF DIAGNOSTICS

From the foregoing paragraphs the following potential uses for diagnostics have been identified:

Identification - of primary organism - eg *Septoria*
- species/subspecies
- pathotype - eg PCN

Identification - of presence of primary infection at very low infection levels (ie presymptomatic expression) - eg late blight

The availability of cheap, easy to use and totally dependable diagnostics for field use would provide ideal tools to improve economics of crop production, reduce unnecessary use of pesticides, improve food quality and safety, and protect the total agriculture/horticulture environment. Additionally, as diagnostic methodologies became cheaper so sampling intensity could increase such that in some instances (eg PCN) patch spraying methods could be used for control. This would further reduce costs of inputs and reduce environmental pressures on agrochemicals.

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SEROLOGICAL METHODS IN CROP PROTECTION

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ABSTRACT

Antibody based, or serological, methods have made a big impact in many areas of crop protection since the late 1970s. Adoption of the technology has led to improvements in the identification, diagnosis and quantification of plant pests and pathogens as well as in the determination of pesticide residues. However progress in the use of serological methods has not been uniform across different disciplines and this paper aims to explore the background, advantages and limitations of immuno-diagnostic methods by particular reference to work carried out in our laboratory over the past ten or so years.

IMMUNOASSAYS

Antibody production

Diagnostic antibodies can be derived from three basic routes, two of which (the production of polyclonal antisera and monoclonal antibodies) have been reviewed by Harlow and Lane (1988). Recent advances in the production of antibody fragments by recombinant molecular genetic means are discussed in a subsequent paper. Polyclonal antisera are produced by immunising animals, typically rabbits, with selected immunogens and subsequently collecting blood samples (or eggs in the case of laying immunised chickens) after suitable time periods. Other animals such as sheep and goats have also been used, particularly if large volumes of antisera are required. Blood samples are allowed to clot and serum can be simply decanted off and may be used whole or can be purified to yield antibody containing fractions, depending on the required use.

A desire to produce more defined reagents led to the development of methods for the production of monoclonal antibodies opening up many new possibilities in immunodiagnostics. The technique involves fusing antibody secreting lymphocytes, obtained from the spleens of immunised mice or rats, with cultured myeloma cells. The resultant hybrid cells (hybridomas) can be selected for and share both the antibody secreting properties of their parent cell-lines. Individual hybridoma cell lines, derived from a single cell, by dilution plating, which have the ability to be grown in cell-culture can then be established. Those clonal cell-lines that retain the ability to secrete antibody will produce only a single (monoclonal) antibody which can be harvested from the culture medium. A number of schemes have been devised to produce and then purify monoclonal antibodies in large amounts and include both culture and *in vivo* (ascites) methods.

Assay formats

A bewildering array of immunoassay formats are available for use in crop protection and they can be classified in many different ways according to various criteria. Reviews of many of the options and protocols for use can be found in Hampton *et al.*, (1990) and Harlow and Lane (1988). Firstly, assays may be performed in a liquid phase or on one of the many solid phases now available. Liquid phase reactions are most commonly carried out in a gel such as agar and rely on visualisation of rings or bands of precipitated antigen/antibody complexes. In single diffusion techniques, one reactant (usually the antigen or test extract) diffuses out from a central well into an agar medium containing the other reactant (usually the antibody) resulting in rings of precipitin around the test well. In double diffusion techniques, such as the well known Ouchterlony method, antibody and antigen diffuse towards each other from separate wells punched out from the agar resulting in bands or lines between the wells. In crop protection these methods have been principally used in plant virology and particularly for determining degrees of relatedness between plant viruses.

There are now many different solid phases available on which immunoassays may be performed each with their own advantages and limitations. Agglutination assays, in which beads, particles or cells are coated with antibodies and added to test solutions provide very rapid and simple methods for the detection of multi-valent antigens. A positive reaction results in the visible aggregation and clumping of cross-linked complexes of particles and antigen, often within seconds or minutes. Agglutination assays may be performed using sensitised latex and other beads, red blood cells and bacterial cells and are often carried out on glass slides or simple cards. The recent development and adoption of brilliantly coloured latex particles has made visualisation of the reaction products much easier. Such assays are particularly suited for the rapid non-quantitative detection and identification of plant viruses and bacteria in small sample numbers and can be performed in the field.

Other solid phases in use include nitrocellulose membranes and microtitre wells, diazotised paper, membranes of other materials and various beads and particles. Linkage of antibodies may be through covalent or non-covalent binding and each of the different materials are often available in various forms. However the most commonly used solid phases are the widely used polystyrene or polyvinylchloride microtitre plates configured in the familiar eight by twelve well format (also available in strips of eight or twelve wells).

Numerous other assay formats have been devised that utilise solid phase supports and the references already mentioned should be consulted for further detail. These formats include dot immunobinding or immunoblotting assays (DIBA), western blotting, radio-immunoassay (RIA), immunofluorescence microscopy, immune-electron microscopy (IEM) and immuno-gold electron microscopy. Other more specialised immunoassay formats also exist and include more recently developed clinical methods such as time resolved fluoroimmunoassays and others which have yet to be widely utilised within crop protection. However, the most widely used and significant immunoassay format is the enzyme-linked immunosorbent assay or ELISA which is described more fully below.

ELISA based assays utilise enzyme labelled antibodies, antigens or secondary reagents as a detection system and immunoassays may also be classified on the basis of the detection system

adopted. Colorimetric detection of immunological reactions can be achieved by enzyme labelled reagents yielding coloured soluble products in microtitre wells or precipitation of insoluble but coloured products onto membrane surfaces. Alternatively, probing membrane bound reactants with reagents such as antibody labelled colloidal gold particles also provides a colorimetric end-point for dot and western blots. Other detection systems include radioactivity, biotinylation and the use of fluorochromes.

Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assays can be split into the two basic groups, non-competitive sandwich assays and competitive assays. In general, the former type is used for the detection of plant pests and pathogens and the latter for small molecules (pesticides and mycotoxins) within the crop protection sphere although not exclusively so. Non-competitive sandwich assays can be further sub-divided into direct and indirect systems depending on whether the specific detecting antibody is itself labelled with an ezyme (direct) or whether a secondary system such as an enzyme labelled anti-antibody reagent is used to detect the specific antibody (indirect). In general, antibodies demonstrate a higher degree of specificity in direct ELISA systems than in indirect ones (Van Regenmortel and Dubs, 1993). This last fact demonstrates one important characteristic of ELISA that the response of the assay for a given antibody or serum can be markedly affected by the choice of assay format. Indeed some, particularly monoclonal antibody, reagents will actually not work at all in some assay formats. Thus it is a common experience that some monoclonal antibodies work well in ELISA but will not work in western blotting.

One of the most common ELISA formats, widely used in plant virology, is known as the double antibody sandwich ELISA (DAS-ELISA). In this direct assay, antibodies are coated onto the surface of plastic microtitre plate wells and unbound reagent washed out. Test extracts are then added and incubated for a suitable period of time (2-4 hours) and any unbound material is again washed out of the wells. The third step is to add an enzyme labelled specific antibody conjugate which will bind to any target antigen bound by the capture antibody. The enzyme conjugated antibody may or may not be the same antibody as was used to coat the plate but must react to separate or repeating components (epitopes) of the target to allow a "sandwich" to be developed. Any unbound enzyme antibody conjugate can then be washed away and any residual enzyme, indicative of bound target and hence a positive reaction, can be detected by addition of a suitable substrate and any coloured product formed can be read spectrophotometrically or detected by eye. Reactions may either be performed as end-point assays ie stopped and read after a fixed point in time or the initial rate of reaction can be measured using a kinetic plate reading system. A number of different enzymes have been used in ELISA with alkaline phosphatase and horse-radish peroxidase being the most commonly encountered. A number of variations from the basic DAS-ELISA theme have been developed each with their own uses, advantages and disadvantages.

It is possible to omit the coating antibody stage and to coat the ELISA well with the test extract directly which is referred to as a plate trapped antigen (PTA-ELISA) assay. The target antigens can then be detected using a direct or indirect system. A third variant is to use an indirect detection system and a trapping antibody to give a four-step ELISA system. In a typical example, plates are coated with antibodies from one species (eg rabbit) and the bound

target antigen detected with antibodies, commonly monoclonal, of a second species (eg mouse). The second antibody can then be detected by addition of an enzyme conjugated anti-mouse immunoglobulin antibody which will not react with the coating or trapping antibody layer. Such an assay is known as an triple-antibody sandwich ELISA (TAS-ELISA) and its uses and merits are discussed below. Many other non-competitive ELISA formats have been devised and are described in the references quoted.

Competitive ELISA assays are commonly carried out in two modes. The first example is known as an antigen capture ELISA in which a known amount of labelled antigen is mixed with the test sample, containing an unknown amount of target antigen, and the mixture added to an ELISA well coated with a specific antibody. Any target antigen in the test sample will compete with the (enzyme) labelled antigen for the coating antibody resulting in less bound label remaining in the well, following the washing away of unbound reactants. A variation of this assay (antibody capture assay) is to coat the ELISA well surface with a sample of pure target antigen and to add a mixture of the test solution (containing an unknown amount of target antigen) and a constant amount of specific antibody. Any antigen in the test solution will inhibit the binding of the specific antibody to the coating (solid phase bound) antigen and the amount of bound antibody remaining in the well after washing can be determined either directly, if an enzyme labelled antibody was used, or indirectly using a secondary reagent such as an enzyme labelled anti-species antibody. In both competitive assays described, increased amounts of target antigen result in less bound label remaining in the ELISA well and a consequent and proportional reduction of colour in the well (in the case of colorimetric enzyme assays). In the case of sandwich assays the opposite is true, i.e. the amount of colour in the well is proportional to the amount of antigen present in the test sample. In both cases, standard curves based on a dilution series of pure antigen (if available) can be prepared and the assay made quantitative.

Limits of detection in ELISA vary greatly but levels of 1-10 $ng.ml^{-1}$ can often be achieved. Amplification of the signal can also be carried out by a variety of means, such as the use of chemiluminescent enzyme substrates or by substrate cycling reactions (Hampton *et al.*, 1990) which can yield a further increase in the limit of detection. Automation of ELISA is relatively straightforward, with suitable equipment widely available, permitting large sample throughputs and low costs per sample.

APPLICATIONS

Plant viruses

The impact of immunodiagnostic methods has had a far greater impact on plant virology than perhaps any other discipline in plant pathology and crop protection. The widespread development and adoption of ELISA in particular has greatly facilitated routine virus testing by reducing test times and permitting large scale surveys and sampling programmes. The impact on certification schemes for healthy seeds and other planting material has been particularly marked. Thus Dutch inspection services laboratories are able to carry out ELISA testing of upwards of four million samples annually in support of their seed potato certification scheme and the virus indexing of top-fruit propagation material can often be accomplished by a

two day ELISA test in comparison with grafting techniques which took months or even years to carry out. Good reviews of the use of immunoassays in plant virus diagrams can be found in Clark and Bar-Joseph (1984), Hampton *et al.*, (1990) and Van Regenmortel and Dubs (1993).

Four reasons can be put forward to explain the success of immunoassay technology in plant virology when compared with other areas of crop protection. Firstly, workers have been able to borrow and refine techniques from the extensive clinical virology world and secondly the technology itself, particularly ELISA, lends itself to large scale relatively low cost screening exercises of the kind required for the improvement of plant health. Thirdly, virus particles themselves are very simple structures consisting as they do of only one or at most a few, often immunogenic, protein species. Lastly and most importantly, plant virus taxonomy itself was partly erected on the basis of immunological cross-reactivity and thus by definition, immunoassays can be developed for the diagnosis and detection of these viruses. For the same reason it has also been possible to develop further immunological reagents, such as monoclonal antibodies, and develop diagnostic assays that can work within existing and accepted taxonomies.

Monoclonal antibodies (MAbs) have also been widely adopted in plant virus immunodiagnostics (Torrance, 1995) for a number of reasons. Firstly it has been possible to develop MAbs that discriminate closely related virus strains, such as those that are defined by vector specificity or host range, which has permitted detailed epidemiological studies of these viruses. Thus it is possible to identify strains of barley yellow dwarf virus transmitted by different aphid species (Barker, 1990) and between host adapted strains of beet mild yellows virus (Smith *et al.*, 1996). A second reason for the widespread adoption of MAbs in plant virus diagnosis relates to the production of plant virus antisera and MAbs. Plant viruses, being obligate parasites, need to be purified from infected plant material prior to immunising animals. Considerable effort has been expended on the development of virus purification techniques with some viruses being much more difficult than others to purify. Inevitably, virus preparations contain some plant proteins as contaminants which raise anti-plant antibodies following immunisation. Assays relying on polyclonal antisera for their specificity exhibit varying degrees of response to plant sap proteins to give a "background signal", depending on the purity of the original virus preparation, which can make interpretation of test results problematic.

The use of monoclonal antibodies eliminates these background reactions and the availability in perpetuity of the reagent obviates the need for further laborious virus purifications, previously necessary when finite supplies of polyclonal antisera ran out. However it is not always easy to develop entirely MAb based sandwich assays for plant viruses, for a number of reasons, and in our laboratory, we have standardised on an indirect two species TAS-ELISA for routine plant virus diagnosis where suitable MAbs are available. Thus relatively poor quality rabbit polyclonal antisera can be used to coat ELISA wells and rat or mouse MAbs used to generate the required specificity in the assay. The use of commercially available enzyme labelled anti-rat or anti-mouse secondary antibody also provides cost-effective continuity of supply of uniform high quality reagents for detection and eliminates the need to produce the enzyme antibody conjugates required by direct ELISA systems. More recently, we have demonstrated that some of the assay components may be combined in so-called "cocktail-ELISA" without

loss of sensitivity and thus can also reduce the number of steps in the test procedure. It is important however that considerable effort is taken in the screening of potential new MAbs to ensure that diagnostic reagents will exhibit the degree of specificity required and in particular do not "miss" strains of a virus if specificity at the virus level is required.

Some viruses still remain extremely difficult if not impossible to purify and immunological reagents remain unavailable. The molecular expression of plant viral proteins in bacterial or other vectors and the production of antibodies to isolated preparations of the expressed viral proteins might provide and alternative route, as was demonstrated by Nikolaeva *et al.*, (1985) for citrus tristeza virus. Amplification methods for plant virus ELISA systems have also extended the usefulness of the technique by increasing the limits of detection. It is now possible to detect the presence some viruses in individual vector insects (Torrance, 1987) which has facilitated epidemiological and disease forecasting studies. In routine use, substrate cycling amplification appears to yield between a four and sixteen-fold increase in sensitivity when compared with an indirect TAS-ELISA (Henry *et al.*, 1992 and this publication). Recent parallel development of other solid phase immunoassays such as nylon-membrane based and latex agglutination assays for plant viruses has also been noticeable. Both assay formats are suited to field sampling and field testing and in our laboratory are being used to facilitate pre-harvest field sampling of potato tissue in support of seed potato production (Barker *et al.*, 1992).

Fungi and bacteria

Good reviews of the use of immunodiagnostics for the detection and diagnosis of fungal plant pathogens can be found in Dewey and Thornton (1995) and protocols for use can be found in Schots *et al.*, (1994 : see Barket *et al.*,1994). Immunodiagnostics methods for bacterial plant pathogens are covered in Hampton *et al.*, (1990). Fungal immunodiagnostics are also the subject of as paper by Dewey elsewhere in this publication but a few general points are relevant here. The adoption of immunodiagnostic methods by fungal plant pathologists has in no way been as marked as in viral plant pathology, with publications on the development of fungal immunodiagnostics far outweighing those describing the successful adoption and routine use of such methods. A number of reasons can be put forward to explain this difference. Firstly fungal plant pathogens are clearly hugely more complicated targets than plant viruses and complications such as the differential detection of different life stages become important. Secondly the field of clinical mycology is much smaller than clinical virology and thirdly fungal plant diagnosticians are perhaps not trained in biochemical methods. Lastly and most importantly, the taxonomy of fungal plant pathogens is largely based, for the good reason that they can be easily observed in culture and under the light microscope, on morphological features rather than biochemical ones. For this reason it is perhaps not surprising that it has proved difficult to generate diagnostic antibodies that match existing fungal taxonomies particularly where different taxa are not clearly defined and intermediate forms exist.

In particular, we have found it very important when developing immunoassays for fungal plant pathogens that the taxa to be delineated fall into natural and clearly distinct groups and that taxonomists with a "feel" for the groups involved are consulted at the outset. Fungal taxonomy is also rapidly evolving and the planning of diagnostic R&D needs to take account of current thinking on the taxa involved. Thus we have successfully raised species-specific

MAbs to *Colletotrichum acutatum*, the cause of strawberry blackspot disease and a quarantine organism for the UK, which is morphologically very similar to other *Colletotrichum* species but regarded by experts as a discrete and "natural" species (Barker *et al.*, 1994; Cook *et al.*, 1995). It became evident during this work that the MAbs raised to *C. acutatum* were specific to the conidia of the fungus which highlights one potential problem with fungal immunodiagnostics which is that stage-specific antibodies may be developed that only recognise part of the life-cycle. For this reason, immunoassays for the strawberry blackspot pathogen require a "bio-amplification" step, needed to trigger sporulation in infected tissue, prior to detection by ELISA. A MAb based ELISA protocol has now been developed for the routine detection of *C. acutatum* in strawberry planting material by Hughes and Lane (unpublished) in our laboratory. This ELISA method replaces the need for laborious examination of each strawberry petiole under the dissecting microscope and provides a positive identification of the sporulating organism. The immunoassay has proved to be reliable in extensive validation trials and has been successfully used in 1995 to carry out a nationwide survey of 250 samples each of 300 strawberry petioles tested as bulked sub-samples of 50 petioles. There is also evidence that the ELISA is capable of reducing the total test time from six to three days and recent evidence (Cook *et al.*, 1995) demonstrated that it might be possible to produce diagnostic MAbs capable of recognising mycelial structures. The development and adoption of the strawberry blackspot immunoassay has however demonstrated the potential of ELISA in routine fungal plant pathogen diagnosis but it should be pointed out that the whole exercise took several years to complete from initial development to validation and eventual deployment. Work in our laboratory has also demonstrated that fungally directed MAbs can be generated and screened that demonstrate very broad specificity to a wide range of fungi and have been developed for the purpose of detecting moulding of food and feedstuffs (Banks *et al.*, 1994).

The use of immunodiagnostics in plant bacteriology is also covered elsewhere in this publication and is described in Hampton *et al.*, (1990). Suffice to say that immunoassays are widely and successfully used, particularly immunoflourescence techniques, but problems remain in generating antibodies of the required specificity for diagnostic use for some taxa.

Invertebrates

Progress has been made in the development of immunoassays for the detection and identification of insect pests although actual applications are so far limited. Thus Chen and Kitto (1993) developed a species-specific immunoassay for the detection and identification of the grain weevil, *Sitophilus granarius*, in wheat and likewise, Stuart *et al.*, (1994) have developed an ELISA method for another storage pest, the beetle *Trogoderma granarium*. Immunoassays have also been successfully used to replace bioassays in the determination of the insecticide resistance status of some insect pests. Thus Devonshire *et al.*, (1986) developed an immunoassay for the esterase conferring insecticide resistance in the aphid *Myzus persicae*. Similarly, Trowell *et al.*, (1995) have developed and employed an immunoassay, capable of identifying eggs or larvae of insecticide resistant and susceptible species of *Helicoverpa* spp., for the management of pesticides in cotton crops. The development of diagnostics for plant parasitic nematodes is covered elsewhere in this publication but the work of Robinson *et al.*, (1993) in the development of MAbs for the differentiation of potato cyst nematodes is typical.

Pesticides and mycotoxins

The application of immunodiagnostics for pesticide and mycotoxin determination is covered elsewhere in this publication and has been reviewed by Weiler (1990) and Gee *et al.*, (1995). Considerable effort is expended on the detection of pesticide residues in the food chain and in the environment for regulatory and other purposes. Analysis often involves costly methods such as GC-MS and individual analyses may cost tens to hundreds of pounds per sample. The adoption of immunoassay methods for the analysis of small molecules in clinical settings sparked off considerable interest in their potential role in pesticide analysis.

Molecules with molecular weights less than about 1000 are not normally immunogenic but some are said to be haptenic in that they have the ability to bind to an antibody but can not induce an immune response. Antibodies to haptens are generated by coupling the target molecule to a carrier, such as a protein, and immunising animals with the resultant conjugate. Antibodies and immunoassays have been successfully raised and developed to a wide range of pesticides using simple sample preparation methods and exhibiting sensitivities equivalent to or surpassing conventional methods. It is perhaps surprising that the take-up of the technology has perhaps been slower than many people expected. A number of reasons can be put forward to explain this. Firstly, much of the effort currently expended on pesticide residue analysis depends on multi-residue methods capable of the simultaneous detection of a wide range of target analytes in unknown samples. ELISA type methods are much more suited for survey type work for individual specific analytes though indeed work of this type is carried out by regulatory authorities. A second problem lies in that antibodies generated to pesticides often cross-react with analogues and often show a differential response. This leads to problems in interpretation of results when there is a possibility of more than one related compound being present in a test sample. Another common problem with quantitative immunoassays for pesticides is that they may have to be used on a wide variety of foodstuffs etc and matrix effects often complicate analysis and extensive validation is required to overcome these effects. Lastly, in reality, the cost of commercially available pesticide immunoassay kits is not insignificant and presumably reflects the high development costs involved.

However it is clear that the technology has a growing role to play in pesticide (and mycotoxin) residue analysis but it is likely that immunoassays will simply fit into the array of methods available to the analyst as appropriate. Some of the areas that ought to be suited for immunoassay uptake are listed in Table 1.

COMMERCIAL AVAILABILITY

Immunoassay kits whether suitable for laboratory or field use are available from a growing number of commercial sources (Anon, 1990). Antibodies to over 100 different plant viruses are currently available including one latex agglutination kit suitable for field testing of potato virus Y. Kits for fungal and bacterial plant pathogens are also commercially available including field test kits for fungal turf-grass and horticultural pathogen diagnosis as well as test kits aimed at providing a more rational approach to the use of fungicides on major arable crops. Similarly kits for pesticides and mycotoxins are becoming widely available from a

Table 1. Typical key areas for pesticide analysis by immunoassay

1.	Analysis of compounds " difficult" by conventional methods.
2.	Residue data collection in support of pesticide registration.
3.	Pre-screening of test samples to eliminate negative samples prior to conventional confirmation of the positives.
4.	Large scale monitoring and survey work for specific compounds
5.	Affinity chromatographic, alternative, sample clean-up methods.
6.	Monitoring residue levels in growing crops, soils and premises as a management tool for growers.
7.	R&D on the fate of pesticides in the environment

number of companies. However it should be remembered that the development of immunoassays is expensive and the potential market is highly fragmented including as it does some 800 plant viruses, 3000 plant pathogenic fungi and perhaps 1000 commonly used pesticide active ingredients. Thus it is likely that kits and particularly field test-kits will only ever become commercially available for the most important pathogens, pests and analytes.

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USE OF MOLECULAR TECHNIQUES FOR THE DETECTION AND DIAGNOSIS OF PLANT PATHOGENS

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ABSTRACT

Considerable progress has been made over the last few years in the development of DNA-based techniques for detection of microorganisms. Identification of plant pathogens involving extraction of nucleic acid and comparison by RFLP and RAPD PCR analysis is now, in many cases a routine procedure. Of great value to plant pathologists is the development of PCR tests which allow detection of very low levels of pathogen in DNA extracted from host plants or soil. This paper describes the basic procedures for DNA-based identification of pathogens and gives examples where appropriate.

NUCLEIC ACID EXTRACTION

Good quality high molecular weight nucleic acid is desirable for most of the procedures outlined in this paper. Fortunately, there are now many published extraction protocols available. For fungal DNA extraction from pure culture, the method described by Raeder & Broda (1985) is possibly the most widely used. DNA is extracted from rapidly growing 3-5 day old liquid cultures following filtration and freezing in foil envelopes in liquid nitrogen. After freezing, the mycelium is pulverised prior to lyophilization. For large scale total DNA extractions, 300 mg of freeze-dried mycelial powder is resuspended in lysis buffer and extracted as described. DNA extracted in this way is suitable for RFLP analysis.

Mitochondrial DNA can be purified from total nucleic acid extractions using *bis*-benzimide caesium chloride gradients centrifugation. An assessment of quality and yield of nucleic acid can be made following electrophoresis on agarose gels.

For PCR procedures, extraction of DNA from small quantities of mycelium scraped from the surface of agar plates is often adequate. However increasingly, PCR diagnostic techniques require the extraction of pathogen nucleic acid directly from infected plant material or soil. Various techniques have been described that vary in their complexity depending on the tractability of the starting material. One of the major problems encountered during preparation of nucleic acid for PCR is the co-purification of inhibitors, often thought to be polyphenolic compounds.

At their simplest, extraction procedures can involve single tube procedures such as heating a small amount of plant tissue in 100 mM Tris-HCL pH 7.4 or 8.4, 1M KCL and 10 mM EDTA for 10 minutes at 95°C and using the resulting supernatant for enzymic amplification (Thomson & Dietzgen, 1995). This method is suitable for the detection of both DNA and RNA viruses in a variety of plant species.

Direct extraction of DNA from soil has also been successful for detection of *Verticillium dahliae* (Volossiouk *et al*., 1995). In this method, soil organisms are disrupted by grinding soil in liquid nitrogen making use of the natural abrasives present in the soil. Addition of skimmed milk powder reduces degradation and adsorption of DNA to soil particles. DNA is then extracted from disrupted cells using sodium dodecyl sulphate-phenol and collected by ethanol precipitation prior to dilution and use in PCR.

Plant material with a high polyphenolic content requires an extended purification protocol. Detection of *Cylindrocarpon heteronema* in apple wood has only been made possible following passage of the purified DNA through a Qiagen (Diagen) column to remove inhibitors (Brown *et al.,* 1993). Similar treatment was required for extraction of pathogen DNA from *Colletotrichum acutatum* infected strawberry plants (Sreenivasaprasad *et al.,* 1996).

Another novel and effective clean-up method involves the use of magnetic beads where a single-stranded DNA probe, which is complementary to an internal part of the target gene, is used to coat magnetic beads. Following hybridisation in a suspension of soil DNA, magnetic extraction of the beads separated the hybrid DNA from all other soil DNA, humic acids and other interfering soil components. Captured DNA was then used as template for PCR (Jacobsen, 1995).

POLYMERASE CHAIN REACTION (PCR)

Amplification of DNA fragments diagnosing sickle cell anaemia was described by Saiki and co-workers in 1985. The basic method relied on the use of a thermo-stable DNA polymerase isolated from a thermophilic bacterium *Thermus aquaticus* and consequently called *Taq* polymerase.

PCR has found a wide range of applications including detection of human pathogens such as human immunodeficiency virus (Ou *et al.*, 1988), enteroviruses (Rotbart, 1990), fungi in clinical specimens (Hopfer *et al*., 1993) and detection of environmental organisms such as coliform bacteria (Bej *et al*., 1990) and *Legionella pneumophila* (Starnbach *et al*., 1989). In recent years the technique has also been successfully applied to a rapidly expanding list of plant pathogens covering fungi, bacteria, viruses and mycoplasmas.

Essential to the development of a successful species or strain specific PCR test is the identification of DNA sequences that are unique or characteristic for those species or strains.

Successful amplification of DNA fragments is dependent upon a range of specific requirements and reaction conditions.

DNA containing 'target' sequences to be amplified is isolated from an appropriate source as indicated above. The quantity of DNA is often less important than quality as the PCR technique is extremely sensitive, often detecting as little as femtogram quantities of DNA. This is obviously critically important when attempting to detect plant pathogens where pathogen DNA may only represent a very small proportion of the total nucleic acid extracted from the source material and the 'target' DNA within the pathogen may only be, for example 200-300

base pairs from a genome of perhaps 5 x 10^7 base pairs. However, the technique is also very sensitive to unidentified inhibitors which can co-purify during DNA extraction from various sources. These inhibitors can, at worst, prevent any amplification during the PCR and often reduce the sensitivity of the test. For this reason it is essential to understand the inhibitor characteristics of the host plant or source material before developing a PCR based diagnostic test.

Oligonucleotide primers, usually in the size range of 10-25 bases and complementary in sequence to the flanking regions of the target DNA to be amplified, are required. The length of the oligonucleotide primers is important as this can affect both the sensitivity and specificity of the reaction. Shorter primers are more likely to anneal to DNA in a non-specific manner thereby leading to amplification of unwanted fragments. If two primers are used, which is normally the case with species-specific PCR, care should be taken to ensure that the theoretical annealing temperatures (2°C for each A and T; 4°C for G and C) are similar for each primer.

The reaction mixture (100 µl), in addition to target DNA (approximately 20 - 50 ng) and oligonucleotide primers (0.4 µM) requires deoxynucleotide triphosphates (200 µM of each) 10 µl of *Taq* x 10 buffer and 2.5 units of *Taq* DNA polymerase (or appropriate quantity of another thermostable polymerase).

The procedure for a typical PCR is outlined in Figure 1. Double-stranded DNA, containing target sequences, is heat denatured at 94°C (c. 1.5 minutes) thereby separating the two strands. The reaction temperature is lowered to a suitable level (30-72°C; c. 2 minutes) to allow annealing of the oligonucleotide primers to the single-stranded template and then raised to 72°C (c. 3 minutes) for synthesis of new strands of DNA to occur. At the end of one thermal-cycle, the quantity of target DNA has doubled. The process is repeated, usually between 25-40 times with the quantity of DNA increasing exponentially until either the reaction mixture components become limiting or the *Taq* polymerase is denatured.

Analysis of the PCR amplified products is usually achieved by separation of fragments on an agarose gel followed by visualisation after staining with ethidium bromide. PCR products can also be sequenced directly. This gives very detailed information on the identity of the target organism but is not really feasible other than for economically important samples or where sample numbers are very low.

Applications

a) The internal transcribed spacer regions of the rDNA gene block

The nuclear ribosomal gene unit (rDNA) is universally found in living cells and contains both highly conserved genes and also variable regions (Figure 2). It is also present in high copy number but is thought, in most cases, to behave like a single gene in that all copies of the repeat unit within an organism have the same DNA sequence.

Many workers have now exploited the conserved sequences of the 18S, 5.8S and 28S using primers designed by White *et al.*, (1990) to amplify the variable internal transcribed spacer regions 1 and 2 (ITS1 and 2). Analysis of the sequences of ITS1 and ITS2 have often identified regions where PCR primers can be designed that may be genus, species and, in some

Target DNA

Heat denature
(94 °C)

Repeat
cycle
(25 - 40
times)

Anneal primers
(30 - 72° C)

Synthesize new
DNA (72° C)

Fig 1. Amplication of DNA by the polymerase chain reaction

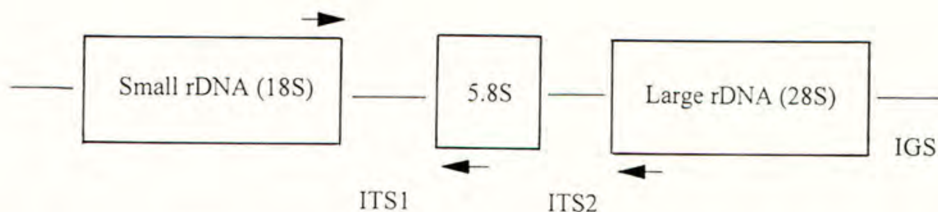

Fig 2. Diagram of the ribosomal DNA repeat unit ITS1 and ITS2 = Internal transcribed spacer region 1 and 2. IGS = Intergenic spacer → indicate commonly used universal primer site.

cases, strain specific. Based on this principle, tests now exist for a range of fungal pathogens including *Cylindrocarpon heteronema* (Brown *et al.*, 1993), *Colletotrichum acutatum* (Sreenivasaprasad *et al.*, 1996). *Mycosphaerella fijiensis* and *M. musicola* (Johanson & Jeger, 1993), *Leptosphaeria korrae* (Ogorman *et al.*, 1994), *Stagonospora nodorum* and *Septoria tritici* (Beck & Ligon, 1995).

The same principle has been used to develop diagnostic tests for mycoplasmas. Based on the 16S rRNA gene specific primers have been synthesised and used to detect peach X-disease (Lee *et al.*, 1994) and three mycoplasma-like organism in infected plants and also, interestingly, in their insect vectors (Namba *et al.*, 1993).

b) Restriction analysis of amplified product

Discrimination between pathogens has also been achieved by amplification of regions of the rDNA gene block followed by restriction enzyme digestion of the product (Cubeta *et al.*, 1991; Chen *et al.*, 1992; Ward, 1993). The advantage of this method is that no prior knowledge of the target organism is required as amplification of DNA fragments is achieved using universal primers (White *et al.*, 1990). The disadvantage is that variation within the sequence of the amplified product has to be detected by use of a range of restriction enzymes recognising four base sites. This can result in a cruder, less well targeted test.

c) Non-repetitive target sequences

Species-specific PCR tests have also been developed based on non-ribosomal DNA sequences. In plant pathogenic bacteria, plasmids have been a useful source of target DNA. In *Xanthomonas campestris* pv *phaseoli* a 3.4 kb plasmid fragment was partially sequenced and primers designed for the detection of common blight in bean (Audy *et al.*, 1994). In *Xanthomonas campestris* cv *citri*, primers derived from a plasmid could distinguish bacterial strains from citrus species causing different diseases (Hartung *et al.*, 1993). Primers homologous to an endocellulase gene and pat-1 gene involved in pathogenicity have been used to differentiate a group of sub-species of the bacterial wilt pathogen of tomato, *Clavibacter michiganensis*. Indeed, a polymerase chain reaction based on primers derived from the pat-1 could distinguish virulent from avirulent strains (Dreier *et al.*, 1995).

For the mycoplasma-like organism associated with lethal yellowing disease of palms in Florida, a primer pair was designed from a cloned 1.3 kb fragment of the MLO genome and used to amplify a 1 kb fragment from a range of infected tissues (Harrison *et al.*, 1994).

d) Reverse-transcription PCR (RT-PCR)

The techniques described above are all used on DNA as the reaction template and would be appropriate for detection of plant viruses with DNA genomes. Many plant viruses, however, have RNA genomes and for this reason, an extra step is required when using PCR as a diagnostic tool. This step involves reverse transcription of the RNA genomes into a single strand of DNA using a suitable enzyme such as AMV reverse transcriptase. This is normally carried out at 42°C for 30 minutes prior to standard PCR thermocycling as described above.

Sequence data are now available for many plant viruses and diagnostic PCR tests have been developed for a number of viruses eg cucumber mosaic virus (Hu *et al.*, 1995), tobacco rattle virus from both tubers and roots (Weideman, 1995) and also for the double-stranded RNA virus associated with La France disease of *Agaricus bisporus* (Romaine & Schlagnhaufer, 1995; Sonnenberg *et al.,* 1995).

e) Immunocapture-PCR (IC-PCR)

Greater sensitivity, and in some cases specificity, has been achieved by combining the precision of PCR with the ability of virus specific antibodies to bind and therefore concentrate virus particles onto a solid surface. Reaction tubes are coated with appropriate sera and then used to trap virions from plant extracts. Components of crude extracts that may otherwise inhibit RT-PCR can be washed away prior to amplification. Examples of successful use of this technique include plum pox virus (Wetzel *et al.,* 1992) and grapevine fanleaf and cherry leafroll (Rowhani *et al.*, 1995).

f) Nested PCR

Increased sensitivity can often be achieved by the use of two pairs of oligonucleotide primers where the annealing sites of the second pair of primers arc contained within the sequence of the amplified fragment of the first primers. This adaptation has been used to improve the sensitivity of detection of *Erwinia amylovora* (McManus & Jones, 1995). Based on the amplification of a fragment of DNA from a plasmid found in *E. amylovora*, a nested set of primers could detect DNA from the equivalent of less than one cell from pure culture. Nested PCR was also used successfully to detect *Pseudomonas syringae* pv *phaseolicola* in bean seed extracts (Schaad *et al.,* 1995).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

RFLP analysis provides a means of discriminating between similar DNA samples at a species or strain level.

Extracted DNA is digested using restriction endonucleases which cleave at specific recognition sites. The resulting restriction fragments are separated according to size by electrophoresis in agarose gels. In some cases, viewing ethidium bromide-strained gels on a UV transilluminator can reveal diagnostic polymorphisms with distinct DNA fragments visible. However, in most cases, the digestion products appear as a smear with fragments ranging up to approximately 25 kb in size. To gain useful information from these gels, the DNA must first be transferred to a nylon or nitrocellulose membrane by Southern blotting (Sambrook *et al.*, 1989) and selective fragments visualized by hybridisation with a suitably labelled DNA probe.

By using different probes, either a very small number of fragments can be visualized, for example using low or single copy number gene probes, or large numbers of fragments, for example using mitochondrial DNA probes.

For distinguishing species of *Trichoderma* Muthumeenakshi *et al.*, (1994) used a nuclear

ribosomal DNA repeat unit probe from *Saccharomyces carlsbergensis* (Verbeet *et al.*, 1983). RFLP analysis of rDNA showed little or no variation in the fragment sizes generated for a single species. In most cases, species could be separated from each other following digestion with a single enzyme, but in some cases two enzyme digestions were required.

While this method easily identified *Trichoderma* species, it has not been successful in some other fungi. *Colletotrichum* contains a number of group species (Sutton, 1992) including *C. acutatum* and *C. gloeosporioides.* Within both of these "species" a number of polymorphisms occur in the rDNA which enable infraspecific groupings to be identified. However, this variation does not allow identification to be made according to the current species concepts.

Mitochondrial DNA (mtDNA), like rDNA, contains conserved regions but is much larger (approximately 20-170 kb) and contains extensive regions of variable sequence. Restriction endonuclease digestion of mtDNA usually results in a relatively large number of fragments (10-25). The size of the mitochondrial genome and the combination of conserved and variable regions often makes it an ideal choice for discrimination between species/strains.

In the case of *Trichoderma* where no or little variation was observed in rDNA within a species, mtDNA RFLPs could distinguish infraspecific elements (Muthumeenakshi *et al.*, 1994). Considerable variation has also been reported for isolates named as both *C. acutatum* and *C. gloeosporioides* (Sreenivasaprasad *et al.*, 1992).

In addition to the rDNA and mtDNA probes described in this section, other probes may produce informative RFLPs. These may include random clones produced without any prior knowledge of function or structure; cloned genes (from the same or a different species); PCR products; or repetitive sequences (see below).

None of these probes will be universally applicable to all systems, but may provide valuable diagnostic information in some cases.

The use of rDNA and mtDNA probes in RFLP analysis produces a unique pattern of fragments which can be used as a fingerprint. However, the term DNA fingerprinting has recently been more closely associated with the use of repetitive sequences or synthetic oligonucleotides as probes.

DNA FINGERPRINTING

DNA fingerprinting relies on the presence of a particular type of repetitive sequence present in all eukaryotic genomes (Tautz & Renz, 1984; Jeffreys *et al.*, 1985). The repetitive sequences are short motifs which are tandemly repeated and sometimes termed minisatellites. The tandem repeats are often dispersed throughout the genome and exhibit a high degree of polymorphism.

Probes can be prepared either by cloning repetitive sequences from a species or by synthesizing oligonucleotides, for example $(GATA)_4$, $(GTG)_5$, $(CA)_8$. Several examples can be found in the literature where DNA fingerprinting has been used to differentiate fungal isolates. Hamer *et*

al., (1989) described a family of dispersed repetitive DNA sequences in the rice pathogen *Magnaporthe grisea* which he called MGR. MGR sequences occurred as approximately 50 copies per genome and were dispersed across all chromosomes. When MGR sequences were used as a probe, RFLP profiles provided a genotype-specific fingerprint and were diagnostic for seven out of eight US pathotypes tested. MGR fingerprints are now being used successfully to map the world population of *M. grisea*.

Synthetic oligonucleotides, complementary to repetitive sequences in the grain legume pathogen *Ascochyta rabiei*, have been used successfully to discriminate various races (Weising *et al.*, 1991), and human minisatellite DNA probes have been used to separate populations of *Colletotrichum gloeosporioides* (Braithwaite & Manners, 1989). A similar approach using human minisatellite DNA was used to separate populations of the Dutch elm disease *Ophiostoma ulmi* (Hintz *et al.*, 1991).

CONCLUSION

The techniques described in this paper can be used to discriminate closely related pathogens and in many cases identify pathogens in extracts made directly from infected plant material or soil.

Limitations still exist for the use of PCR for large scale pathogen testing although it is likely that microtitre plate formats or other adaptations of amplified fragment analysis will soon be available (Hataya *et al.*, 1994; Dale & Dragon, 1994).

Sensitivity remains one of the main advantages of PCR with claims of detection levels typically in the region of 10 femtogram for viral RNA (Romaine & Schlagnaufer, 1995) and 10 bacterial cells when PCR is combined with Southern blotting for product detection (Hartung *et al.*, 1993).

Ironically, sensitivity is also one of the main threats to the successful use of PCR for diagnosis as great care must be taken to ensure that contamination of samples does not lead to false positives. Wide interest in the use of PCR technology throughout the biological sciences will undoubtedly result in development of user-friendly procedures in the near future.

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