Session 3

Diagnostics for Fungal Plant Pathogens

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FACTORS CONTRIBUTING TO SUCCESSFUL PCR-BASED DIAGNOSTICS FOR POTATO AND OTHER CROP PLANTS.

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ABSTRACT

Despite widespread use of PCR to diagnosis infectious diseases, the technology generally has not been adopted by commercial diagnostic laboratories because of cost as well as concerns about reproducibility and quality control. We are attempting to incorporate PCR-based diagnostics into a government extension programme to monitor *Verticillium* species in commercial potato fields. For this a number of factors have been addressed which have limited the application of PCR technology. In this paper we have summarized our routine protocols for extraction of DNA from infected tissues and infested soils, PCR amplification and fractionation of products. We have emphasized typical problems and experimental approaches used to address or avoid them. With few qualifications which are fully discussed, we believe that the five standardized protocols which are presented, now represent a reasonable technology to detect and quantify *Verticillium* pathogens in potato and probably other susceptible crops. When compared with previous biological assays, the new technology is less expensive, much faster, more accurate and can be applied cost effectively and reproducibly on a large scale.

INTRODUCTION

Verticillium wilt and early dying syndrome are serious diseases wherever potatoes are grown. Constant and rapid monitoring of pathogen levels are important since potato cultivars differ widely in levels of resistance to various *Verticillium* species such as *V. dahliae*, *V. albo-atrum* and *V. tricorpus* (Platt, 1986); also climate or cultivar changes can lead to critical shifts in the occurrence or predominance of species (Celetti and Platt, 1987). Such observations emphasize the need for an accurate method of species identification and quantification which is sufficiently rapid for a producer or inspector to make appropriate recommendations, especially since control options differ depending on pathogen type. At present the main method of detection has been field inspection, even though *Verticillium* wilt can have the same symptoms as excess chemical top-killer, drought stress and mature plant senescence. Current laboratory methods, which are somewhat more accurate are time consuming (up to 7 weeks) and labour intensive. Results are at best semi-quantitative and most tests fail to distinguish possible species adequately.

In recent years we have developed PCR-based diagnostics to monitor *Verticillium* species in potato and other crops. Such assays are fast, very sensitive, and can be applied quantitatively. An important component of this research has been adaptation for the large scale use required by governmental and commercial testing laboratories. In this context we have worked closely with Agriculture and Agri-Food Canada research personnel in Ontario and Prince Edward Island,

major potato growing regions of Canada. Successful development of large scale PCR-based diagnostics must be based on the following principles. The assay must be very direct in order to minimize labour and cost requirements. The assay also must yield reproducibly quantifiable results over a broad range of pathogen levels and potential environments. This need for efficiency, quantitative reproducibility and sensitivity demands the use of controls which are more stringent than is frequently required for mere identification. In the present paper we have investigated factors which our experiences suggest are most critical for effectiveness and have made specific recommendations which may help others to take PCR-diagnostics to the fields.

MATERIALS AND METHODS

Extraction of DNA from potato tissues

Leaves or one inch segments of a stem were removed from plants, sealed in plastic and, whenever possible, placed directly on ice until stored at -20°C. Tubers were stored at 4°C. In our optimized protocol 0.5 gm of frozen tissue was diced and ground in liquid nitrogen by using a mortar and pestle for 5 min or until a fine powder remained. The tissue was homogenized in 5 ml of SDS extraction buffer (0.3% SDS, 140 mM NaCl, 50 mM sodium acetate, pH 5.1) and 5 ml of water-saturated phenol (Steele *et al.*, 1965); this mixture was incubated at 65°C for 10 min with occasional mixing and the phases were separated by centrifugation (10,000 xg, 10′, 10°C). The nucleic acid in 1.5 ml of the aqueous phase was precipitated with 2.5 volumes of ethanol at 20°C overnight. The precipitate was collected by centrifugation at 4°C (8,000 g, 10 min), dissolved in 300 µl distilled H_2O and 150 µl of ammonium acetate (7M) were added (5 min, 4°C). The nucleic acid was reprecipitated with 2.5 volumes of ethanol for several hours or overnight. The precipitate was collected by centrifugation at 4°C and the pellet was washed once with ethanol, followed by centrifugation and dried. The dried pellet was dissolved in 100 µl of water and stored at -20°until use (Figure 1).

Extraction of DNA from soil

Soil samples were stored dry at 4°C or frozen (-20°C) for long-term storage. In our optimized protocol 0.25 gm of soil (1oam, sand, clay, gravel) was ground with liquid nitrogen using a mortar and pestle for 5 min or until a fine powder remained. The powdered soil was suspended in 0.5 ml of skim milk powder solution (0.1 gm of milk powder in 25 ml of H_20) by vigorous vortexing; for quantitative assays internal control template DNA (usually 500 pg) was also added at this time. Soil and debris were removed by centrifugation at 4°C (12,000 x g. 10 min) and the supernatant was mixed with 2 ml of SDS extraction buffer (0.3% SDS in 0.14 M NaCl, 50 mM sodium acetate [pH 5.1]) by vortexing. An equal volume of water-saturated phenol solution was added; the phases were mixed by intermittent vortexing for 2 min. at room temperature and then separated by centrifugation (12,000 x g. 10 min). The nucleic acid in the aqueous phase was precipitated with 2.5 volumes of ethanol at 20°C for several hours or overnight when convenient. The precipitate was collected by centrifugation at 4°C, and the pellet was washed twice with ethanol with centrifugation between each rinse, and dried. The dry pellet was dissolved in 250 µl of water and stored at -20°C until assayed (Figure 2).

Standard PCR amplification

Five µl aliquots of DNA extract were used per reaction tube. PCR amplification (Figure 1) was conducted using 50 µl of PCR reaction mixture containing PCR buffer (normally 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 0.1 mg per ml bovine serum albumin (BSA), 0.2 mM of each deoxyribonucleotide triphosphate, 12.5 pico-moles of each oligonucleotide primer (Hu *et al.*, 1993, Moukhamedov *et al.*, 1994, Robb *et al.*, 1993), 2 units of TaqDNA polymerase and the sample DNA. For studies in which internal control template was not included during extraction (i.e. soils), control plasmid DNA (0.2-1.0 pg) usually was added to the reaction mixture (Hu *et al.*, 1993); for radioactive studies 0.5 µCi [a^{32}P]dATP or dCTP were added to each tube. After the addition of 60 µl of light mineral oil the solution was mixed by vortex and the phases were separated by centrifugation in a microfuge for 1 min. Using a temperature cycler, the DNA was amplified with 30 reaction cycles consisting of a 1 min denaturation step at 95°C, a 1 min annealing step at 37-65°C (depending on the primer sequence) and a 2 min elongation step at 72°C. A stringent annealing temperature (58-60°C) was carefully chosen for species specific assays. For subsequent analyses by gel electrophoresis, 5 µl aliquots were analyzed directly or, when the signal strength was very low, the sample was first concentrated by precipitation with 2.5 volumes of ethanol containing 2% potassium acetate at -20°C overnight. In the latter case, the reaction mixture was diluted with 50 µl of H_20 for further analysis by gel electrophoresis.

"Nested" PCR amplification

Five µl aliquots of DNA extract were used per reaction tube; usually the extract was first diluted 50-fold to reduce or avoid inhibiting substances. PCR amplification was conducted as described for the standard amplification protocol. The primers used in the first reaction recognized a longer target DNA sequence (Figure 2) and could be more general in specificity (Volossiouk *et al.*, 1995). Sixty µl of light mineral oil were added and vortexed. The phases were separated by centrifugation in a microfuge for 1 min. Using a temperature cycler, the DNA was amplified with 30 reaction cycles consisting of a 1 min denaturation step at 95°C, a 1 min annealing step at 37-65°C (depending on the primer sequence) and a 2 min elongation step at 72°C. For the second phase of amplification, 5 µl of the first amplification reaction mixture were used per tube and the amplification was repeated using the standard amplification protocol and the second set of species specific oligonucleotide primers (Figure 2). These primers were usually very specific and the annealing was carried out at a highly stringent temperature (58-60°C).

Fractionation of PCR amplification products by agarose gel electrophoresis

Either a vertical or a horizontal gel apparatus was used (Maniatis et al., 1982); commercial or homemade horizontal mini gel systems were found to be fast and inexpensive. An agarose gel was most convenient and adequate for most analyses. Acrylamide gels or a larger apparatus were substituted for more complex fragment mixtures.

For a standard 2% agarose gel, 0.4 gm agarose was added to 20 ml of 10-fold diluted TBE buffer (0.9 M TRIS, 0.9 M boric acid, 25 mM EDTA, pH 8.3) and dissolved by heating in a boiling water bath, autoclave, or microwave oven. The open ends of the plastic gel tray were taped, then the tray was leveled with a bubble level, and the agarose solution (precooled to about 50°C) was

poured. Immediately a slot maker with the required number of teeth was inserted. After the gel set (about 30 min), the tape was removed and the gel was placed in the electrophoresis tank. Buffer (20-fold diluted TBE) was added to cover the gel to a depth of about 1 mm (about 150 ml), and the gel was pre-run at about 15 mA for 10 min. Two µl of loading dye (5% SDS, 25% glycerol, 0.025% bromophenol blue) were added to 5 µl of sample and the mixture was heated to 65°C for 1-3 min before loading. Electrophoresis was carried out at about 80-90V or 15-20 mA (conditions will vary with gel apparatus) for about 2h or until the dye marker approached the bottom of the gel slab.

a) For non-radioactive gels. The gel was stained about 40 min with ethidium bromide (0.5 µg/ml) solution and excess stain was rinsed away with water. For qualitative analysis the bands were visualized with a UV transilluminator (300 nm). To quantify the signal, a photograph of the gel was analyzed using a densitometer or a CCD camera imaging system was used to capture the image and calculate band intensities (Figure 2).

b) For radioactive gels. While agarose gel electrophoresis could also be used, radioactive products on an 8% polyacrylamide sequencing gel (e.g., Maniatis et al., 1982) were easier to handle and provided higher resolution. Autoradiography was carried out using fast X-ray film with an exposure of about 2h at -70°C. For quantitative analyses the gel plate was oriented carefully with the autoradiograph and the relevant bands were cut out. The radioactivity in each band was usually determined by liquid scintillation counting. If available, a "filmless" phosphorescence imaging system was used instead to significantly reduce the cost and labour. In each case, a standard curve based on known amounts of internal control template (Hu *et al.*, 1993) was used to determine the amount of nucleic acid which was present from the PCR product ratio (Figure 1).

RESULTS AND DISCUSSION

Sample collection

The first critical step was in the proper handling and storage of the sample prior to extraction. If at all possible leaf and stem samples were wrapped and frozen immediately or stored on ice for as brief a period as possible before freezing. We found that inhibitors of the PCR-reaction accumulated very rapidly in green tissues and natural desiccation rapidly decreased the "freezer life" of samples. Tubers were placed in cold storage as soon as possible. Soil samples were also dried and frozen quickly to prevent degradation.

Extraction of *Verticillium* DNA from infected plant tissue

While many extraction procedures provide high quality DNA, they are usually labour-intensive and not suitable for large-scale diagnostics. Hence, a more direct method was sought for use in the PCR assays. Direct extraction procedures based on SDS/phenol or hexadecyl trimethyl ammonium bromide (CTAB, Rogers and Bendich, 1985) often represent a simple, cost effective and reliable method to purify whole cell nucleic acid (Figure 1). We tested both approaches to extract plant and fungal DNA's from infected potato stems and tubers. For either tissue we found that CTAB extracts generally contained less natural inhibitor, but SDS-phenol extracts often contained more DNA. Therefore, for quantitative diagnostics we recommend SDS-phenol

extraction combined with subsequent treatments to eliminate inhibitory effects. We examined a variety of modifications to eliminate inhibitors or overcome their effects. For example, extractions with organic solvents such as acetone, acetonitryl, ether, or ethanol and inorganic precipitants such as lithium chloride or ammonium acetate were examined. Only precipitation in the presence of high ammonium acetate (7.5M) was beneficial in many cases so this step was introduced routinely, into our standard nucleic acid extraction protocol. Under most circumstances further inhibition problems could be solved by sample dilution (i.e. 50-fold for leaves and stems, 100-fold for tubers). Necrotic tissues sometimes required 1000-fold dilution levels. Results obtained from infected potato stems extracted using this protocol are illustrated in Figure 1.

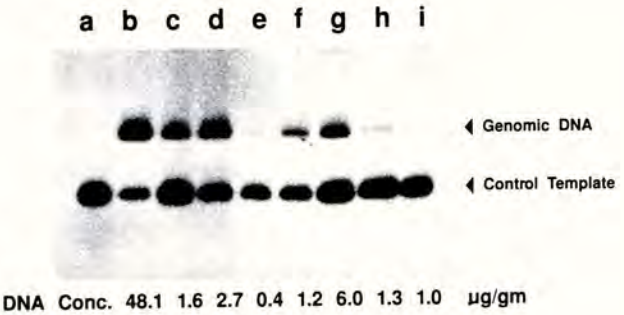

Figure 1. Quantification of *V. albo-atrum* DNA in stems of severely diseased potatoes. Plants were extracted using the SDS-phenol method and the extracts were diluted 50-(lanes d-f) 200-(lanes b,c) or 500-(lanes g-i) fold depending on the level of inhibitors. Extracts were amplified using the standard PCR protocol with V. *albo-atrum* specific primers, internal control DNA and [$a^{32}P$] dATP, fractionated on an acrylamide gel, photographed and quantified using a scintillation counter as described in the Methods section. Lane a contains internal control template DNA.

The other major limitation to the SDS-phenol method was adequate disruption of cell walls to allow the release of DNA. Various disruption methods were examined to develop a suitably direct, but effective method of DNA preparation which could be used with fungal and plant tissues. Grinding in liquid nitrogen, which minimized degradation during tissue disruption and allowed direct utilization of frozen samples without thawing, was adopted. The extended grinding time (i.e. 5 min) was critical in quantification for complete release of fungal DNA.

Extraction of *Verticillium* DNA from soil

Despite its undisputed potential for monitoring pathogens in the environment, including soils, the extended processing time involved in separating the cells from soil colloids is a major limitation, particularly when quantitative measurements are desirable. Problems related to DNA degradation and adsorption have been recognized for more than a decade (e.g. Novitsky, 1986) and contaminants such as humic acid and phenolic compounds, commonly found in soils, reduce or inhibit enzymatic reactions and even the specificity of hybridization analysis (Steffan and Atlas, 1991; Picard *et al.*, 1992). Over the last two decades, many methods have been proposed to avoid or minimize these factors, but these have been complex, labour intensive (e.g., Holben *et al.*, 1983) and difficult to apply on a large scale. To address such limitations, we explored the possibility that our direct plant extraction procedure could be modified for use with soil (Volossiouk *et al.*, 1995). The use of liquid nitrogen for cell disruption appeared ideal to avoid degradation by soil nuclease activities and preservation of samples by freezing again was very convenient and could be used directly with this approach. Other disruption methods including vortexing with glass beads, boiling, or repeated cycles of freeze/thaw were examined, but the results were surprisingly poor. Some signal was visible after vortexing with glass beads but none was observed with extracts using boiling or freeze/thaw. As a result, additional disruption procedures were abandoned.

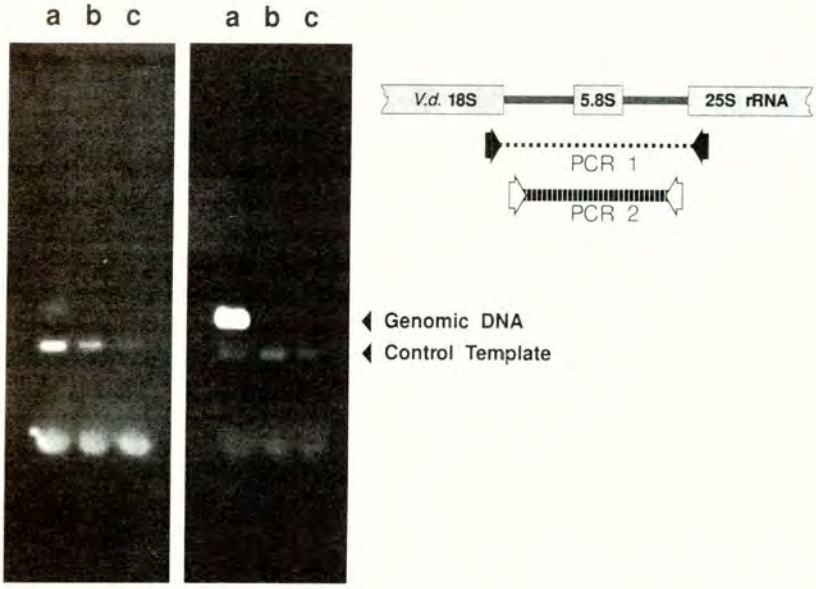

Figure 2. Application of "nested" PCR amplification for the detection of *V. dahliae* microsclerotia in soil. An internal control template was added to farm soil (0.25g) containing 1 µg of microsclerotia which was extracted and PCR amplified using the standard (lane a in left gel) protocol (only PCR 2 reaction) or nested (lane a in right gel) PCR protocol (PCR1 followed by PCR 2) before fractionation by agarose gel electro- phoresis. A soil extract without microsclerotia and a reaction with an equivalent aliquot of control template are included in lanes b and c, respectively. A 30 cycle amplification was used in each reaction.

While simple grinding provided the most promising results, losses and inhibition clearly were present with most soil samples and further improvements were sought. Four rationales were examined in greater detail (Volossiouk *et al.*, 1995): the use of stronger solvent conditions to more effectively dissolve the DNA; the use of other additives and organic solvents in order to remove inhibitory substances; the use of carrier molecules to reduce losses due to degradation; and finally, because more alkaline conditions are often used in DNA preparation procedures (e.g. Birnboim and Doty, 1979), an alkaline SDS buffer was substituted to observe the effect of alkaline denaturation. With most attempts improvements were not observed and with alkaline buffers the inhibition was much worse. However, in many instances the use of Denhardt's solution or addition of skim milk powder (0.01-0.1%) to the sample were equally effective in substantially improving the signal (Figure 2). In our standard soil protocol we routinely added skim milk powder as a convenient and cost effective macromolecular carrier in the extraction phase providing an effective reduction in nucleic acid degradation and adsorption. Farm soils also contained substances which were inhibitory to PCR but a 50-fold dilution of soil extract before PCR-amplification relieved inhibition in most cases (Volossiouk *et al.*, 1995). Results obtained from infested soil using this protocol are illustrated in Figure 2.

Internal control template

An internal control DNA template (Figure 1) which also was amplified by the specific primer set was added to the PCR reaction to detect inhibitory compounds or an occasional failed PCR-reaction (Hu *et al.*, 1993). Control templates also were critical for quantification (Hu *et al.*, 1993). The choice of quantification method appeared flexible and could be selected to complement the available equipment. We have used extensively a densitometer, video capture equipment or scintillation counting with good results. We also successfully used a phosphorimager (Model GS 250 Molecular Imager, BioRad Laboratories, Ltd., Hercules, CA) in place of a scintillation counter. The use of isotope was generally less cost effective both with respect to reagents and labour, but appeared to provide a higher degree of accuracy. The use of phosphorimaging equipment probably represented the best compromise, but also represented a very substantial initial cost.

When large amounts of control template were synthesized for long-term or large-scale use, it was convenient to clone the sequence in an efficient cloning vector such as pBR322 or pTZ19R (Maniatis *et al.*, 1982). Plasmids containing the control sequences were prepared using the methods of Holmes and Quigley (1981). CeCl/EtBr gradient centrifugation (Radloff *et al.*, 1967) has been our method of choice for further purifying this DNA. When used, the purity was high and the absorbency at 260 nm usually could be used to determine the amount of DNA. One absorbency unit at 260 nm usually was taken as equivalent to 40 µg of DNA. For long-term storage, the control template was stored as an ethanol precipitate; for shorter terms, it was dissolved in glass distilled water in small aliquots and stored frozen. Repeated freeze-thawing of the template solutions or storage at higher temperatures was avoided to minimize degradation and contamination through bacterial growth, etc.

Sensitivity and "Nested" PCR procedures

While in principle, a normal PCR assay can detect as little as a single target molecule, in many circumstances the observed amplification was reduced substantially by inefficient priming, inhibiting substances in the extract, or even competing reactions due to less specific priming. A common problem particularly for tubers or soils, was a very small amount of target while inhibiting substances required a high dilution for efficient amplification. Under these conditions a signal was not detected at all. Two approaches were examined to improve sensitivity and circumvent these limitations. A simple modification which led to a 10-fold gain in sensitivity was to analyse the entire amplification reaction mixture rather than just 10% of it, as described in the standard protocol. In this circumstance, the reaction mixture was first precipitated with 2.5 volumes of salted ethanol and then dissolved in a minimum volume of loading buffer (usually 5 µl H_2O + 2 µl dye solution) for application to the analytical gel. When more sensitivity was desirable, an increase in the number of cycles could be used to amplify the signal, but this often resulted in elevated background and artefacts, at least in part due to recombination *in vitro*. A "nested" or two-step PCR amplification (e.g., Haqqi *et al.*, 1988) offered a better solution (Volossiouk *et al.*, 1995) . In this case two different sets of primers were used. A larger target sequence was amplified during the first PCR polymerase chain reaction and a shorter target, within the first product, was amplified during the second step (Figure 2). Such an approach greatly amplified the signal strength, usually without a concomitant increase in non-specific fragments or other artefacts (Figure 2).

Quality control of PCR-based assays

As pointed out in recent reviews (e.g. Kitchin and Bootman, 1993; Victor *et al.*, 1993), despite widespread use of PCR to diagnose diseases, the technology generally has not been adopted by routine diagnostic laboratories. Unfortunately, the power of PCR is also its greatest potential weakness and misleading results due to contamination with DNA from external sources have resulted in scepticism. Quality control to avoid contaminating signals must be more rigorous and is extremely critical if "nested" primers are to be used. For qualitative identification and most quantitative problems, routine care, including use and storage of reagents in small aliquots, separate pipettes for routine reagents to avoid contact with DNA, and the use of aseptic conditions wherever possible, appeared to be sufficient. Our first attempts with "nested" PCR methods, however, resulted in a high frequency of intense false positive signals. A detailed examination of the problem revealed that the major source of contaminants was actually the local deionized water sources, all of which contained trace amounts of *Verticillium* genomic DNA's and even internal control template DNA's. Presumably this resulted from cellular growth in the water supply including the deionization resin cartridge. We subsequently prepared uncontaminated water by distilla- tion. Previously, we had avoided this type of water because some suggestions in the literature cautioned against this source. This has not been a problem in our hands; accordingly, in this study we routinely used distillation as the source of water for PCR assays. With a good water source, our routine precautions have been adequate even with "nested" PCR assays. While articles on quality control procedures (e.g. Kitchin and Bootman, 1993, Victor *et al.*, 1993) often recommend a relatively long list of precautions, the only factors which we have found to be essential, beyond normal good laboratory practices, were as follows:

a) Control reactions without DNA template were always included to detect the presence of contaminating DNA in the reagents.
b) Water which was used for extraction solutions or PCR reagents was obtained (e.g., glass distillation) and tested to be free of target DNA.
c) Reagents were stored frozen in relatively small amounts, avoiding repeated use as much as possible.
d) PCR reagents were aliquoted with dedicated pipettors which never came in contact with nucleic acid excluding the oligonucleotide primers.

The methods, observations and suggestions found in this paper evolved from a collaborative study with government agencies, the joint purpose being to introduce PCR-based diagnostics into an extension programme to monitor *Verticillium* species in commercial potato fields. In comparison with traditional phytopathogical testing we have no doubt that PCR-based methodologies are more sensitive, more accurate and less labour intensive. In our experience the protocols which we have described ensure competitive cost efficiency and a reasonable level of reproducibility over a broad range of pathogen levels and environmental variables. Important features included in the five optimized protocols were:

a) A single step extraction procedure, based on SDS/phenol which required no prior purification steps other than grinding in liquid nitrogen.
b) The use of a simple milk powder-based solvent with soil samples which minimized nucleic acid degradation and losses due to adsorption.
c) Sample dilution as a method which minimized or eliminated the effects of inhibitory substances without further purification steps.
d) Use of internal control templates which compensated for differences in efficiency and inhibition with alternate samples.
e) "Nested" or two step PCR amplification as a means of increased sensitivity, which minimized detection limits and permitted the required levels of sample dilution.

Further gains in time and cost reduction can be expected through the use of multiplex assays and eventually by automation; however, many of the factors addressed in the present study would still apply. Moreover most of these recommendations have broad applicability to a wide range of plant pathogens.

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PRODUCTION AND USE OF MONOCLONAL ANTIBODIES FOR THE DETECTION OF FUNGI

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ABSTRACT

Genus-and species-specific monoclonal antibodies raised to fungi are now being used successfully in immunoassays for the detection and tracking of fungi in plants and soils but their employment for routine diagnostic purposes in crop protection is still limited and few fungal immunoassays are available commercially. The most commonly occurring monoclonal antibodies, IgM antibodies, have proved to be very useful in the development of fungal immunodiagnostic assays. In general, they bind rapidly and recognize heat stable, carbohydrate epitopes on glycoprotein molecules. These antigens are localized in cell walls and the extracellular matrix of fungal hyphae and are produced at all stages of growth both *in vitro* and *in vivo.* Extraction of fungal antigens from some plant tissues and soils remains a challenge.

INTRODUCTION

For many fungal diseases, there is a need for assay systems, such as immunoassays, that can be used to confirm visual symptoms and detect early and latent infections before symptoms are apparent. The assays need to be quick, specific, sensitive, easy to use and of low cost; they will never entirely replace classical methods but they should enable advisors and growers, who may have little mycological expertise, to screen large numbers of samples either on site or with minimal facilities. The use of immunoassays for viral diseases has had a long successful history but employment of immunological techniques for the detection and quantification of fungi has, until recently, not been successful. This is because it is difficult to raise antisera to fungi that do not cross-react with related and unrelated fungi and extracts from host tissues. This problem has now been circumvented by raising monoclonal antibodies. The advent of hybridoma technology (Kohler and Milstein, 1975) has made it possible to raise and select clones of hybridoma cells that secrete highly-specific antibodies i.e. monoclonal antibodies (MAbs). A number of workers have raised genus-, species- and isolate-specific MAbs to plant pathogenic fungi and have used these to develop immunoassays but, despite their success, few are available commercially.

The initial costs and time involved in raising and selecting hybridoma cell lines that secrete fungal-specific monoclonal antibodies are high but running costs are low; selected cell lines can be cultured to produce unlimited quantities of a specific MAb and preserved indefinitely. MAb-based immunoassays can be replicated easily allowing many samples to be processed at one time; they can be also made 'user friendly' for on-site field studies and used to quantify specific fungal biomass in mixed microbial environments such as soils or in plant tissues. Furthermore, they can be adapted for determining sequential growth of pathogens in soils (Thornton, 1996a).

The process of raising fungal-specific monoclonal antibodies is not difficult but requires dedicated cell tissue culture facilities, accompanying skills and time. The costs involved in raising monoclonal antibodies to a specific fungus vary with the degree of pathogenicity of the fungus and the level of specificity required. In general, we have

found that it is much easier and, therefore, quicker and cheaper, to raise specific MAbs to saprophytic and necrotrophic fungi than to specialized pathogenic fungi, and that cell lines secreting genus-specific MAbs are found to occur far more frequently than those secreting species-or isolate-specific MAbs.

RAISING AND SELECTING HYBRIDOMA CELL LINES SECRETING FUNGAL-SPECIFIC MONOCLONAL ANTIBODIES.

Unfortunately, the process of raising fungal-specific MAbs is still relatively inefficient in that, whatever fungal fragment or extract is used as the immunogen, a large percentage of the hybridoma clones produced secrete non-specific MAbs. No one immunogen appears to be more specific than another but we do know that a number of the species-specific fungal MAbs that have been raised recognize glycoproteins present in both cell walls and in extracellular fluids (Dewey, 1992; Cole *et al.*, 1991; Cole *et al.*, in press). We commonly use, as immunogens, extracellular antigens obtained by washing the surfaces of solid slant cultures with phosphate buffered saline (Dewey, 1992; Bossi & Dewey, 1992; Thornton *et al.*, 1993). The great advantage of this method is that it is simple, requires little preparation and no processing. The washings can be injected directly into the mouse without freeze-drying. If necessary, such washings can be frozen and thawed without the apparent loss of antigenicity.

Recently, we have tried to improve the efficacy of our immunizations by using a co-immunisation technique first developed by Barclay and Smith (1986) for bacteria. Mice are injected with antigens from the target fungus together with antibodies previously raised to a related fungus or extracts of host material. This blocks the formation of cross-reactive antibodies thereby reducing the number of hybridoma cell lines secreting non-specific MAbs. Consequently, the time and money spent on secondary and tertiary screening assays is reduced considerably. We found this technique to be particularly helpful in raising specific MAbs to the fungal pathogen causing apple canker, *Nectria galligena* (Dewey *et al.*, 1995); antisera to the related fungus *Fusarium solani* f.sp. *pisi* was used as a blocker. Using this technique, it is should now be possible to raise and select MAbs that will recognize the most abundant pathogen-specific fungal antigens that can be extracted easily from infected tissues, thereby ensuring that highly-sensitive immunoassays can be developed for use in early detection of pathogens. To do this, mice would be immunized with extracts from tissues with early infections together with, as a blocker, antisera raised to extracts from healthy plant tissues.

In general, IgG antibodies present in antiserum recognize taxonomically more specific antigens than do IgM antibodies and the use of only the IgG fraction from antisera has helped in the development of antisera that can be used for detection purposes (see Dewey, 1992). Nevertheless, it must be stressed that some fungal IgM MAbs are highly specific and useful; they recognize carbohydrate epitopes that appear to occur as repeat epitopes on glycoprotein molecules and are, thus, highly sensitive allowing detection levels in the nanogram to picogram range (Dewey *et al.*, 1992). We have also found that the binding of some of these IgM MAbs occurs rapidly, in seconds rather than hours, allowing incubation times to be reduced to a minimum (Dewey *et al.*, 1992, Thornton, pers. comm.).

In developing immunodetection assays, there are a number of parameters that must be considered first and prioritized. Sensitivity, specificity, speed, suitability (i.e. field or lab-based) and costs are important criteria. The minimum level of specificity required in the final immunoassay should be defined at an early stage otherwise a considerable amount of time and money can be wasted in the process of selecting a suitable MAb. Species-specificity is not always the most important criterion. For example, in the

development of a sensitive immunoassay for *Pseudocercosporella herpotrichoides,* for use in fungicide trials, differentiation from other taxonomically-unrelated fungi commonly found infecting the stem-based region of cereals was more important than raising a MAb that did not recognize other species of *Pseudocercosporella* (Priestley & Dewey, 1993). A greater immunological similarity was found between *P. herpotrichoides* and the other stem based pathogens of cereals, particularly *Microdochium nivale*, than with other fungi (Dewey, 1988). Inevitably, in raising MAbs and developing immunoassays some problems arise which are hard to anticipate. In the above case, we found that the secondary antibody-peroxidase conjugate bound non-specifically to extracts from young seedlings, despite the use of several different blocking agents. This non-specific binding was almost certainly due to the presence of the lectin, wheat germ agglutinin. The problem was finally overcome by using an acidic extraction buffer in a double antibody sandwich assay fromat (DAS-ELISA). Using this system, the pathogen could be detected in extracts from inoculated seedlings 6 to 8 days after inoculation, that is some 12 to 14 days before symptoms became apparent.

TYPES OF IMMUNOASSAYS

Several different assay formats exist but the most common are the ELISA-based systems. Most of the ELISA tests developed for fungi are, with the exception of the commercial assays, all simple indirect assays in which the micro-titre wells are directly coated with the test sample. These assays are sometimes referred to as plate-trapped antigen assays (PTA-ELISA). Fungal antigens, particularly glycoproteins, which appear to be the immunodominant molecules, bind strongly to certain makes of micro-titre wells; we find 1 x 12 well microstrips produced by Labsystems, Finland, to be particularly effective. When the wells have been coated with fungal carbohydrates or glycoproteins, then washed and dried, they can be stored dry at 4°C for several years (Dewey, 1992). The disadvantage of using antigen-coated wells is the relatively long binding step, 5 hours or more (generally an overnight step) needed to ensure maximum binding of the antigens to the wells but the simplicity of such assays is attractive. Fungal carbohydrates and most glycoproteins are heat stable, which means than many of the cross-reactive proteinaceous antigens can be precipitated from the test sample by heat treatment before the antigen mixture is used to coat wells (e.g. soil extracts Thornton *et al.*, 1993). We have found that assays involving antigen-coated wells work particularly well where the fungus is present on or near the surface of the infected tissue such as rice grains (Dewey *et al.*, 1989,1990) and where passive release by overnight soaking is sufficient to enable detection at very low infection levels. Such methods could prove useful in the detection of fungal spores on petals or seed-borne pathogens that are present at or near the surface of the seed coats. Most of the commercial enzyme immunoassays are DAS-ELISA tests in which one of the antibodies is generally a specific MAb and the other polyclonal (i.e. antiserum). Sensitivity can sometimes be a limiting factor. We have found that biological amplification methods are particularly helpful for detection of grain or seed-borne fungi; test samples are soaked in buffer or water, in microtitre wells, overnight at room temperature instead of at 4°C. This allows fungal propagules to germinate and secrete antigens that bind to microtitre wells. Similarly, biological amplification methods have been used for the detection of soil-borne pathogens such as *R. solani.* In these assays soil samples are incubated in a selective medium for 48h and then aliquots of the cell-free fluid are tested by ELISA (Thornton *et al.*, 1993). Only a few ELISA kits are available commercially. The best known are those developed by AgriDiagnostics for the detection of turf grass pathogens (Miller *et al.*, 1992), now sold by Neogen (Lansing, Michigan, USA) and AGDEN, Auchincruive, in the UK. They have produced ELISA-kits for the detection of *Phytophthora, Pythium, Sclerotinia and Rhizoctonia.* Unfortunately, the cost of each individual assay is high which limits their use in crop protection. A few companies such as Sigma, Bioreba (Nyons, Switzerland)

and AGDEN sell antibodies to specific fungi, both antisera (PAb) and MAbs, for use in detection assays.

Magnetic bead systems provide a promising alternative to solid support systems using micro-titre wells for capturing fungal antigens. Thornton (in press) has developed a magnetic bead immunoassay for the detection and quantification of *R. solani* in soils. Soil extracts are incubated with a mixture of IgM and IgA murine MAbs specific to *R. solani.* Water-soluble antigens are bound by the antibodies and the complex is extracted using commercially available beads (Dynabeads, Dynal, Oslo, Norway) pre-coated with rat antibodies that specifically recognize mouse IgM antibodies. The beads plus the complex are pulled out of solution by placing in a magnetic field and then the complex is detected by sequential exposure and washing of the bead/complex to commercial goat anti-mouse IgA (α-chain specific) alkaline phosphatase conjugate antibodies and the substrate p-nitrophenol.

A few Dot-blot and dip-stick assays have been developed for fungi. Some test systems use nitrocellulose or nylon membranes and others use polyvinylidene difluoride (Immobilon P, Millipore). The reporter antibody conjugate is, generally, an enzyme conjugate but gold conjugates, which can be silver enhanced, have been used by a number of workers and are thought by some to be more sensitive; the latter have proved most useful in the early detection of *Humicola lanuginosa* on the husks of rice grains (Dewey *et al.*, 1989). Agri-Diagnostics have used the specific MAbs raised to turf grass pathogens (see above) to develop on-site dot-blot field tests that can be completed in 10 minutes. These tests are sandwich assays in which the antigen, present in turf grass extracts, is trapped with one antibody already bound to the membrane and detected with a second antibody enzyme conjugate followed by the substrate. These assays involve the use of both polyclonal and monoclonal antibodies. Again, purchase cost of the assay systems precludes their widespread use. Cahill and Hardham, (1994), using antibodies previously raised to zoospores of *Phytophthora cinnammomi,* have developed an excellent multiple dip-stick assay for detection of this pathogen in infected soils and roots; this assay system will soon become available commercially. Preliminary tests using the system to detect the pathogen on infected material in the UK have shown that it can be very useful (Case *et al.*, pers. comm.).

Tissue printing or squash blot systems hold considerable promise. Arie *et al.*, (1993) have developed a combined culture/immunoblot assay for the detection of pathogens in soils in which fungal propagules in the soil grow during the incubation period and release antigens that diffuse through the gel onto the membrane. These membrane -bound antigens are then immunolabelled. Thornton (1996a) has used a blot system to visualize the progress of *R. solani* in soils in the presence and absence of host seedlings and/or the biocontrol agent *Trichoderma harzianum.*

Immunofluorescence techniques are very useful for visualizing fungal spores and hyphae on and in plant tissues but they are never likely to become widely used for mass screening because they involve microscopy and a UV light source. Nonetheless, they may prove useful in epidemiology for the enumeration of airborne fungal spores settling on leaves or petals (Salinas and Schots, 1994) or trapped on tapes. Using a modified tape in a Berkhard spore trap, we have been able to collect and distinguish spores of *Botrytis cinerea* from other airborne spores using the genus-specific MAb BC-KH4 (Figure 1, Dewey and Kennedy, unpublished). Using this system with species-specific MAbs, it should now be possible to determine the most vulnerable periods for infection and disease onset for a number of foliar diseases.

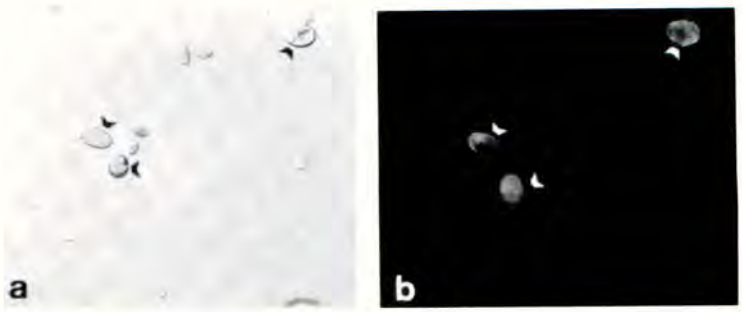

Figure 1. Spores of *Botrytis cinerea* (arrows) and other airborne spores trapped on a modified tape and immunolabelled with the *Botrytis* MAb, BC-KH4, viewed a) with bright light and b) with UV light. Note: only *Botrytis* spores are immunolabelled and visible under UV light.

EXTRACTION OF ANTIGENS FROM PLANT TISSUES AND SOILS

Many workers experience difficulties in extracting fungal antigens from infected tissues and soils. We have found, for example in assays for *Botrytis cinerea* using the MAb BC-KH4, that, although immunolabelling studies at the EM level have shown that the antigen is clearly expressed in diseased tissues at an early stage (Cole *et al.*, in press), detection of the antigen in extracts from pre-symptomatic tissues by ELISA is poor (Dewey, unpublished). The BC-KH4 antigen, in the early stages of infection appears to bind to the plant tissues. Other workers have found similar problems. Avila *et al.* (1995) found in the detection of *Pythium ultimum* in hypocotyls of sugar beet, using a species-specific that, although the antigen could be clearly detected in plant extracts by Western blotting, it could not be detected by ELISA. They suggested that the problem may be related to host compounds that interfere either with the binding of the fungal antigen to the microtitre wells or with the antigen-antibody reaction. It is also possible that fungal antigens may not be released from plant tissues unless a detergent is present in the extraction buffer. Buffers used for extraction of fungal antigens vary; phosphate buffered saline (PBS, pH 7.2) is commonly used for antigen coating of wells where carbohydrate epitopes are involved and bicarbonate buffers (pH 9.6) for systems employing antibodies recognising proteinaceous epitopes either PTA-ELISAs or DAS-ELISAs. The pH of extracts should always be checked because, if it is lowered considerably as is common in extracts from soils, it will affect the binding properties of the antigen to the microtitre wells (Thornton, pers. comm.). Most systems involve physically grinding or macerating the infected tissue but there is no consensus as to the most effective method of releasing the fungal antigens.

FUTURE PROSPECTS

MAb-based immunoassays are proving to be very useful as research tools particularly for tracking the spread of a specific fungi within plant tissues and in soils (Dewey *et al.*, 1995; Beckman *et al.*, 1994; Thornton, 1996b). Thus, it is almost certain that the sales of fungal-specific antisera and MAbs for research purposes will increase but growth and development of commercial kits is more questionable. The costs and time involved in transferring a research assay into a commercial kit are significant and are generally reflected in the purchase price of the kits. Sometimes, the costs are hidden; some kits, such as those developed by Ciba-Geigy and Du Pont for the detection of *Septoria tritici* and *Staganospora (Septoria) nodorum*, are used as a service to support the sale of fungicides. It is possible, therefore, that, in the foreseeable future, commercial immunoassay kits will only be sold for the early detection of diseases of high value crops where they are most cost effective.

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A NOVEL APPROACH FOR IMMUNOMONITORING AIRBORNE FUNGAL PATHOGENS.

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ABSTRACT

Monoclonal antibodies (MAB) were raised to germinating spores of *Alternaria brassicae*, the cause of dark leaf and pod spot in oilseed rape. A species-specific MAB, 34/7/G (IgG_1), was used to quantify the number of airborne spores in wind tunnel experiments. Spore samples were obtained with a novel spore trap providing samples compatible with direct enzyme-linked immunosorbent assay (ELISA) processing after the spores had been allowed to germinate. The number of germlings well^{-1} correlated well with the signal in ELISA allowing the detection of as few as 5 germlings well^{-1}. In wind tunnel experiments using airborne *A. brassicae* a close relationship was found between the estimated spore number using ELISA and the actual spore number when counted.

INTRODUCTION

There is a need for more accurate and reliable early warning schemes in disease forecasting to potentially improve disease control through more appropriate timing of fungicides and also avoid their unnecessary use. Pathogens can be well established before symptoms are visible and consequently control measures based on disease detection by direct observation of symptoms may be less efficient than they would have been had the risk for disease been detected earlier. For air-dispersed fungal pathogens the risk of a disease epidemic may be estimated by monitoring the amount of inoculum present at critical phenological stages of the crop. Current methods of estimating exposure to airborne inoculum are labour-intensive, time-consuming and the results may be biased according to the taxonomical skills of the personnel. Immunodiagnostic techniques have been used to identify fungal pathogens in soil samples and plant tissue. However, there has been little application of such techniques for detecting and quantifying air-dispersed fungal propagules.

METHODS AND RESULTS

Development of the approach

Conventional ELISA techniques for the detection of fungal spores rely on the extraction of target antigens from the sample. This is necessary because several types of fungal spores are too large to be effectively retained in ELISA wells and may be lost during washing steps and also because current spore traps do not provide samples compatible with direct ELISA processing. The amount of target antigen is either detected using a non-competitive ELISA by immobilising the extract on the surface of the reaction vessel or by competition ELISA using a reference target antigen as standard. The lack of standardised extraction methods and the possibility of antigen denaturation on antigen adsorption may introduce considerable variability in results or cause a complete failure of the assay (Soderquist & Walton, 1980; Friguet *et al.*, 1984; Jemmerson, 1987; Spangler, 1991).

The process of spore germination was found to immobilise the antigen in the reaction vessel. The use of germinating spores thus avoids antigen extraction and circumvents problems associated with antigen immobilisation. However, germinating spores were found to express endogenous alkaline phosphatase activity (EAPA), the enzyme used as a reporter molecule in the detection ELISA. EAPA was inhibited when spores were exposed on germination to a 60 % (v/v) aqueous solution of glacial acetic acid (AA) for 10 min at room temperature. Contrary to a report by Ponder and Wilkinson (1981), AA was not found to denature epitopes expressed by the antigen.

An amplification ELISA (AELISA) based on multiple, alternating applications of specific and secondary antibody was developed to increase the sensitivity of the assay. The rationale behind this technique was that the secondary antibody when applied in molar excess may bind the antigen-complexed MAB with only one paratope leaving the second free which can then bind another MAB during a second incubation of the specific MAB. This MAB which does not have to bind the antigen is then bound by further secondary antibodies during another incubation with the secondary antibody leading to assay amplification by increasing the epitope/reporter enzyme ratio.

Monoclonal antibody production

A female Balb\c mouse (10 weeks old) was given 5 intraperitoneal injections of homogenates of 0.9 - 5 x 10^5 germinating *A. brassicae* spores suspended in 0.5 ml of 0.85 % (m/v) saline. Two days after the final boost, the spleen cells were fused with SP2/0-Ag14 myeloma cells in a ratio of 1 : 10. Hybridomas were plated on macrophage-sensitised 96-well microtitre plates and culture supernatants (CSN) were screened by ELISA against spores which had been allowed to germinated in the ELISA wells overnight (Schmechel, 1996).

After the first screening, 18 clones (7 x IgG_1, 7 x IgM, 4 x IgG/IgM) were selected and tested for their degree of specificity. Unlike most antibodies, MABs 34/7/G (IgG_1) and 73/11/D (IgG_1/IgM) reacted only with the homologous fungus. Both MAB did not react with *Alternaria alternata*, *A. brassicicola*, *A. infectoria*, *A. linicola*, *Botrytis cinerea*, *Cladosporium herbarum*, *Epicoccum nigrum*, *Erysiphe graminis*, *Fusarium avenaceum*, *F. culmorum*, *F. poae*, *F.tricictum*, *Phoma lingam*, *Cylindrosporium concentricum*, *Metarhizium anisopliae*, *Stemphylium botryosum* and *Verticillium dahliae*. *Aspergillus* and *Penicillium* were also tested without determining their species affiliation. The binding of either MAB was eliminated by heat treatment or oxidation of the antigen by periodate suggesting the recognition of epitopes expressed at a junction of proteinaceous

and periodate-sensitive saccharide moieties of a glycoprotein. MAB 34/7/G was found to be more sensitive and chosen for further experimentation. Figure 1 shows the relationship between the optical density (OD) and the number of germlings of *A. brassicae* per well. The relationship was establish with the AELISA described below. MAB 34/7/G in combination with the AELISA is able to reliably quantify germling densities between 5 and 400 germlings well^{-1}. Antigen concentrations below 5 germlings well^{-1} are not high enough to generate OD values of ≥ 2.5 times the negative control values as the positive threshold level. Antigen concentrations of 400 germlings well^{-1} represent the upper detection limit of the assay.

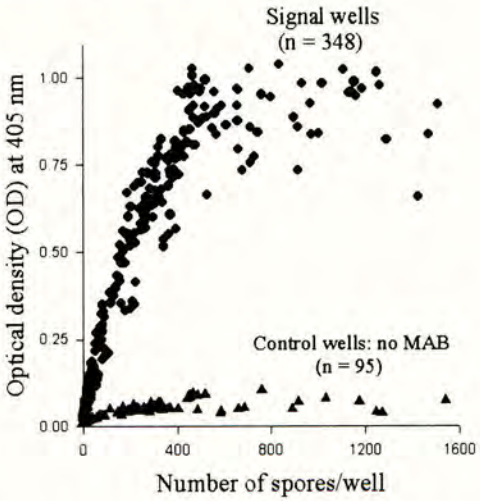

Figure 1 Relation between the number of *A. brassicae* germlings well^{-1} and optical density (OD) in ELISA using MAB 34/7/G after a substrate incubation time of 1 h.

Amplification ELISA (AELISA) format

In order to establish highly specific detection assays with high sensitivity the following AELISA format was developed:

1. Sensitisation of ELISA plates with fungal spores in appropriate germination buffer overnight to allow germination to facilitate effective antigen retention in the reaction vessel.
2. Inhibition of EAP activity using AA at 60 % for 10 min.
3. Washing of ELISA plates in phosphate-buffered saline (PBS) containing 0.05 % (v/v) Tween 20 for 3 min, 3 times.
4. Blocking of unoccupied binding sites on the reaction vessel surface with PBS containing 1 % (m/v) Nido milk powder for 30 min at 37 °C.
5. Washing as in step 3.
6. Incubation of CSN of MAB diluted 5 times with PBS containing 0.05 % (v/v) Tween 20 and 1 % (m/v) Nido milk powder (PBSTN) at 37 °C for 15 min.

7. Washing as in step 3.
8. Incubation of secondary antibody (alkaline phosphatase conjugate, SIGMA: Catalog No. A-8025) diluted 1000 times with PBSTN at 37 °C for 30 min.
9. Repetition of steps 5 - 8.
10. Washing as in step 3.
11. Incubation of the antigen-antibody-complex with *p*-nitrophenyl phosphate in diethanol-amine buffer at a strength of 1 tablet per 10 ml (SIGMA: Catalog No. 104-105). Determination of the OD (405 nm) after 1 h using an ELISA plate reader.

Development of a spore trap providing samples compatible with direct ELISA processing

Commonly used spore samplers such as Burkard spore traps or rotorod traps collect spores on a transparent adhesive-coated plastic tape. Spores are identified using light microscopy and the concentration of spores in the air can be estimated from spore counts, the nominal sample volume of the trap and the sampling time. Rotorods can collect relatively large numbers of spores because of their high volume sampling rate. This type of trap was therefore selected to be developed for use with immunological sampling analysis.

A rotorod trap was modified by replacing the adhesive-coated tape used as collection material by ELISA strip modules (Nunc: Immuno Module PolySorp, Catalog No. F8 469078). The modules, containing 8 ELISA wells, were attached to the rotating arms of the rotorod using 2 rubber bands which covered the third and fourth top wells leaving 6 wells per strip available for spore collection (Plate 1). The shape of the modules and the rotating arms were adjusted so that the majority of the spores were collected evenly across the bottom of the wells. The collection area per well was 56.6 mm^2 and the speed of rotation was about 2800 rev/min giving a nominal sample volume for the trap of 537 l min^{-1}. The trap was tested in Rothamsted's wind tunnel using spores of *Lycopodium clavatum* as a model particles. The collection efficiency of the trap was calibrated using a miniature Burkard spore trap as reference trap. The average collection efficiency of the novel trap was 0.7 for *L. clavatum* at wind speeds between 1 - 3 m s^{-1} and 0.4 for *A. brassicae* at wind speeds between 0.5 - 3 m s^{-1} (Schmechel, 1996). The difference indicates that spore shape may be important for this type of trap as the aerodynamic size of the two spore types are similar (Gregory, 1973; McCartney *et al.*, 1993). The configuration of the novel trap is shown in Plate 1.

The strips can easily be detached following sampling and processed by ELISA without any sample handling or manipulation beyond germinating the spores. Figure 2 shows a comparison of the number of spores of *A. brassicae* per well when counted and estimated using MAB 34/7/G, the AELISA and a standard curve derived form spore numbers between 1 and 400 per well. Although there was a good correlation between the counted spore number and the OD in ELISA, $r^2 = 0.87$, the actual spore number was underestimated using the ELISA technique. This was probably due to spores accumulating in specific areas in the bottom of the ELISA wells leading to mutual inhibition of growth resulting in fewer epitopes per spore. The unequal distribution of the spores was probably because the configuration of the trap was developed with *L. clavatum* rather then *A. brassicae* spores. An adjustment of the angular arrangement of

the modules may improve the accuracy of the ELISA estimation.

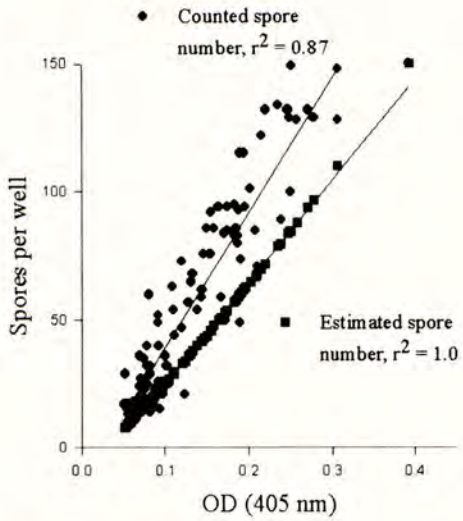

Figure 2 Comparison of the number of spores well^{-1} when counted (●) and estimated by AELISA using MAB 34/7/G (■). The substrate was incubated for 1 h.

CONCLUSIONS

An immunological approach based on MAB and ELISA has been developed to identify and quantify viable airborne fungal inoculum. Wind tunnel experiments led to the development of a novel spore trap which provides samples compatible with direct ELISA processing. A good correlation between actual and estimated airborne spore numbers of *A. brassicae* was obtained. An amplification ELISA has been developed which detected as few as 5 spore germlings per ELISA well.
This approach harmonises sampling-, processing- and analyzing system of air-samples. It may potentially reduce the variability of results by reducing the experimental error leading to improved vertical (between-days) and horizontal (between-laboratories) comparability and reproducibility of estimates of airborne fungal inoculum. This may not only support the development of standardised methods but also enhance the diagnostic and predictive value of forecasting models.

ACKNOWLEDGEMENTS

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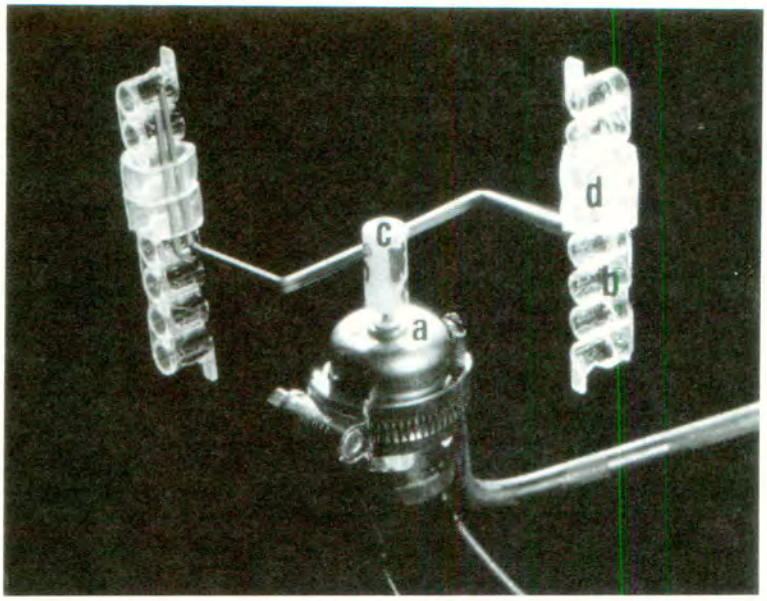

Plate 1 Configuration of the novel spore trap: (a) 12 V electric motor, (b) modified ELISA strip module, (c) modified angular support arm, (d) 6 mm wide rubber bands.

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DEVELOPMENT OF A MULTIPLEX PCR SEED HEALTH TEST TO DETECT AND DIFFERENTIATE THREE PATHOGENS OF BARLEY

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ABSTRACT

The fungal pathogens *Pyrenophora* spp. cause major economic losses of barley crops world-wide. Conventional seed health tests are time-consuming and labour-intensive. The PCR could provide an effective alternative. RAPD-PCR, followed by cloning and sequencing of products, enabled specific primers to be designed for use in PCR. Two sets of primers, PT2F & PT2R and PM1F & PM1R, are specific for *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* respectively. Further work will include the development of a set of specific primers for the detection of *P. graminea* and the incorporation of the three primer sets into a multiplex seed health test for *Pyrenophora* spp.

INTRODUCTION

Barley diseases caused by *Pyrenophora* spp. result in severe economic loss for growers in many different countries. Three distinct types of disease are found: leaf stripe symptoms, caused by *P. graminea*; net blotch, caused by *P. teres* f. sp. *teres*; and the spot form of net blotch, caused by *P. teres* f. sp. *maculata*.

These species have different forms of disease transmission. *P. graminea* is transmitted entirely by seed and can not cause disease by direct leaf infection, whereas local transmission from plant debris is more significant for *P. teres* spp. Hence unambiguous identification of the pathogens is necessary for development of effective control strategies. Traditionally, seeds are tested for the presence of *Pyrenophora* spp. by incubating seeds on agar or blotting paper for a week (Rennie & Tomlin, 1984). The morphological characteristics of the pathogen are then observed using a microscope. However, these techniques are both time-consuming and labour-intensive and fungal identities can sometimes only be confirmed after prolonged pathogenicity testing.

Therefore a need exists to develop a simple, rapid and effective test for the identification of *Pyrenophora* spp. New DNA-based technologies have great potential for use in such tests and have already been successfully applied to some systems. Two forms of DNA-based disease diagnosis were considered for this system. Firstly, the use of species-specific probes (e.g. Johanson *et al.*, 1994) and secondly, utilisation of the polymerase chain reaction (PCR). The latter has the advantages of rapidity and extreme sensitivity as well as the potential for simultaneous detection of several pathogens in a multiplex reaction containing several sets of primers (Cadieux *et al.*, 1993). Hence, a PCR-based approach was taken here.

The aim of this work was to develop a multiplex PCR seed health test to allow unambiguous identification of *Pyrenophora* spp. on barley. Initially, two strategies were taken to design specific primers for use in PCR. Firstly, DNA fingerprinting techniques. These are based on random PCR amplification with arbitrary primers and have proven to be powerful tools for genetic analysis elsewhere, particularly as no prior sequence information is required (Welsh & McClelland, 1990). Random Amplified Polymorphic DNA (RAPD) has been used both in the development of species-specific DNA probes (Johanson *et al.*, 1994) and in the identification and characterisation of pathotypes (Kelly *et al.*, 1994). Preliminary work by Reeves & Ball (1991) suggested the potential value of RAPDs in differentiation of *Pyrenophora* spp. on barley. In our study, the generation of RAPD profiles was followed by identification of species-specific bands. These were then cloned and sequenced. The sequence information was used to design species-specific primers for use in PCR.

A second approach was to design specific primers based on sequence information from the Internal Transcribed Spacer (ITS) region of ribosomal genes. This has been successfully done in other organisms to develop specific diagnostic tests (Xue *et al.*, 1992). Sequence information for the ITS regions was obtained by PCR using the universal primers designed for this purpose (White *et al.*, 1990).

The specificity of the designed primers in PCR was investigated under different reaction conditions to optimise the PCR. Those primers that specifically detected DNA from a particular *Pyrenophora* species could then be used in the design of a multiplex seed health test.

MATERIALS AND METHODS

Source and preparation of fungal DNA

Pyrenophora spp. were isolated from barley seed that had a wide geographical origin, including Denmark, Sweden, USA and the UK. Surface-sterilised seeds were incubated on Potato Dextrose Agar (PDA) plates at 22° C for 7 days. After visual identification of the seed-borne pathogens and saprophytes, the *Pyrenophora* and fungal saprophytic isolates were each individually sub-cultured onto PDA.

A loopful of mycelium was inoculated into 50 ml of Potato Dextrose Broth and incubated for 7-10 days at 25° C, 100 rev/min. DNA extraction was performed according to Raeder & Broda (1985). The DNA was quantified using a TKO 100 Mini-Fluorometer (Hoefer Scientific Instruments) and diluted to 10 ng/µl.

PCR amplification

All PCR reactions were based on a standard set of conditions. Each 25 µl reaction contained the following: 1 x PCR buffer (Perkin Elmer Cetus); 0.2 µM each primer (Genosys Biotechnologies); 200 µM each dNTP (Pharmacia Biotech); 1.5 - 4.0 mM $MgCl_2$; 0.625 units Amplitaq ® polymerase (Perkin Elmer Cetus); and 10 ng DNA template. Reaction volumes were made up to 25 µl with sterile distilled water. The cycling conditions for different reactions are summarised in Table 1.

Table 1. Cycling conditions for RAPD-PCR and specific PCR with ITS primers.

	RAPD-primer PCR	ITS primer PCR
Denaturing step	94 ° C, 1 min.	92° C,1 min
Annealing step	35 ° C, 1 min	58° C, 1 min
Extension step	72 ° C, 1 min	72° C, 1 min
Number of cycles	45	30
Final extension step	72 ° C, 5 min	72° C, 5 min
$MgCl_2$ conc. (mM)	4.0	1.5

For the specific primers, optimisation of the PCR cycling conditions was done by manipulation of $MgCl_2$ concentration and annealing temperature. PCR products were analysed by electrophoresis on a 1.4 % agarose TBE gel at 100 V for up to 5 hours, before detection by ethidium bromide staining.

Southern Analysis

DNA was transferred to a nylon membrane as described by Sambrook *et al.*, (1989). DNA probes were made by cutting DNA bands from agarose gels and eluting the DNA using the Qiaquick (Qiagen) system as directed by the manufacturer. The DNA probes were non-radioactively labelled and hybridised using the digoxigenin system (Boehringer Mannheim), according to the instructions given.

Cloning and sequencing

Some DNA amplification products obtained in RAPD-PCR allowed differentiation between *Pyrenophora* spp. These were cloned into a Promega pGEM1 vector. The ends of the fragments were sequenced using the M13 Universal sequencing primer in association with the ABI Prism ™ Dye Terminator Cycle Sequencing Ready Reaction Kit, containing Amplitaq ® DNA polymerase, according to the manufacturer's instructions. Sequencing of fragments from the internal transcribed spacer region of ribosomal genes was also done, using the universal ITS1 (TCCGTAGGTGAACCTGC GG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al.*, 1990).

Primer selection

RAPD-PCR was performed using primers (Genosys Biotechnologies) ranging from 10 to 17 nucleotides in length. Primers for specific PCR were designed using DNAstar software based on sequence information obtained as described above.

Sensitivity determination

A dilution series of DNA was prepared for each species of *P. teres*, ranging from 100 ng/µl to 100 fg/µl. 1 µl of each dilution was added to 24 µl of PCR reaction mixture containing specific PCR primers. Following amplification the products were analysed

on 1.4 % agarose TBE gels to determine the minimum amount of DNA that could be detected.

RESULTS & DISCUSSION

Fifty-four RAPD primers were screened under the standard PCR conditions for their ability to differentiate between the *Pyrenophora* species. Three of these gave consistent and reproducible fingerprints, as summarised in Table 2 below:

Table 2. RAPD primers

Primer Name	Primer Sequence	Name of Species	Size of Species Specific DNA amplified Product
M13-20	GTAAAACGACGGCCAGT	*P.teres*	1.3 kb
Primer 60	CGGTTTGGTC	*P.teres*	1.4 kb
Primer 72	GGACGATTCG	*P.teres* f. sp. *maculata*	1.9 kb

Primer M13-20 was additionally screened against 28 *P. graminea* and 107 *P. teres* f. sp. *teres* isolates as part of a trial on seed from an International Seed Testing Association (ISTA) comparative test. *P.teres* f. sp. *teres* was distinguished by the presence of the 1.3 kb band. These isolates were also identified microscopically and the results compared to those from the M13-20 screen. The microscopic identification supported the results of the DNA identification. Hybridisation work demonstrated that whilst this band is only present in *P. teres* f. sp. *teres* it shares some homology with other bands amplified from both species. This may be due to several priming events occurring in the same stretch of DNA or to the amplification of a repeated sequence. However, there is no homology with the amplified products of any of the fungal saprophytes investigated, including the closely related *Cochliobolus sativus*.

On the basis of these results, specific primers were designed for PCR using sequence information. Primers PM1F and PM1R are specific for *P.teres* f. sp. *maculata*, whereas primers PT2F and PT2R are specific for *P. teres* f. sp. *teres*. Initially, the PCR was performed at 1.5 mM $MgCl_2$ and at an annealing temperature calculated based on the GC content of the primers. These parameters were then manipulated to optimise the PCR. The results of the PCR optimisation are summarised in Table 3.

Table 3. Optimised PCR conditions for each set of specific primers.

	PM1 F & R primers	PT2 F & R primers
Denaturing step	94 ° C, 1 min	94 ° C, 1 min
Annealing step	70 ° C, 1 min	67 ° C, 1 min
Extension step	72 ° C, 1 min	72 ° C, 1 min
Number of cycles	30	30
Final extension step	72 ° C, 5 min	72 ° C, 5 min
$MgCl_2$ conc. (mM)	2.5	2.5

Sensitivity analyses show that the PM1 F & R primers will specifically detect *P. teres* f. *maculata* at DNA levels as low as 100 pg. Initial results suggest a similar degree of sensitivity for the detection of *P. teres* f. sp. *teres* by the PT2 F & R primers. Both sets of primers were also screened against a range of saprophytes and other species of *Pyrenophora*, including *P. avenae* and *P. hordei*. In all cases, amplification was of a specific band from the target species' DNA only, this being a 1.9 kb DNA amplification product from *P. teres* f. sp. *maculata* and a 1.4 kb DNA amplification product from *P. teres* f. sp. teres. The results are summarised in Table 4.

Table 4. Isolates of fungal DNA screened with specific primers

SPECIES	Number of isolates screened	Amplification with Primers PM1 F & R	Amplification with Primers PT2 F& R
P.teres f. sp. *maculata*	5	YES	NO
P.teres f. sp. *teres*	19	NO	YES
P.graminea	13	NO	NO
P.avenae	7	NO	NO
P.hordei	2	NO	-
Saprophytes	12	NO	NO

Primers are currently being designed from the ITS region sequence information. Sequencing of the ITS region was done for 5 isolates of each of the three *Pyrenophora* species, using primers ITS1 and ITS4. Initial results show that there is a high degree of sequence conservation both within and between species. However, there is some interspecific sequence variation from which it may be possible to design specific primers for detection of *P. graminea.*

Future work will concentrate on how specific primers can be incorporated into a multiplex seed health test. *Pyrenophora* spp. are found in the outer regions of barley seed and thus it should be possible to obtain DNA by soaking the seeds and using the resulting liquor in PCR. However, preliminary experiments are presenting problems with PCR inhibition by seed products. It will also be necessary to develop a set of PCR conditions in which three specific primer sets can amplify their target DNA simultaneously. The two sets of specific primers designed so far have different optimal conditions for amplification. Initial results indicate that neither primer set amplifies effectively and specifically under the other's optimal conditions. Such difficulties must

be resolved to develop an efficient and reliable seed health test. A long-term aim of this project is for automation of the system and elimination of the gel electrophoresis step, thereby making it a more rapid and cost-effective seed health test.

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DEVELOPMENT OF A DNA DIAGNOSTIC FOR DISEASE DETECTION BASED ON THE ß-TUBULIN GENE

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ABSTRACT

Integration of modern disease diagnostic methods into a decision framework supporting more effective fungicide use requires robust methodology, coupled with good disease quantification. PCR technologies offer many new diagnostic opportunities, but methods currently available lack the ease and reliability of ELISA-based immunoassays. This paper describes initial attempts to combine specific PCR amplification of a ß-tubulin gene fragment of *Rhynchosporium secalis* and its hybridization to a labelled oligonucleotide probe to provide an ELISA- based assay format. A poly-A tailed double stranded, PCR fragment was captured on poly-T coated magnetic beads and hybridized to a biotinylated probe. Successful hybridizations were detected using streptavidin alkaline phosphatase linked to a colour detection system. Positive hybridizations yielded only low signal intensities when compared with hybridization to a single-stranded PCR product. It is suggested that the double stranded ß-tubulin target sequence either reannealed too rapidly preventing maximum hybridization, or assumed a secondary structure, which buried the biotin so that it was no longer accessible to streptavidin.

INTRODUCTION

PCR technologies have created many new diagnostic opportunities, and identification of pathogens using this methodology is now almost routine. However, to guide fungicide use diagnosis requires not only correct disease identification, but also accurate quantification to allow both fungicide choice and dose rate to be matched with disease level. Unfortunately, the robustness of ELISA-based immunoassays is not yet matched in PCR methods, although considerable effort is now being directed towards developing simplified formats, which also allow accurate identification and quantification of PCR products. Where antigens are produced only shortly before symptom expression, DNA-based diagnostics may provide earlier pre-symptomatic detection than that achieved by immunoassays, allowing better exploitation of the curative properties of some fungicides.

Our work so far has concentrated on the detection of benzimidazole resistance in cereal pathogens, especially in *Rhynchosporium secalis* (Wheeler *et al.*, 1995), but specificity within the ß-tubulin target gene should permit detection of benzimidazole resistance to be coupled with disease identification and measurement. Nested-PCR

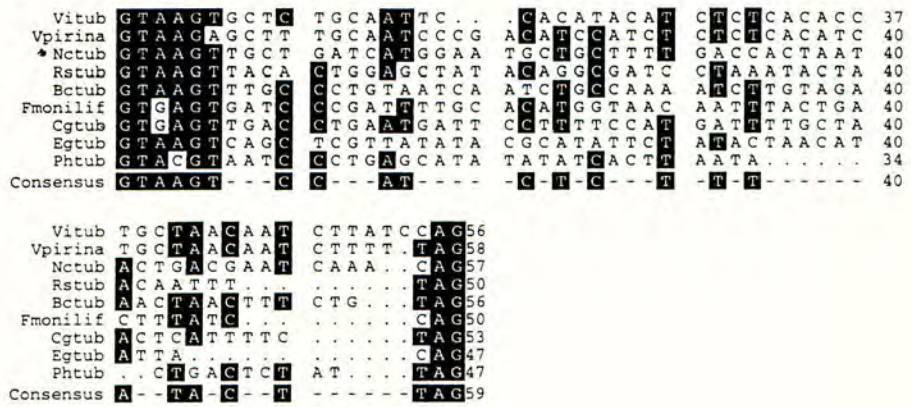
allows direct amplification of target ß-tubulin gene fragments from plant material without the need to isolate DNA. Detection of this target DNA using allele-specific oligonucleotide probes, has been limited to dot blots on charged nylon membranes, which cannot readily be quantified. This paper explores initial attempts to transfer membrane-based technology into a micro-titre plate format.

METHODS AND RESULTS

Detection specificity

DNA sequence analysis of the ß-tubulin gene from several fungi identifies considerable sequence identity within the amino-acid coding region, but exposes extensive variation within introns (Figure 1). Although some fungi lack intron 6 this intron has been particularly useful for specific detection of benzimidazole resistance. By designing an outer nested-PCR primer to match either an internal sequence within intron 6, or covering the equivalent of the splice region where intron 6 is lacking, we have specifically amplified a ß-tubulin gene fragment from several cereal pathogens, including distinguishing between different *Fusarium (Giberella)* spp. (Lu Yuejian, personal communication, 1995).

Figure 1. Intron 6 sequences of the ß-tubulin gene from several fungi.



Eg tub = *Erysiphe graminis* (Sherwood & Somerville, 1990)
Nc tub = *Neurospora crassa* (Orbach *et al.*, 1986)
Cg tub = *Colletotrichum graminicola* (Panaccione & Hanau, 1990)
Bc tub = *Botrytis cinerea* (Yarden & Katan, 1993)
V1 tub = *Venturia inaequalis* (Koenraadt *et al.*, 1992)
F.monilif = *Fusarium moniliforme* (Lu Yuejian, unpublished)
V.pirina = *Venturia pirina* (Hollomon & Ishii, unpublished)
Ph. tub = *Pseudocercosporella herpotrichoides* (Baggett, unpublished)
Rs. tub = *Rhynchosporium secalis* (Wheeler *et al.*, 1995)

Capture and hybridization of target ß-tubulin sequence

To explore the capture on magnetic beads of a ß-tubulin target gene fragment and its hybridization to an oligonucleotide probe, we amplified a 349 bp poly A-T tailed PCR fragment from genomic DNA from *R. secalis* strain 1130. This strain is benzimidazole sensitive, and has the wild type sequence:

196		198		200
TCT	GAT	GAG	ACC	TTC
Ser	Asp	Glu	Thr	Phe

around ß-tubulin amino acid codons 198 and 200, which are known to be involved in benzimidazole resistance (Hollomon & Butters, 1994). Conditions for the PCR reaction have been described elsewhere (Wheeler *et al.*, 1995). The outer pair of primers used in the nested PCR were:

R1 5' G G C A A C T T T C T C T G T T G T T C C A T G 3'

R3 5 A G G A T C G C C T G T A T A G C T C C A G T G T 3'

The inner primers were:

RN1 5' C A A G G T C T C C G A C A C C G T G G T A G A 3'

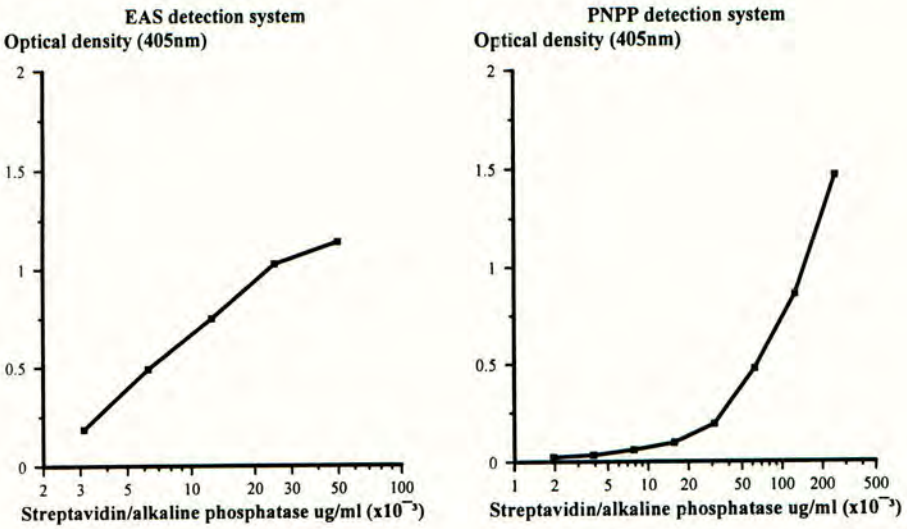
RN2-A 5' A^{25} G A A A G A G T G G G C T C C A C G G C T G G T 3'

Double-stranded hybridization

Solution hybridization to the biotinylated 15-mer oligonucleotide probe (2ng) (TCTGATGAGACCTTC), and capture of the target DNA fragment on magnetic beads (Dynal, Oslo, Norway) was carried out essentially according to the method described by Suzuki *et al.*, (1993) for detecton of HIV. PCR reaction mixture (8 µl) containing amplified target (about 200 ng DNA) was mixed with hybridization buffer (0.75 M NaCl; 0.075 M Na citrate, pH 7.0) containing 2 ng probe. DNA was denatured by heating at 100°C for 10 min, and transferred to fresh Eppendorf tubes containing 20 µl poly-T coated magnetic beads prepared according to the manufacturer's instructions. Reactions were cooled to room temperature, and beads washed (10 mM Tris-Cl, pH 7.6; 150 mM NaCl; 1 mM EDTA; 0.001% Tween 20) to remove unbound components. Streptavidin alkaline phosphatase (100 µl GibcoBRL, Paisley, UK; 1 µg ml^{-1}) in TBS (0.01 M Tris pH 7.5; 0.15 M NaCl) was added to each Eppendorf tube, and removed after three 10 min washings with buffer. Colour was developed using either *p*-nitrophenylphosphate (PNPP) as substrate for the alkaline phosphatase, or an ELISA amplification system (EAS, GibcoBRL, Paisley, UK) according to the manufacturer's instructions. EAS amplifies the alkaline phosphatase signal by coupling through NADPH-NADH to produce a deep red formazan colour. Reactions were stopped by addition of 0.3N sulphuric acid (50 µl) and read in a micro-titre plate reader (Dynatech Instruments, Billingshurst, UK) at 490 nm (EAS) or

405 nm (PNPP). Control reactions were either without target ß-tubulin DNA or probe. A standard curve of colour production was prepared for each detection system using different dilutions of streptavidin alkaline phosphatase (Figure 2).

Figure 2. Standard curves for EAS and PNPP detection of alkaline-phosphatase.



Single stranded hybridization

To obtain a single stranded PCR product, a biotinylated RN2 primer was used instead of a poly A-tailed one. The PCR product (10 µl) was bound to streptavidin coated magnetic beads (Dynal M-280) according to the manufacturer's instructions for direct solid-phase sequencing, and denatured with freshly prepared 0.1N NaOH. The non-biotinylated sense strand was removed by washing, and the remaining biotinylated antisense strand hybridized at room temperature (15 min) to a digoxygenin labelled 15-mer probe (Genosys Biotechnologies, Texas; 2ng) instead of the biotinylated one used in the double stranded experiment. Anti-digoxygenin coupled alkaline phosphatase (Boehringer Mannheim, Germany; 0.75 units ml^{-1} in TBS) was added to each Eppendorf tube and removed after 10 min by washing with wash buffer. Colour was developed using EAS.

Detection of hybridization

Although the EAS detection system produced a somewhat higher background, it was at least 10 times more sensitive than PNPP. All hybridization reactions produced optical density readings significantly above control levels (Table 1).

Table 1. Hybridization of an allele-specific oligonucleotide probe to single stranded and double stranded PCR ß-tubulin target fragments.

	Optical Density$_{405}$
	EAS
No probe	0.065
No template	0.014
Double stranded PCR product	0.219*
Single stranded PCR product	1.379*

* Significantly different from control (Students "t" test)

Signals generated from a double stranded PCR fragment were much lower than those using a single stranded product. Increasing the quantity of probe, magnetic beads, streptavidin alkaline phosphatase, or probe length to 21-mer, all failed to increase signal intensity. Other experiments using a PCR product generated with a biotinylated RNI PCR primer and RN2-A as the other primer, and monitoring capture by poly-T using streptavidin alkaline phosphatase, confirmed that the PCR product was captured as expected.

DISCUSSION

The lack of any significantly increased signal after adding additional probe, magnetic beads, or alkaline phosphatase, suggests that under the conditions used in our experiments, probe hybridization events to the double stranded PCR product were saturating. Since the poly-T sites were likely to be well in excess of the available poly-A sequences on the target DNA, and poly A-T duplex formation occurs at temperatures below 40°C, poor signal intensity did not result from a failure to capture sufficient target DNA. Instead, the double stranded target DNA may have reannealed before the probe could hybridize to sufficient sites, or assumed a secondary structure which buried the biotin attached to the probe so that it was no longer accessible to the streptavidin.

These results suggest that successful transfer of hybridization-based diagnostic

technologies to magnetic beads, or direct capture on poly-T coated to micro-titre plates to provide good quantification will either require some methodology that delays reannealing of the double stranded PCR product to allow better access of the probe, or that a simple procedure must be found to generate a single stranded PCR product without the need to denature it with NaOH. We are actively exploring approaches to overcome these difficulties.

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DETECTION OF CROP FUNGAL PATHOGENS BY POLYMERASE CHAIN REACTION TECHNOLOGY

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ABSTRACT

We have developed polymerase chain reaction (PCR) assays specific for the wheat pathogens *Stagonospora nodorum* (formerly *Septoria nodorum*), *Septoria tritici* and the banana pathogens *Mycosphaerella fijiensis* and *M. musicola*. We have integrated enzyme-linked immunosorbent assay (ELISA) technology into one of these PCR-based assays to develop a microtiter plate format for quantification of disease pressure. These assays are therefore very well suited for automation and large-scale application.

INTRODUCTION

The determination of nucleotide sequences of many plant pathogens has made possible the development of PCR assays for the detection and differentiation of several pathogens. Due to its enormous sensitivity, specificity and short development time PCR provides a good alternative to other diagnostic methods. Lee and Taylor (1990) showed PCR can be used to amplify specific DNA fragments from a single ascospore. We have previously demonstrated PCR can be used to identify the presence of a fungal pathogen before the onset of visible disease symptoms (Beck & Ligon, 1995). In addition, PCR primers can be designed to differentiate fungi at the species level. This provides a very high level of specificity in these assays. Species-specific PCR assays can be developed more rapidly and at lower cost than similar serological assays. For these reasons, plant disease diagnostics based on PCR technology are likely to become a prominent part of integrated pest management.

DEVELOPMENT OF SPECIES-SPECIFIC PCR PRIMERS

Although the rDNA genes of related organisms are generally highly conserved, the internal transcribed spacer (ITS) regions are usually less well conserved (White *et al.*, 1990). PCR primers made to the nuclear subunit rDNA regions flanking the ITS regions were used to amplify and clone potential polymorphic sequences from several pathogenic fungal species (Figure 1). The ITS sequences were compared and species-specific primers were designed to the polymorphic ITS regions. These ITS-derived primers were used to amplify specific fragments from the fungus from which they were designed, thus allowing the detection of the pathogens. The primers also successfully amplified similar-sized fragments from crop tissues infected with the respective fungi. Previously, this technique has been used to detect viral (Rojas *et al.*, 1993) and fungal (Elliott *et al.*, 1993) plant pathogens. We have applied this method to develop PCR assays for the detection of *S. nodorum*, *S. tritici*, *M. fijiensis* and *M. musicola*.

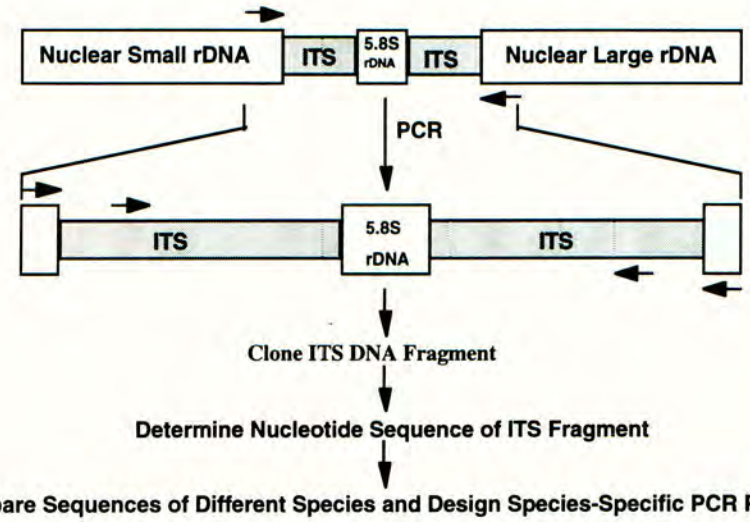

Figure 1. Designing PCR primers for diagnostics. Locations on nuclear rDNAs of PCR primers. The arrowheads represent the 3' end of each primer.

DETERMINATION OF PRIMER SPECIFICITY

After the design and synthesis of the primers, they were tested for their ability to PCR amplify specific ITS fragments from the purified genomic DNAs from which they were designed. The primers were also tested for their ability to detect the targeted pathogen in infected plant tissue and for their inability to cross-react with purified DNA from other indigenous pathogens.

S. nodorum-specific primers

The *S. nodorum*-specific primers JB433 (5'-ACACTCAGTAGTTTACTACT-3') and JB434 (5'-TGTGCTGCGCTTCAATA-3') amplified a 448-bp fragment from all of the *S. nodorum* isolates tested in Figure 2. The *S. nodorum*-specific primers did not produce any amplification products when purified genomic DNA was tested from the following cereal pathogens: *Pseudocercosporella herpotrichoides, Pseudocercosporella aestiva, Ceratobasidium cereale, Drechslera sorokiniana, S. tritici, Septoria glycines* and *Septoria passerinii* (Figures 2 & 3). In addition, the *S. nodorum*-specific primers amplified a 448-bp fragment from wheat tissue infected with *S. nodorum* and from wheat tissue infected with both *S. nodorum* and *S. tritici*, however, not from healthy wheat (Figure 3). Furthermore, a wheat sample visually assessed and determined to be infected with *S. tritici* produced a faint amplification product with the *S. nodorum* primers (Figure 3).

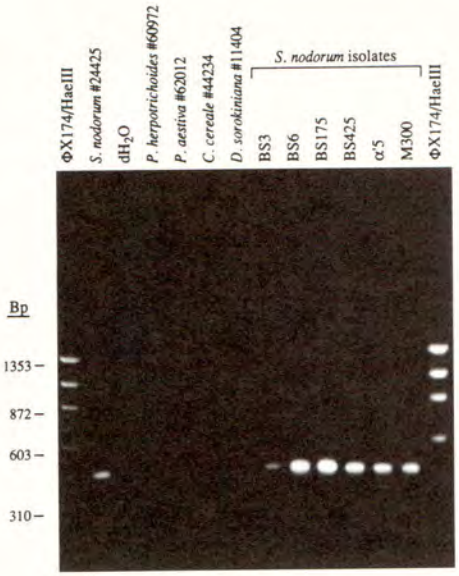

Figure 2. Agarose gel of PCR products from *S. nodorum* - specific primers amplified from fungal DNA of cereal pathogens and *S. nodorum* isolates.

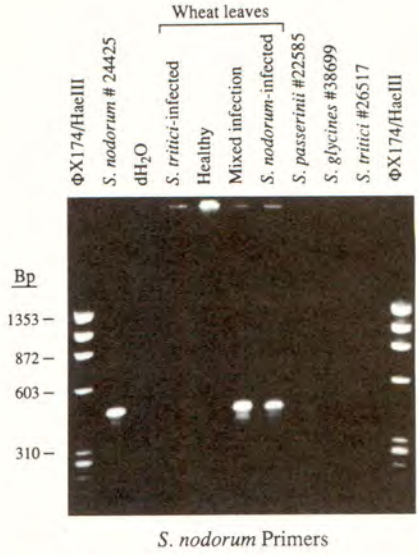

Figure 3. Agarose gel of PCR products from *S. nodorum* - specific primers amplified from infested wheat leaves and other *Septoria* spp. DNAs.

S. tritici-specific primers

All the *S. tritici* isolates that were tested (Figure 4) produced a 345-bp amplification product using the *S. tritici*-specific primer, JB446 (5'-CGAGGCTGGAGTGGTGT-3'), and the nuclear small rDNA-specific primer, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (White *et al.*, 1990). The *S. tritici*-specific primers did not amplify DNA fragments from any of the purified genomic DNAs from the cereal pathogens listed above for *S. nodorum* testing (Figures 4 & 5). These primers also did not amplify DNA fragments from the phylogenetically similar pathogens, *M. fijiensis* and *M. musicola* (Figure 4). In addition, the *S. tritici*-specific primers amplified a 345-bp fragment from wheat infected with *S. tritici*, but not from healthy wheat or wheat infected with *S. nodorum* (Figure 5).

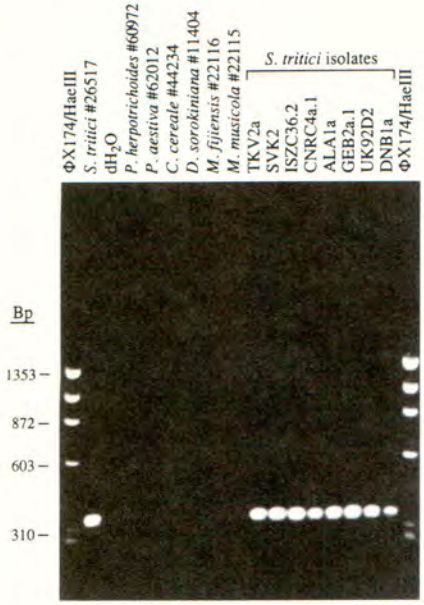

Figure 4. Agarose gel of PCR products using JB446 and ITS1 primers with fungal DNA of cereal and banana pathogens and *S. tritici* isolates.

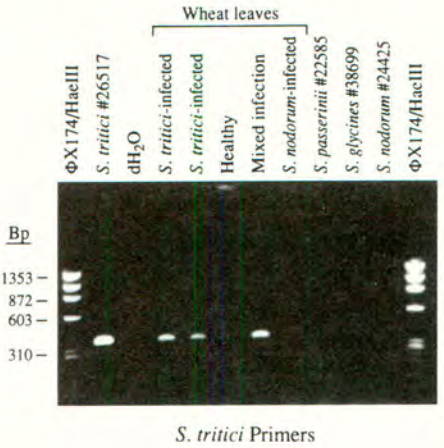

Figure 5. Agarose gel of PCR products using the *S. tritici* - primers with fungal DNA from *Septoria* spp. and DNA from wheat leaves infected with *Septoria*.

Additional PCR assays

The same methodology was used to develop PCR primers specific for the banana pathogens *M. fijiensis* and *M. musicola.* These were able to detect and differentiate the causative agents of Sigatoka.

DEVELOPMENT OF HIGH-THROUGHPUT, QUANTITATIVE PCR ASSAYS

We have investigated several different methods for quantifying PCR products. The *S. nodorum* diagnostic primers were integrated into a quantitative colorimetric assay format as described by Nikiforov *et al.* (1994). This colorimetric assay incorporates a phosphorothioate-modified primer which is also biotin-labeled, and an unmodified second primer into the polymerase chain reaction (Figure 6). The double-stranded PCR product was made single-stranded by treatment with the enzyme T7 gene 6 exonuclease. The PCR strand which contains the phosphorothioate-modified primer is protected from the hydrolytic activity of this enzyme, whereas the unmodified primer strand is degraded. The single-stranded PCR product was hybridized to a capture primer immobilized on a 96-well plate. A horseradish peroxidase-labeled antibody specific for biotin was used to detect the amount of hybridized PCR product colorimetrically.

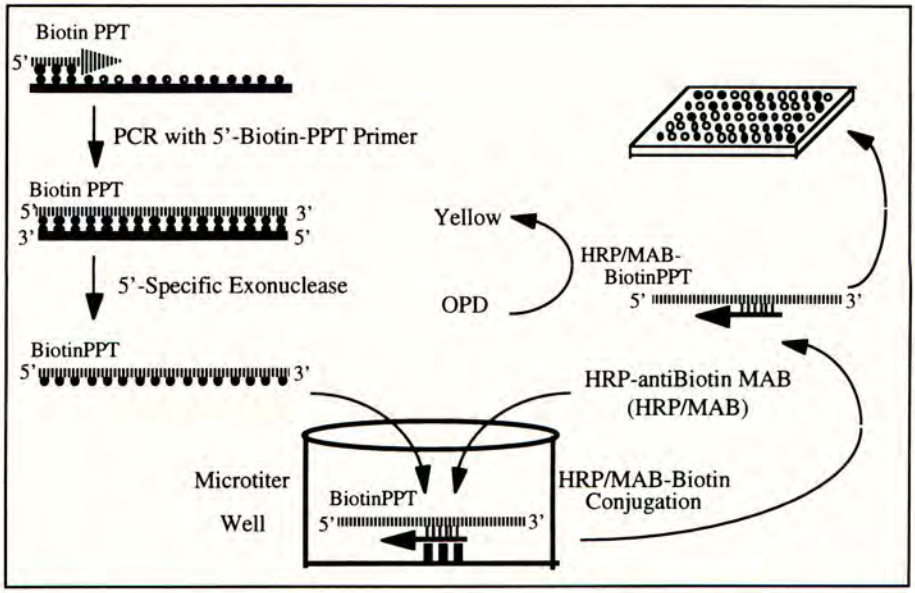

Fig. 6. Colorimetric quantification of PCR products using the method of Nikiforov *et al.* (1994).

The 5' *S. nodorum*-specific primer (JB433) was modified to contain a biotin label and four phosphorothioate bonds at the 5' end. An additional primer, ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White *et al.*, 1990) was used for capturing the PCR product produced from amplification with the *S. nodorum* primers. Purified genomic *S. nodorum* and wheat extracts were assayed by PCR. *S. nodorum*-specific primers resulted in the amplification of a 448 bp fragment from *S. nodorum* genomic DNA and from wheat extracts numbered 3, 4 and 5 (Table 1). No amplification products were produced with the water control or with wheat extract number 9 (Table 1). Subsequent colorimetric analysis of the PCR products from the amplification with the *S. nodorum*-specific primers produced the colorimetric results in Table 1. All of the amplification products produced with the *S. nodorum*-specific primers produced a positive colorimetric value. Furthermore, the colorimetric value decreased as the amount of infecting fungus decreased. The colorimetric background was below A_{492}= 0.100 with the water control and healthy wheat extract.

ELISA values were also determined on the same wheat extracts (Table 1) using Ciba's "Septoria Watch"/"Septotest" assay (Smith *et al.*, 1994). As with the PCR assay, significantly positive ELISA values were found for wheat extracts numbered 3, 4 and 5 (Table 1). Comparison of the ELISA values and colorimetric PCR values showed a strong correlation between the two assays (Figure 7).

Table 1. Comparison of ELISA and PCR amplification of DNA for the detection of *S. nodorum* in wheat extracts.

Extract#	ELISA[a]	Colorimetric[b]	EtBr Gel[c]
S. nod. DNA	NT[d]	1.389	+++
dH20	NT[d]	0.068	-
9	0.086	0.039	-
3	0.621	0.376	+
4	1.761	0.849	++
5	3	1.031	+++

[a]ELISA values are in OD_{650} (optical density at 650 nm) units.
[b]Colorimetric values are in OD_{492} units.
[c]-, no visible band; +, faint band; ++, clear band; +++, strong band.
[d]Not tested

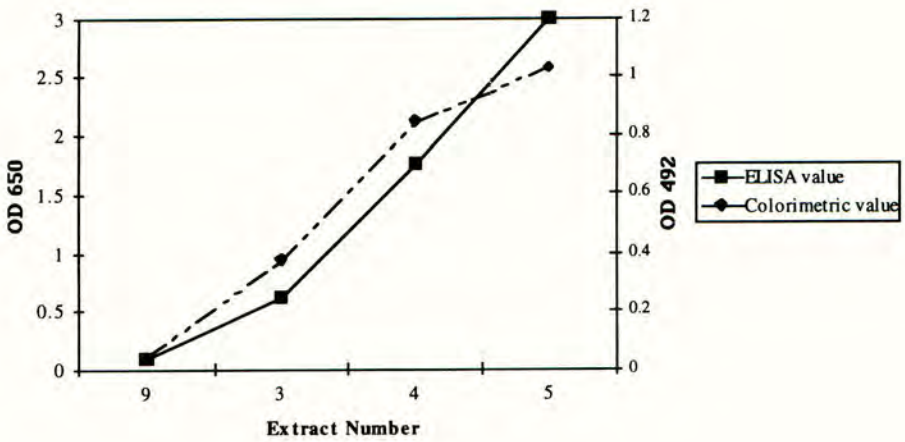

Figure 7. Comparison of ELISA and PCR amplification of DNA for the detection of *S. nodorum* in wheat extracts.

We also adapted the method described by Holmstrøm *et al.* (1993) using our *S. nodorum* primers (Figure 8). This method is based on using a biotin labeled PCR primer and incorporating digoxigenin-11-dUTP during the PCR extension step. After thermocycling, the PCR products are captured in microtiter wells containing immobilized streptavidin. Subsequently, alkaline phosphatase conjugated antibody specific for digoxigenin is bound to the incorporated digoxigenin, and a colorimetric result is produced by addition of *p*-nitrophenyl phosphate.

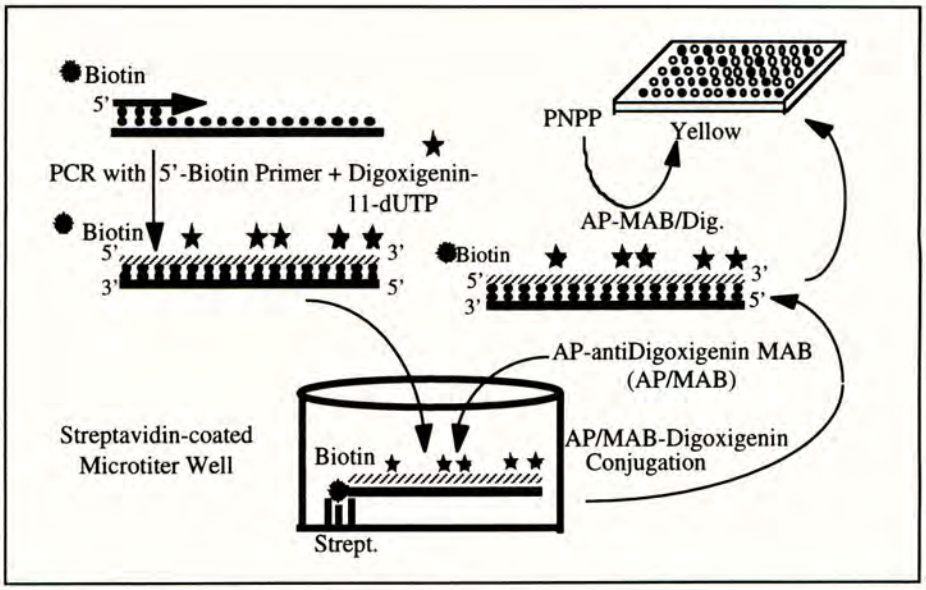

Figure 8. Colorimetric quantification of PCR products using the method of Holmstrøm *et al.* (1993).

We have attempted to quantify PCR products using several different methods. By adapting ELISA-type detection methods for PCR products into our assay, we have a microtiter plate format for assessing disease pressure quantitatively. The microtiter plate format permits high throughput analysis of plant samples.

CONCLUSIONS

Crop disease diagnostics provide valuable information for government officials, field advisors, distributors and farmers for implementing integrated pest management practices (Binder *et al.*, 1995). PCR has been used for the amplification of pathogen-specific DNA sequences in highly sensitive methods for detecting various pathogens in plants (Henson *et al.*, 1993). Current developments of PCR-based diagnostics are to make it more quantitative, detect multiple pathogens in a single PCR, optimize the extraction method to improve sensitivity and implement the assays in a high throughput format.

Although we have been successful in integrating ELISA-type detection methods into PCR assays, the disadvantage of using this technology for quantifying PCR products is the amount of processing required after completion of the thermocycling. Additional methods which allow the amplification product to be read immediately after PCR or allow real time determination of the amplification of the product need to be developed. This would reduce the time required for sample analysis.

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