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PCR as a Tool for the Investigation of Seed-borne Disease

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14.1 Introduction to Seed-borne Disease

Seed-borne diseases are defined as diseases which have one or more stages of their life cycle associated with the seed. For example, nearly all the major fungal pathogens of rice have been reported to be seed-borne. The range of organisms infecting seed is extensive (Tables 14.1–14.3). However, very little is known about the role and importance of seed-borne fungal inoculum in the dissemination and development of even the most common diseases. Ninety per cent of all food crops are propagated by seed, and the nine most widely grown crops in world agriculture (wheat, sugar beet, rice, maize, barley, groundnut, soybean, common (*Phaseolus*) bean and sorghum), are all affected by seed-borne diseases.

The importance of seed as a vector of a range of diseases is well established (Neergaard, 1977), and there is a significant economic impact of seed-borne disease, particularly in under-developed countries where routine chemical treatment of seed is prohibitively expensive, and individual farmers can suffer huge yield reductions. For example, an average 4% overall crop loss of the wheat harvest in the US could include individual farmers' losses of over 80% in an infected field. Similarly, an average 2% overall crop loss of the rice harvest in the Philippines could include individual farmers losses of over 58% in an infected field.

Seed diseases and seed-borne diseases can be caused by a number of different factors: microorganisms (including viruses, bacteria, fungi and nematodes), physiological factors (including nutrient deficiency, growth in an unsuitable environment, phytotoxicity, ageing and congenital disorders) and mechanical factors (due to processing or handling equipment and insect injury).

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Table 14.1. Relative frequency of different organisms as seed-borne pathogens (Richardson, 1996).

	No.	%
Fungi	915	72
Bacteria	111	9
Viruses and viroids	238	19
Nematodes	13	1

Table 14.2. Major seed-borne organisms prevalent in Nepal (Rajbhandary and Shrestha, 1992).

Host	Causal agent	Disease	Loss (%)
Rice	*Alternaria padwickii*	Pink kernel	
	Bipolaris oryzae	Brown spot	5–20
	Pyricularia oryzae	Blast	10–30
	Xanthomonas campestris pv. *oryzae*	Bacterial blight	10–25
Wheat	*Alternaria alternata*	Black point	
	Bipolaris sorokiniana	Brown spot	
	Tilletia caries	Bunt	<5
	T. laevis	Bunt	<5
	T. indica	Karnal bunt	
	Ustilago tritici	Loose smut	5–10
Maize	*Bipolaris maydis*	Leaf blight	10–20
	B. turcicum	Leaf blight	10–20
	Fusarium moniliforme	Kernel rot	10–30
Crucifers	*Alternaria brassicae*	Leaf spot	5–20
	A. brassicicola	Leaf spot	5–20
	Sclerotinia sclerotiorum	Stalk rot	5–15
	Xanthomonas campestris pv. *campestris*	Black rot	10–30
Soybean	*Cercospora kikuchi*	Purple seed stain	
	Colletotrichum dematium	Anthracnose	
	Soybean mosaic virus	Hilum bleeding	

Table 14.3. Important seed-borne fungal pathogens in Nepal (Manandhar *et al.*, 1992).

Genus of pathogen	Total species	Total hosts	Pathogen species of economic importance	Host
Alternaria	11	35	*A. brassicae* and *A. brassiacola*	Crucifers
			A. padwickii	Rice
			A. triticina	Wheat
Aspergillus	2	17	*A. flavus* and	Groundnut, maize
Bipolaris			*B. oryzae*	Rice
			B. sorokiniana	Wheat
Botryodiplodia	1	1	*B. theobromiae*	Maize
Botrytis	1	3	*B. cinerea*	Chickpea
Cercospora	2	2	*C. kikuchii*	Soybean
			C. oryzae	Rice
Colletotrichum	3	5	*C. capsici*	Chilli
			C. lindemuthianum	Beans
			C. truncatum	Soybean
Diplodia	1	1	*D. maydis*	Maize
Drechslera	12	16	*D. tritici-repentis*	Wheat
Fusarium	9	33	*F. moniliforme*	Rice and maize
			F. oxysporum	Legumes
Phoma	1	9	*P. glumarum*	Rice
Protomyces	1	1	*P. macrosporus*	Coriander
Pyricularia	2	2	*P. oryzae*	Rice
Tilletia	3	2	*T. indica, T. laevis* and *T. tritici*	Wheat
Ustilaginoidea	1	1	*U. virens*	Rice
Ustilago	3	3	*U. maydis*	Maize

With the increasing distribution of germplasm between researchers and countries, it is important that germplasm banks and international trade do not harbour and distribute seed-borne pathogens, as fungal pathogens can travel on, in or with seed batches. Major plant diseases that have been spread around the world through trade (though not necessarily in seed) include powdery mildew (on grapes), citrus canker, potato wart, wheat flag smut and Dutch elm disease. In order to reduce such transmission routes, safeguards have been introduced to prevent the spread of pests and pathogens while allowing safe, uninterrupted seed movement. These safeguards vary widely from country to country, but include rules and regulations, import permits, phytosanitary certificates, inspections, treatments, isolation, passage through quarantine greenhouses and seed health testing. Seed health tests seek to establish the presence of a pathogen in the seed and to make an estimate of the extent of this infection within the seedlot (Reeves, 1995).

14.2 Problems Encountered in the Study of Seed-borne Disease

Imported seed and the transport of plant varieties from one geographical region or ecological zone to another is increasing, and this practice may introduce new diseases where they had not been encountered previously. Seed-borne inoculum may give rise to progressive disease development in the crop, and symptoms of disease may not be apparent immediately in the germinating seed. The causes of poor germination are often not identified by farmers and this is further complicated by the fact that the incidence of fungal infection of seed is not necessarily translated into subsequent incidence of disease. Seed that is infested with a fungal pathogen may be discoloured and poorly filled and thus have a poor market value, and seed that appears healthy may harbour low populations of the pathogen that later serve as an inoculum source. Little work has been undertaken to date to determine the relative significance of different sources of inoculum in disease development for even the most widely grown crops. It is, therefore, important to: (i) understand the structure of fungal populations; (ii) determine whether individual strains can be identified; (iii) determine how quickly the pathogenic strains adapt to their plant hosts; (iv) determine how rapidly they might adapt to changes in the cultivars grown; and (v) ascertain whether alternative hosts are significant to the life cycle or to disease dissemination. There are already relatively straightforward methods to differentiate between different species in a pathogen complex but it remains particularly difficult to differentiate between virulent and non-virulent strains of the same pathogen species.

14.3 Methods Available in the Study of Seed-borne Disease

Traditional methods in the recognition and detection of seed-borne organisms include: (i) direct observation; (ii) microscopic examination of imbibed seeds; (iii) examination of cultured organisms removed by washing; (iv) examination of seed after incubation (including the blotter test and the agar plate test); and (v) examination of growing plants ('growing-on' test). Each of these requires three steps: extraction, isolation and identification (Reeves, 1995). Such methods can be adapted to discriminate between particular species through exploitation of nutritional requirements. For example, Gnanamanickam *et al*. (1994) used selective media to detect *Xanthomonas oryzae* pv. *oryzae* in rice seed. However, despite the semi-selectivity of the media chosen, the sensitivity of the seed assay remained inadequate because even the faster growing *X. o. oryzae* strains were not recovered unless present in relatively high concentrations (2×10^5 to 1×10^6 c.f.u. ml^{-1}) in the seed extract. Detection of seed-borne *X. o. oryzae* on semi-selective media has been a challenge because the pathogen grows very slowly, and growth is

suppressed by contaminating bacteria found on seed. The more traditional methods suffer the disadvantage that they are slow and the sensitivity and accuracy of diagnosis can be a problem.

The last decade has seen great advances in diagnostic technology, especially in the development of rapid and sensitive methods (Pearce and Holderness, 1996; Richardson, 1996). Recently, there has been a change in emphasis from a more traditional morphological approach to identification of plant pathogens (for example, spore size, shape and colony colour) to a more functional approach based on aspects of the life cycle (for example, mechanism of spore production, DNA relationships and physiological attributes). This has come about firstly through immunodetection techniques (including polyclonal antisera and monoclonal antibodies, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy), and then methods currently in use and under development for the differentiation of different strains of filamentous fungi, which include the use of secondary metabolites, mycotoxins/antibiotics, protein electrophoresis, isoenzyme electrophoresis, fatty acid analysis (Paterson and Bridge, 1994) and DNA-based technology. DNA-based methods have included restriction fragment length polymorphism (RFLP) and pulse-field gel electrophoresis (PFGE) methods (Kasuga *et al*., 1993), and PCR amplification of various genome regions including rRNA genes and random amplified polymorphic DNA (RAPD) (Schots *et al*., 1994).

Ball and Reeves (1991, 1992) reviewed the use of ELISA, PCR, RAPD and similar methods to identify pathogens on seed, but the effort to date has been concentrated on the detection of bacteria and viruses. There is currently much effort being devoted to the further development of such techniques; Slack *et al*. (1996) have prepared a comparison of PCR, ELISA, and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. Seed-borne organisms, however, can provide particular problems not necessarily encountered with organisms in pure culture. For example, RAPD cannot be used directly to detect seed-borne organisms because of non-specific hybridization of the primer which may occur with extraneous DNA from seed extracts (Reeves, 1995), and although PCR techniques have been used for the characterization and identification of fungi, little attention has been given to the detection of seed-borne pathogens (Reeves, 1995). Lange (1986) examined nucleic acid techniques for the detection of seed-borne viruses, and their use for the detection of plant pathogenic bacteria (Rasmussen and Reeves, 1992) and seed-borne diseases (Reeves, 1995) has been reviewed. Blakemore *et al*. (1994), by testing fungi isolated from seeds, noted that such methods are not yet at a stage for incorporation into seed health tests, but because of their high sensitivity, these techniques will continue to be used to detect microorganisms which are difficult to culture or difficult to identify.

14.4 PCR Methods Available for the Study of Seed-borne Diseases

The ability to amplify DNA from crude mycelial preparations is an important factor in the identification of filamentous fungi from plant material, as is the ability of the PCR reaction to use small amounts of material and even partly degraded materials of poor quality. Due to these features many potential sources of fungi can be used, such as unpurified genetic material or material taken directly from soil, growing mycelia, museum specimens and spores (Foster *et al*., 1993). The first stage in the analysis of seed-borne organisms is the culture of the organism from the seed; this is necessary, because PCR can detect dead bacteria and fungi which would not be a problem in seed. Where only one organism is present, or where a species-specific test is to be used, this can be undertaken in small volumes (500 µl) of a rich liquid growth medium over 72 h at 30°C. DNA can then be extracted using a range of different techniques including that of rapid extraction (Cenis, 1992; Raeder and Broda, 1985; Zolan and Pukilla, 1986). Specific DNA fragments can be amplified by PCR and products separated by gel electrophoresis. Banding patterns can be used to distinguish between species or varieties (Ball and Reeves, 1991). PCR can be carried out with RAPD primers (Williams *et al*., 1990); these are very short primers, not having identified DNA sequences, and usually 8–12 bases long (Caetano Anolles *et al*., 1992) which will hybridize to a large number of arbitrary sites in the genome (Fox, 1993). RAPD bands aid identification where this is primarily difficult. Examples are shown in Figs 14.1 and 14.2.

Higher specificity than that obtained with RAPD can be achieved by using variable non-translated repeat (VNTR)-derived oligonucleotides as PCR primers. This molecular method for differentiating fungal populations, and for differentiating between isolates, has been widely established and tested in the field (for example, see Bridge *et al*. 1997 and Pearce *et al*. 1996). Representative oligonucleotide primers can be selected to give either a high or a low discrimination between strains. Amplification products obtained from a range of isolates can then be used to group different strains of the same species. Following initial work with *Sarocladium oryzae* and *Bipolaris oryzae* (Fig. 14.3), the method has been tested against other common rice pathogens including *Gerlachia oryzae*, *Fusarium moniliforme*, *Pyricularia oryzae* and *Rhizoctonia solani* (Bridge *et al*., 1997; Pearce *et al*., 1996).

PCR-based methods have also been used in the investigation of air-borne spread of fungal pathogens. For example, a PCR-based method has been developed for the identification of *Tilletia indica*, the causal agent of Karnal bunt of wheat (Smith *et al*., 1996). Oligonucleotide primers were designed from a sequence derived from cloned *Dra*I fragments of mitochondrial DNA and were used to analyse teliospores which had been germinated from a seed wash extraction method of infested grain. The results demonstrated

that *T. indica* could be reliably detected at an infestation level of five teliospores per 50 g grain.

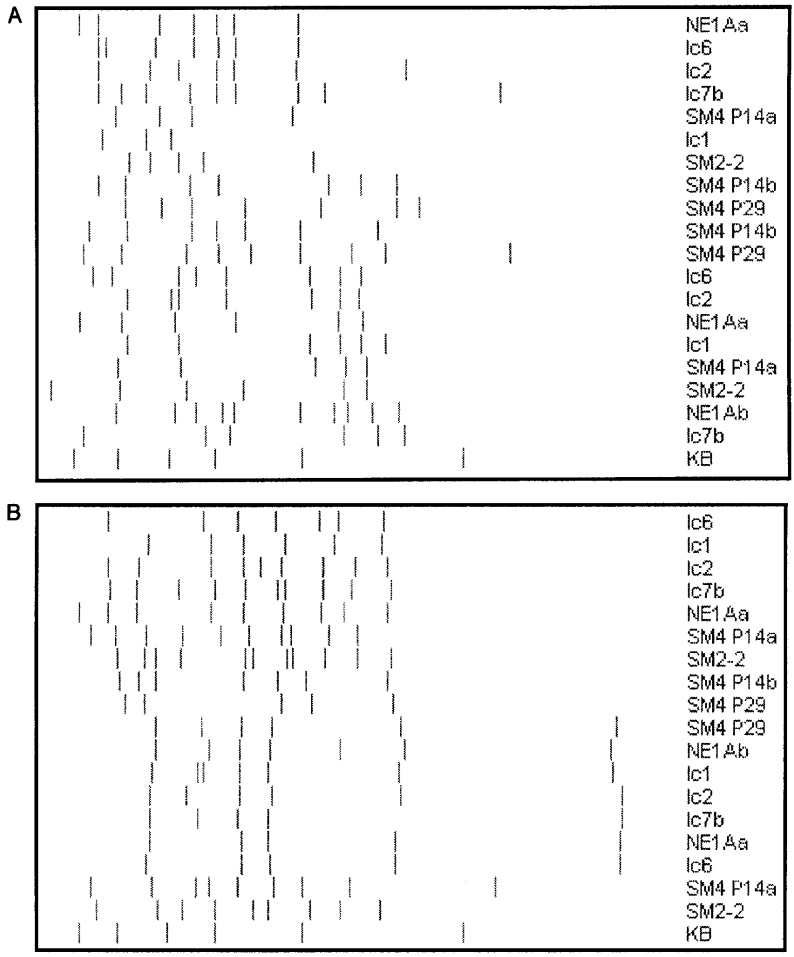

Fig. 14.1. Banding patterns obtained from *Sarocladium oryzae* from RAPD primers. *Panel A*: the first nine horizontal lanes show banding patterns for DNA from nine different strains amplified using primer A11. The next ten lanes show patterns from ten isolates amplified using primer no. 71. The last lane shows the molecular size markers. *Panel B*: the first nine horizontal lanes show DNA from nine isolates amplified using primer A13, lane 10 shows one isolate amplified using primer A14, and lane 11 shows one isolate using primer A13. The next seven were amplified using primer A14. The last lane shows the molecular size markers.

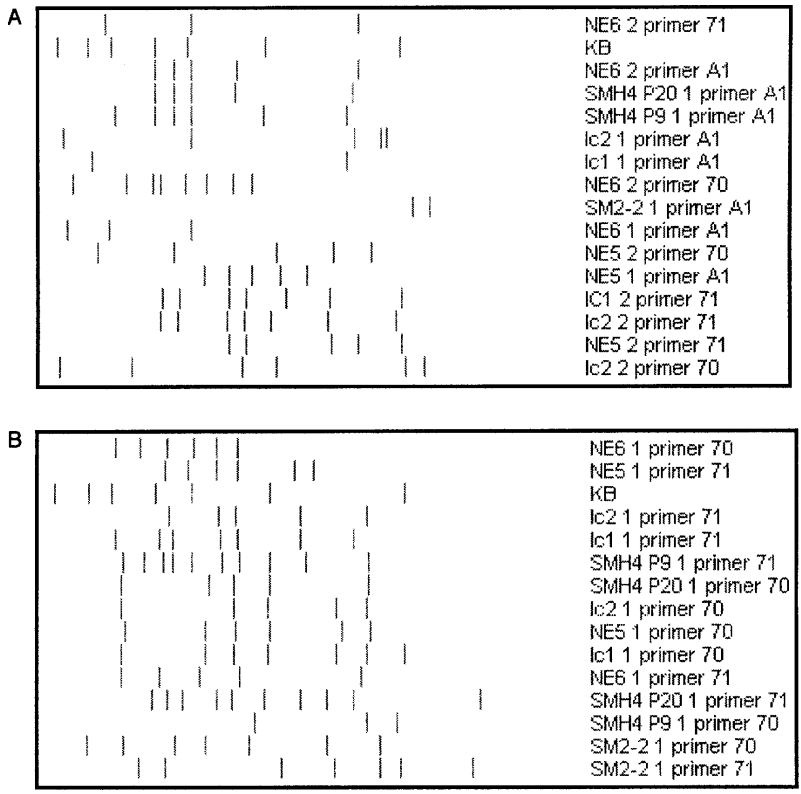

Fig. 14.2. *Panel A*: isolates of *Bipolaris oryzae* amplified with one of three RAPD primers. Size markers are shown in lane 2. *Panel B*: ten isolates of *Bipolaris oryzae* amplified with two RAPD primers. Size markers are shown in lane 3.

14.5 Applications of PCR in the Analysis of Seed-borne Disease

14.5.1 Fungi

The utility of PCR as a specific and sensitive assay for plant pathogen identification is well documented (Henson and French, 1993; Smith *et al*., 1996). PCR and its applications in fungal disease diagnosis have also been described on many occasions (for example, Annamalai *et al*., 1995). PCR has also been shown to be a useful tool in the assessment of seed-borne inoculum, in measuring seed-borne inoculum potential, the extent of transmission from seed to crop, the extent and intensity of disease in the crop and seed-borne inoculum in relation to yield reduction and crop losses (Pearce and Holderness, 1996). PCR methods have been used in numerous other studies for identifying fungal pathogens from seed, including the

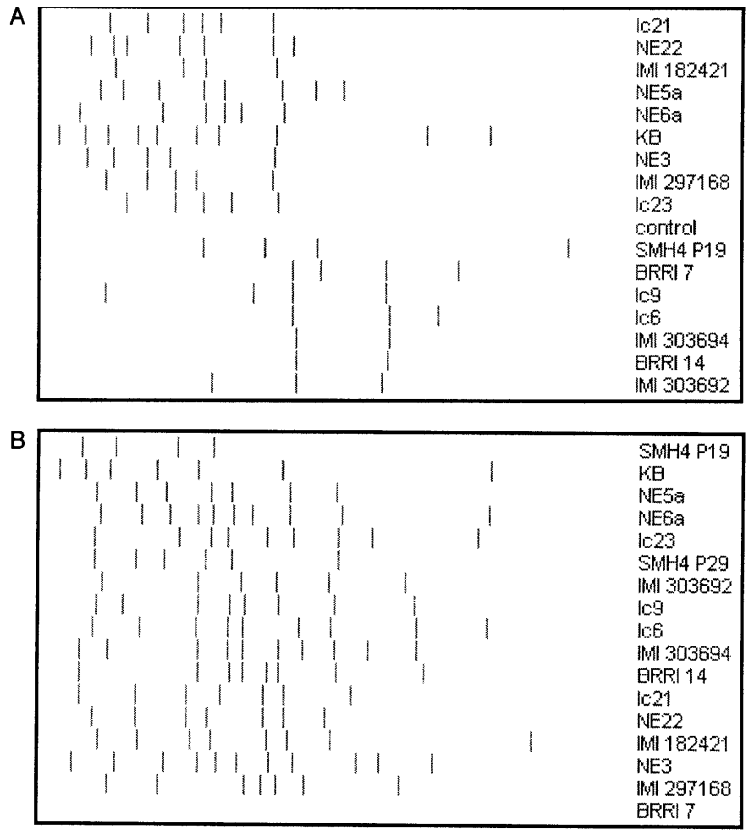

Fig. 14.3. Eight isolates of *Sarocladium oryzae* and eight isolates of *Bipolaris oryzae* tested with VNTR primers. *Panel A*: Primer GF, *S. oryzae* lanes 1–3, 7–9 and 11. *B. oryzae* lanes 4 and 5 and 12–17. Size markers are shown in lane 6. *Panel B*: Primer RY, *S. oryzae* lanes 1, 5 and 6. *B. oryzae* lanes 2–4, 7–11 and 17. Size markers are shown in lane 2.

detection of *Pyrenophora* species, *Fusarium moniliforme*, *Stenocarpella maydis*, and the *Phomopsis/Diaporthe* complex (see Blakemore *et al*., 1994). A PCR-based method has been developed for the identification of *Tilletia indica*, causal agent of Karnal bunt of wheat, in seed wash (Smith *et al*., 1996) and to develop an assay to detect *Fusarium poae* in wheat (Parry and Nicholson, 1996). The latter assay was highly sensitive; it was capable of detecting *F. poae* at less than 1% seed infestation and it overcame problems of conventional isolation methods such as competition from other fungi. The key advantage here is that the method can detect specific pathogens in the presence of more aggressive species.

14.5.2 Other pathogens

PCR has been used in studies identifying other pathogens from seed including the detection of *Pseudomonas syringae* pv. *pisi* (Rasmussen and Wulff, 1990), the halo blight bacterium *Pseudomonas syringae* pv. *phaseolicola* in phaseolus bean seeds (Prosen *et al*., 1991; Tourte and Manceau, 1991), and in the recognition and detection of the *Xanthomonas* pathogens that cause cereal leaf streak in seed.

Maes *et al*. (1996) have suggested that PCR-based techniques could be useful for the identification of bacteria in seed, and Bariana *et al*. (1994) have developed a method for detection of five seed-borne legume viruses in one sensitive multiplex PCR test. PCR has been used to develop a rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and a nested-PCR (Hartung *et al*., 1996).

14.6 Limitations of the PCR Method

The PCR method has a great deal of potential in seed pathology, but there are also a number of problems and limitations associated with its use. Limitations of PCR reactions described elsewhere in this book will also apply to the study of seed pathology. In particular, there are a number of variables within the PCR itself which must be optimized for each primer–species combination. The dNTP concentration must be optimized and increasing the $MgCl_2$ concentration from 1 to 10 mmol l^{-1} can have a dramatic effect on the specificity and yield of the amplification (Saiki, 1990). Studies with *Bipolaris* and *Sarocladium oryzae* isolated from rice seed *Oryza sativa* have shown that there can be both an increase and a decrease in the number of VNTR bands produced when the annealing temperature is raised from 35 to 47°C (Bridge *et al*., 1997; D.A. Pearce unpublished work). High primer concentrations can promote mispriming and the accumulation of non-specific PCR products when different buffers are used. Other variables which require optimization include cycle temperatures and durations, primer concentration and sample concentration and purity. Such problems are compounded when the reaction mixture becomes more complicated, for example, Bariana *et al*. (1994) stated that the multiplex assay was less robust and required very specific conditions. As several factors all affect the specificity and efficiency of DNA amplification, optimization is essential. No single protocol will be suitable for all applications, and each new PCR application will require optimization.

The problems associated with PCR reactions in terms of consistency and accuracy of consecutive reactions require the preparation of adequate controls for each PCR run. Such controls should include a strain with a known banding pattern (from a previous run) and an internal positive control to ensure that a negative result is not the result of failure of the PCR reaction, such as may be caused by inhibitors in the sample extract. It is also important

in seed pathology to conduct some form of pathogenicity test to back up the data. This is important, as the PCR reaction works equally well for dead and for living material, and so may amplify plant DNA. Virulence analysis and pathogenicity testing can also determine whether the increased occurrence of a particular strain is due to increased transmission in the seed or to a higher virulence. Care must be taken to prevent extraneous DNA from contaminating the reaction. All stock solutions must be fresh and sterile, and the possibility of carry-over contamination must be minimized.

Interference may occur at two stages of the strain analysis, either at the extraction stage when the fungus is isolated from the seed or in the PCR reaction mixture. An example of interference at the extraction stage is given by Gnanamanickam *et al*. (1994) who experienced problems in the detection of *Xanthomonas oryzae* pv. *oryzae* in rice seed. In this case direct isolation from seed is difficult, because *X. o. oryzae* grows slowly on media and does not compete with faster growing contaminant bacteria that may show similar colony characteristics and pigmentation. An example of interference at the amplification stage is where the PCR method is most effective with purified DNA but also works with crude preparations; crude DNA samples from seed may contain large amounts of extraneous DNA and this could result in considerable non-specific hybridization with primer DNA (Vivian, 1992). During multiplex PCR, it is important to select primers which do not anneal to each other; where a mixture of genomic DNA from two different fungi is used as template for different primers, competition between the two amplicons can cause inconsistent results. Chemicals from the environment, or other components of the seed/plant tissue, may interfere with the PCR. Hartung *et al*. (1996) found that a nested PCR improved the sensitivity from the single-stage PCR approximately 50- to 160-fold. However, they also found that the addition of citrus extracts and copper (used in leaf sprays) inhibited nested PCR amplification of *Xanthomonas axonopodis* pv. *citri.*

A number of studies have been conducted to determine the reproducibility of PCR in differentiating fungal populations. For example, Slack *et al*. (1996) compared PCR, ELISA and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. They found that the results of each assay were significantly affected by inoculum dose, cultivar and sampling date. Of the inoculated samples 36.2, 35.8 and 29.1% tested positive by PCR, ELISA and DNA respectively.

14.7 Further Method Development

A current major disadvantage in the use of nucleic acid methods in seed health testing is that of quantification (Reeves, 1995) because it is not yet possible to determine the number of infected seeds in a batch, and there is a need to link presence *per se* with a measurement of potential future disease incidence. Colhoun (1983) has considered the need to be able to measure the

inoculum load on individual seeds in order to calculate threshold levels for seed health tests.

The choice of primers is important and can be improved, as it is suggested that primers with longer sequences of bases (up to about 20) are less likely to be present at random, even in the large genomes of plants (Fox, 1993) and will, therefore, achieve specific hybridizations.

Numerous minor manipulations can be made to the reaction process, in order to increase processing speed and to increase accuracy. For example, the use of cell lysates instead of extracted DNA obviates the DNA extraction step, inactive primers which are activated by the first denaturation step prevent primer dimers from forming and computer analysis packages, for example GELCOMPAR, can speed interpretation of results.

Vera-Cruz *et al*. (1996) have used repetitive extragenic palindromic (REP)-PCR and RFLP analysis to measure haplotypic variation in *X. oryzae* pv. *oryzae* within a single field. Their technique involved the analysis of the genomic structure of the field population by repetitive sequence-based PCR, with oligonucleotide primers corresponding to interspersed repeated sequences in prokaryotic genomes and RFLP with the insertion sequence IS1113. REP-PCR involves the use of specific primers (BOX, ERIC, ERIC2, REP and REP2). Competitive PCR may allow some degree of quantification of pathogens present in seed batches (Nicholson *et al*., 1993).

Amplified fragment length polymorphism (AFLP) may provide greater specificity and reliability, as would DNA probes that can be obtained by excising species-specific bands from a gel (Blakemore *et al*., 1992). Specific primers, obtained by sequencing such a band, could be used to develop a PCR-based test and provide a means of accurate identification and sensitive, rapid detection.

The internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) subunit repeat was sequenced in representative isolates of *Cylindrocladium floridanum* and *Cylindrocarpon destructans* (Hamelin *et al*., 1996). These sequences were aligned and compared with ITS sequences of other fungi in the GenBank database. Two internal primers were then synthesized for the specific amplification of portions of the ITS regions of each of the species. These products were then used to develop a test in which species-specific fragments were amplified directly from infected roots from which one or two of the fungi had been isolated. ITS regions evolve rapidly, and may vary among species within a genus or among populations.

Nested PCR, in which a second round of PCR using primers internal to those of the first round is performed (Schesser *et al*., 1991), may be used to overcome non-specific amplification products from other organisms and from host tissues. Multiplex PCR would allow simultaneous testing for more than one organism by the use of mixtures of primers which are specific for individual organisms. Ligase chain reaction (LCR), like PCR, is a DNA amplification system that is highly specific and very sensitive (Lee, 1993) and even a single mismatch can prevent the reaction. High specificity has,

however, meant that it has not yet been used in plant pathology (Reeves, 1995).

14.8 Future Potential for the Study of Seed-borne Disease

There is a wide range of potential applications of PCR methodology in the analysis of seed-borne disease transmission. The potential exists for the use of the PCR method with washings from seed samples (Vivian, 1992) and without the need for extraction and isolation of the organism (Ball and Reeves, 1991). The revision of species concepts in individual pathogen populations is also being enhanced by the use of such molecular techniques.

Factors limiting the transmission of seed-borne disease can be enhanced in order to reduce disease transmission and enhance yield. In order to do this, it is important to know exactly what influence the alteration of cultural practices is having on the seed-borne element of transmission. These factors could include the impact of climate, storage, seed crop management, location of production, adjustment of cultural practices, chemical protection, protective inoculation, seed treatment and seed health testing (Misra *et al*., 1995). Disease management can involve the adjustment of sowing dates, deep ploughing, balancing host nutrition, intercropping, management of pollen and removal of collateral hosts. Control and monitoring of seed exchange can prevent the spread of pathogens limited to a small area. Other parts of the cropping cycle which can be exploited for disease management include: storage practices, the number of crops grown per year or the order of crops, climate, planting method, seed treatment and plant density.

PCR is, therefore, a useful tool for the investigation of seed-borne disease. PCR can be used in plant pathology to understand the structure of fungal populations, of both pathogens and their non-pathogenic antagonists, determine whether individual races exist; for example, is the *Sarocladium oryzae* that infects rice (*Oryzae sativa*) the same pathogen that attacks Bamboo (*Bambusa* spp.) or is it a different species? (See Bridge *et al*., 1989.) PCR methods may allow pathologists to determine how quickly a particular pathogen is evolving, or adapting to a new host or cultivar, and whether alternative hosts are significant to the pathogen life cycle or disease dissemination.

References

Amatya, P., Dahal, G. and Manandhar, H.K. (1992) Plant pathology in Nepal. In: Mathur, S.B., Amatya, P., Shrestha, K. and Manandhar, H.K. (eds) *Plant Diseases, Seed Production and Seed Health Testing in Nepal.* Proceedings of the

first HMG/DANIDA/FAO training course in seed health testing techniques. DGISP, Denmark.

Annamalai, P., Ishii, H., Lalithakumari, D. and Revathi, R. (1995) Polymerase chain reaction and its applications in fungal disease diagnosis. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 102(1), 91–104.

Ball, S.F.L. and Reeves, J.C. (1991) The application of new techniques in the rapid testing for seed-borne pathogens. *Plant Varieties and Seeds* 4, 169–176.

Ball, S. and Reeves, J. (1992) Appication of rapid techniques to seed health testing – prospects and potential. In: Duncan, J.M. and Torrance, L. (eds) *Techniques for the Rapid Detection of Plant Pathogens*. Blackwell Scientific Publications, Oxford. pp. 193–207.

Bariana, H.S., Shannon, A.L., Chu, P.W.G. and Waterhouse, P.M. (1994) Detection of five seedborne legume viruses in one sensitive multiplex polymerase chain reaction test. *Phytopathology* 84(10), 1201–1205.

Blakemore, E.J.A., Reeves, J.C. and Ball, S.F.L. (1992) Research note: polymerase chain reaction used in the development of a DNA probe to identify *Erwinia stewartii*, a bacterial pathogen of maize. *Seed Science and Technology* 20, 331–335.

Blakemore, E.J.A., Jaccoud Filho, D.S. and Reeves, J.C. (1994) PCR for the detection of *Pyrenophora* species, *Fusarium moniliforme*, *Stenocarpella maydis*, and the *Phomopsis/Diaporthe* complex. In: Schots, A., Dewey, F.M. and Oliver, R. (eds) *Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification*. CAB International, Wallingford, UK, pp. 205–213.

Bridge, P.D., Hawksworth, D.L., Kavishe, D. and Farnell, P.A. (1989) A revision of the species concept in *Sarocladium*, the causal agent of sheath-rot in rice and bamboo blight, based on biochemical and morphometric analyses. *Plant Pathology* 38, 239–245.

Bridge, P.D., Pearce, D.A., Rivera, A. and Rutherford, M.A. (1997) VNTR derived oligonucleotides as PCR primers for population studies in filamentous fungi. *Letters in Applied Microbiology* 24, 421–425.

Caetano Anolles, G., Bassam, B.J. and Gresshoff, P.M. (1992) Primer–template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides. *Molecular and General Genetics* 235, 157–165.

Cenis, J.L. (1992) Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research* 20, 2380.

Colhoun, J. (1983) Measurement of inoculum per seed and its relationship to disease expression. *Seed Science and Technology* 11, 665–671.

Foster, L.M., Kozak, K.R., Loftus, M.G., Stevens, J.J. and Ross, I. (1993) The polymerase chain reaction and its application to filamentous fungi. *Mycological Research* 97, 769–781.

Fox, R.T.V. (1993) *Principles of Diagnostic Techniques in Plant Pathology.* CAB International, Wallingford.

Gnanamanickam, S.S., Shigaki, T., Medalla, E.S. and Mew, T.W. (1994) Problems in detection of *Xanthomonas oryzae* pv. *oryzae* in rice seed and potential for improvement using monoclonal antibodies. *Plant Disease* 78, 173–178.

Hamelin, R.C., Bérubé, P., Gignac, M. and Bourassa, M. (1996) Identification of root rot fungi in nursery seedlings by nested multiplex PCR. *Applied and Environmental Microbiology* 62 (11), 4026–4031.

Hartung, J.S., Pruvost, O.P., Villemot, I and Alvarez, A. (1996) Rapid and sensitive

colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and a nested-polymerase chain reaction assay. *Phytopathology* 86, 95–101.

Henson, J.M. and French, R. (1993) The polymersae chain reaction and plant disease diagnosis. *Annual Review of Phytopathology* 31, 81–109.

Kasuga, T., Woods, C., Woodward, S. and Mitchelson, K.R. (1993) *Heterobasidion annosum* 5.8S ribosomal DNA and internal transcribed spacer sequence: rapid identification of European intersterility groups by ribosomal DNA restriction polymorphism. *Current Genetics* 24, 433–436.

Lange, L. (1986) The practical application of new developments in test procedures for the detection of viruses in seed. *Developments in Applied Biology* 1, 269–281.

Lee, H. (1993) Infectious disease testing by ligase chain reaction. *Clinical Chemistry* 39, 729–730.

Maes, M., Garbeva, P. and Kamoen, O. (1996) Recognition and detection in seed of the *Xanthomonas* pathogens that cause cereal leaf streak using rDNA spacer sequences and polymerase chain reaction. *Phytopathology* 86, 63–69.

Manandhar, H.K., Shrestha, K. and Amatya, P. (1992) Seed-borne fungal diseases. In: Mathur, S.B., Amatya, P., Shrestha, K. and Manandhar, H.K. (eds) *Plant Diseases, Seed Production and Seed Health Testing in Nepal*. Proceedings of the first HMG/DANIDA/FAO training course in seed health testing techniques. DGISP, Denmark.

Misra, J.K, Gergon, E.B. and Mew, T.W. (1995) Storage fungi and seed health of rice: a study in the Philippines. *Mycopathologia* 131, 13–24.

Neergaard, P. (1977) *Seed Pathology*. Macmillan Press, London.

Nicholson, P., Rezanoor, H.N. and Su, H. (1993) Use of random amplified polymorphic DNA (RAPD) analysis and genetic fingerprinting to differentiate isolates of race O, C and T of *Bipolaris maydis*. *Journal of Phytopathology* 139, 261–267.

Parry, D.W. and Nicholson, P. (1996) Development of a PCR assay to detect *Fusarium poae* in wheat. *Plant Pathology* 45, 383–391.

Paterson, R.R.M. and Bridge, P.D. (1994) *Biochemical Techniques for Filamentous Fungi*. CAB International, Wallingford.

Pearce, D.A. and Holderness, M. (1996) Methods of identification for fungi. *Planning Workshop on Seed Health for Disease Management*, International Rice Research Institute, Los Baños, Laguna, The Philippines, January 22–25.

Prosen, D., Hatziloukas, E., Panopoulos, N.J. and Schaad, N.W. (1991) Direct detection of the halo blight pathogen *Pseudomonas syringae* pv. *phaseolicola* in bean seeds by DNA amplification. *Phytopathology* 81, 1159 (abstract).

Raeder, U. and Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* 1, 17–20.

Rajbhandary, K.L. and Shrestha, K. (1992) Plant diseases in seed production. In: Mathur, S.B., Amatya, P., Shrestha, K. and Manandhar, H.K. (eds) *Plant Diseases, Seed Production and Seed Health Testing in Nepal*. Proceedings of the first HMG/DANIDA/FAO training course in seed health testing techniques. DGISP, Denmark.

Rasmussen, O.F. and Reeves, J.C. (1992) DNA probes for the detection of plant pathogenic bacteria. *Journal of Biotechnology* 25, 203–220.

Rasmussen, O.F. and Wulff, B.S. (1990) Detection of *Pseudomonas syringae* pv. *pisi* using PCR. In: Durbin, R.D., Surico, G. and Mugnai, L. (eds) *Proceedings 4th*

International Working Group on Pseudomonas syringae *Pathovars*. Stamperia Granducale, Florence, pp. 369–376.

Reeves, J.C. (1995) Nucleic acid techniques in testing for seed-borne diseases. In: Skerritt, J.H. and Appels, R. (eds) *New Diagnostics in Crop Sciences*. CAB International, Wallingford.

Richardson, M.J. (1996) Seed mycology – centenary review. *Mycological Research* 100(4), 385–392.

Saiki, R.K. (1990) Amplification of genomic DNA. In: Innis, M.J., Gelfand, D.H., Sninsky, J.J. and White,T.J. (eds) *PCR Protocols: a Guide to Methods and Applications*. Academic Press, New York, pp. 13–20.

Schesser, K., Luder, A. and Henson, J.M. (1991) Use of polymerase chain reaction to detect the take-all fungus *Gaeumannomyces graminis*, in infected wheat plants. *Applied and Environmental Microbiology* 57, 553–556.

Schots, A., Dewey, F.M. and Oliver, R. (1994) *Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification*. CAB International, Wallingford.

Slack, S.A., Drennan, J.L. and Westra, A.A.G. (1996) Comparison of PCR, ELISA and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. *Plant Disease* 80, 519–524.

Smith, O.P., Peterson, G.L., Raymond, J.B., Schaad, N.W. and Bonde, M.R. (1996) Development of a PCR-based method for identification of *Tilletia indica*, causal agent of Karnal bunt of wheat. *Phytopathology* 86, 115–122.

Tourte, C. and Manceau, C. (1991) Direct detection of *Pseudomonas syringae* pathovar *phaseolicola* using the polymerase chain reaction (PCR) In: Durbin, R.D., Surico, G. and Mugnai, L. (eds) *Proceedings 4th International Working Group on* Pseudomonas syringae *Pathovars*. Stamperia Granducale, Florence, pp. 377–379.

Vera-Cruz, C.M., Ardales, E.Y., Skinner, D.Z., Talag, J., Nelson, R.J., Louws, F.J., Leung, H., Mew, T.W. and Leach, J.E. (1996) Measurement of haplotypic variation in *Xanthomonas oryzae* pv. *oryzae* within a single field by rep-PCR and RFLP analyses. *Phytopathology* 86, 1352–1359.

Vivian, A. (1992) Identification of plant pathogenic bacteria using nucleic acid technology. In: Duncan, J.M. and Torrance, L. (eds) *Techniques for the Rapid Detection of Plant Pathogens*. Blackwell Scientific Publications, Oxford, pp. 145–161.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18, 6531–6535.

Zolan, M.E. and Pukilla, P.J. (1986) Inheritance of DNA methylation in *Coprinus cinerus. Molecular and Cellular Biology* 6, 195–200.