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REVIEW ARTICLE

Detecting *Phytophthora*

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Abstract
Species of the genus *Phytophthora* are arguably the most destructive plant pathogens causing widespread damage to many horticultural and ornamental species, and to native ecosystems throughout the world. Globalization has increased the volume of plants being transported over long distances and has increased the spread of *Phytophthora* species. As traditional detection methods such as baiting or direct isolation are incapable of handling the large volume of material to be tested, researchers have developed more rapid and specific antibody and DNA based tests. This review compares the performance of the different types of tests used for detection of *Phytophthora*.

Keywords: *Phytophthora; diagnostics; baiting; ELISA; PCR*

Introduction

Oomycete pathogens of the genus *Phytophthora* are the most destructive plant pathogens known. There are over 82 species in the genus many with a wide host range (Erwin and Ribiero 1996; Blair et al. 2008). The most infamous is *Phytophthora infestans* the causative agent of the Irish potato famine in the 1840s. Today late blight is still a problem wherever potatoes are produced and is estimated to cost US growers about $5bn per annum to control (Judelson and Blanco 2005). Other species of major importance that have emerged in more recent times are *Phytopthora ramorum* the causative agent of Sudden Oak Death, responsible for the widespread death of Tanoaks and oak trees in the United States and which threatens species of oak and other species in Europe (Balci et al. 2007), *Phytophthora alni* responsible for devastating losses to alder stands across Europe (Brasier et al. 2004; Jung and Blaschke 2004), and *Phytophthora kernoviae* a major pathogen of ornamental species in Europe (Brasier et al. 2005). In Australia, *Phytophthora cinnamomi* a species with a very wide host range is responsible for a major epidemic of eucalypt forest and heathland species across the southern parts of the continent. The disease has resulted in the widespread destruction of animal habitats, and placed many species of native Australian plants at risk of extinction (Shearer et al. 2007). *P. cinnamomi* is also a major problem in oak forests in the United States Portugal, France, and Mexico (Cahill et al. 2008).

Phytophthora spreads mainly through the movement of infested soil, water and infected plants and plant material although there are species that are transmitted aerially (Goodwin 1997; Cahill et al. 2008). Especially damaging as a source of inoculum are those plants that are infected but do not show signs of symptoms either because the disease has not yet progressed to the stage where symptoms are evident, or due to suppression of symptom development by the use of fungicides. In 2003/04 several thousand nurseries across the USA received asymptomatic plants infected with *P. ramorum* from suppliers on the west coast. Aside from the death of plants, an embargo on the supply of plants from infected areas in the western United States and Canada threatens the viability of an industry worth over $13bn per annum (Jones et al. 2005). Movement of infected plants also promotes the formation of hybrid species with new pathogenic characteristics, e.g., *P. alni* a hybrid species that is in contrast to its putative parental species is a highly aggressive pathogen to species of the genus *Alnus*

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(Ersek and Nagy 2008) There have been a number of reports of hybrid species of *Phytophthora* in recent years (Brasier 2008).

The volume of plant germplasm transported between countries and continents has increased dramatically over the last two decades fuelled by the globalization of world trade, consumer demand for new varieties of cut flowers and ornamental plants, and the need to develop new varieties for horticulture and forestry applications (Strange and Scott 2005; Cahill et al. 2008; Surkov et al. 2008). Preventing the spread of *Phytophthora* requires the development of detection techniques that are robust, highly specific, and sensitive. In order to deal with the needs of the modern commercial world they also need to be able to provide an answer rapidly. Over the last ten years there has been intensive research into the development of such tools for the detection of various *Phytophthora* species in different plant species and tissues as well as in soil and water. Given the importance of *Phytophthora* as a pathogen, and the plethora of tests that have been developed, it is pertinent to ask how the different test compare with each other, and in what way are they most efficiently used so that we may develop guidelines for efficient detection of *Phytophthora* from a range of substrates and set priorities for future research. Here we review the tests most commonly used for detection of *Phytophthora*, namely isolation, baiting, immunological tests and DNA based tests. We look at the factors that affect the efficiency of the different test formats and compare their performance for detection of *Phytophthora* in plant material, soil, and water.

Techniques for detection of *Phytophthora*

Isolation

Phytophthora can be detected by plating diseased tissue onto selective agar containing antibacterial and antifungal antibiotics allowing outgrowth of *Phytophthora* from the tissue and identification by morphological characters (Tsao 1983; Huberli et al. 2000; Kox et al. 2007). False negatives caused by failure of the pathogen to grow out of the tissue can occur. Huberli et al. (2000) found that 8.6% of *Eucalyptus marginata* plants that tested negative by isolation subsequently tested positive after extensive washing of the tissue segments. The results were ascribed to the removal of growth inhibitory substances by the washing. The same study found that 21% of symptomless plants tested positive by isolation. Hughes et al. (2006b) found that two tissue samples that initially tested negative for *P. ramorum* by isolation, were subsequently found to test positive when baited using fresh tissue, or by extended incubation of the isolation plates. In order to remove excessive polyphenols and stimulate germination of resting spores and thus increasing isolation frequency, bark samples of alders and beech trees are regularly flooded and washed for a couple of days before plating (Jung and Blaschke 2004; Jung 2008). The application of molecular techniques has further highlighted the occurrence of false negatives when testing plant tissue samples for the presence of *P. ramorum* (Bulluck et al. 2006; Kox et al. 2007), or *P. fragariae* (Ioos et al. 2006). In these studies the number of samples that tested positive by PCR was greater than those that tested positive by culture plate isolation (Table 1). The reason for the failure to grow on the selective agar is not known but may be related to antagonism by microorganisms in the tissue sample (Malaczjuk 1983), inhibition by plant phenolics (Huberli et al. 2000) or inhibition of germination of oospores or chlamydospores (Tsao 1983; McCarren et al. 2005).

Baiting

Baiting is commonly used for detection of *Phytophthora* spp. in soil. The technique involves floating pieces of susceptible tissue on a soil water slurry with a high water/soil ratio (Erwin and Ribiero 1996). Zoospores formed by *Phytophthora* in the sample infect the baits which after several days' incubation are then plated onto selective agar as described above. *Phytophthora* species growing out of the bait can be identified by morphological examination, or DNA sequence analysis (Marks and Kassaby 1974; Eden et al. 2000). Depending on the time of year at which the sample is taken, the efficiency of detection ranges from 0 to more than 90%. The host species from which the bait tissue is derived also influences the efficiency of detection (Marks and Kassaby 1974). Although the bait tissue is derived from a host species that is susceptible to the pathogen, different host species give very different efficiencies of detection (unpublished). Isolation of *Phytophthora* from the infected bait requires considerable time, the use of selective media

Table 1. Comparison of PCR and culture isolation for detection of *Phytophthora*.

Target species	No of Positive Detections C+P+[1]	C+P-	C-P+	Reference
P. ramorum	388	6	54	(Ioos et al. 2006)
P. ramorum	5		9	(Bilodeau et al. 2007)
P. ramorum	25	2	2	(Hughes et al. 2006)
P. ramorum	9	1	0	(Kong et al. 2003)
P. ramorum	33			(Schena et al. 2006)
P. kernovia	14	1	1	(Schena et al. 2006)
P. nicotiana	13			(Ippolito et al. 2002)
P. citophthora	2		3	(Ippolito et al. 2002)

[1]C, culture plate isolation; P, PCR; E, ELISA (Incl LFD and immunodipstick assay). + and – indicate positive and negative reactions respectively.

and considerable knowledge of the genus. A major problem is that the presence of fast growing organisms such as *Pythium* tends to inhibit growth of the target species (Nechwatal et al. 2001). The formation of zoospores in the soil/water slurry is not always sufficient for a positive result. Studies using antibody tests (Wilson et al. 2000) have shown that in a significant number (16%) of cases although zoospores were detected in the water the results of the baiting were negative (Table 2). Thus it appears that infection of the bait can be a limiting factor.

The efficiency of detection of *Phytophthora* in soil by baiting can be improved by drying out the soil and rewetting it (double baiting) (Jeffers and Aldwinkle 1987) demonstrating that as with tissue sections the pathogen although present will not always grow out of the sample. Davison and Tay (2005) found that double baiting increased the recovery of positive samples from 1.9 to 2.5% and 6.3 to 7.5% of samples taken from the centre and margins respectively of disease fronts in Western Australia. The quality of the water can also significantly affect the outcome as zoospores are very sensitive to toxic ions present in unpurified water (Tsao 1983). In a study by Gerrettson-Cornell quoted by Tsao (1983) the frequency of recovery of *P. cinnamomi* was 94, 32, and 0% respectively when glass-distilled water, deionized water, and distilled water from a metal still were used. Typically the frequency of recovery from soils in Western Australia is much lower than from soils in the eastern states of Australia (Victoria and New South Wales). Blowes (1980) compared the frequency of recovery of *P. cinnamomi* from soil samples from New South Wales and Western Australia and reported recoveries of 27% and 0.4% respectively. It is not clear whether this is due to differences in soil composition or different climatic conditions.

Table 2. Comparison of ELISA and culture isolation for detection of *Phytopthora*.

	No of Positive Detections			
Target Species	E+C−[1]	E+C+	E−C+	Reference
P. ramorum	70	197	28	(Lane et al. 2007)[2]
P. cinnamomi	8	14	16	(Wilson et al. 2000)[3]
Phytophthora spp	20	100	12	(MacDonald et al. 1990)

[1]C, culture plate isolation; P,PCR; E, ELISA. + and − indicate positive and negative reactions.
[2]This study used the LFD test instead of ELISA.
[3]This study used an immunodipstick to test the baiting water for *Phytophthora*.

Immunodetection

Cahill and Hardham (1994) developed an immunological dipstick assay for detection of *P. cinnamomi* zoospores based on the use of a zoospore specific monoclonal antibody. The antibodies are incorporated into a dipstick format. The dipstick also contains zoospore attractants such as phenols, alcohols and amino acids. The assay could detect as few as 40 zoospores /mL. In a subsequent study Wilson et al. (2000) showed that the efficiency of detection of *P cinnamomi* soil by baiting could be increased by use of the dipstick assay to test the bait water for the presence of zoospores (Table 2).

Commercially available ELISA tests for *Phytopthora* detection based on antibodies to a generic *Phytophthora* antigen have proved useful as a quick means of identifying infected plants although they can only identify to the genus level (McDonald et al. 1990; Ali-Shatayeh et al. 1991). The antibodies also show cross reactivity with some species of *Pythium*. Comparative tests of detection of *P cinnamomi* (McDonald et al. 1990), and *P ramorum* (Bulluck et al. 2006; Kox et al. 2007) by plating and ELISA have shown that ELISA tests do not always detect the presence of the pathogen. There are a significant number of samples where the pathogen has been isolated by plating and/or PCR but was not detected by ELISA (Tables 2 and 3). Ali-Shatayeh et al. (1991) have reported that the sensitivity of detection with the *Phytophthora* ELISA kits varies with the species, and even with isolates of the same species.

A rapid on-site antibody based testing device (Lateral flow Device, LFD) has been developed by Forsite Diagnostics (York, UK) for detection of *Phytophthora* species in plant tissue samples in the field (Lane et al. 2007). In a number of trials, the test proved to be specific

Table 3. Comparison of ELISA, PCR, and culture isolation for detection of *Phytophthora ramorum.*

No of Positive Detections							
E+P−C−[1]	E+P+C−	E+P−C+	E+P+C+	E−P+C+	E−P+C−	E−P−C+	Reference
	20	8	36	3	1	3	(Kox et al. 2007)
10	54		113	13	52		(Bulluck et al. 2006)

[1]E, ELISA; P, PCR, and C, culture plate isolation.

and sensitive for identification of Phytophthora in diseased tissue. An experiment to determine the sensitivity of detection with the LFD showed that it could detect infected tissue in a mixture consisting of one part infected material to 99 parts uninfected material (Lane et al. 2007). In their study on evaluation of techniques for detection of *P. ramorum* Kox et al. (2007) directly compared the performance of the AGDIA *Phytophthora* ELISA test and the LFD test. Of 82 samples analyzed by both methods, the ELISA test gave positive reactions with 14 samples that were negative when tested by LFD. Twelve of these were also negative by *Phytophthora* generic PCR, *P. ramorum* specific PCR and by culture plating. The remaining two samples were positive by both *Phytophthora* generic and *P. ramorum* specific PCR analysis but negative by culture plating. The LFD test gave two positive results that were negative by the ELISA test. Whist both were positive with *Phytophthora* generic PCR, only one was positive for *P. ramorum* specific PCR. However, *P. ramorum* was isolated from both samples. Despite the disagreements, there was very good agreement between both types of tests and they are likely to be a useful initial screen to identify those samples that will undergo further laboratory based testing.

The USDA recommends using ELISA tests as a first screen to reduce the number of samples that proceed through to further testing (Bulluck et al. 2006). As the ELISA tests are not species specific, the identity of the species has to be confirmed by PCR and/or culture plate isolation. Two studies (Bulluck et al. 2006; Kox et al. 2007) have compared the performance of ELISA tests with PCR and culture plate isolation. In both studies the majority of samples that tested positive by PCR and/or culture plate isolation also tested positive by ELISA (Table 3). In both studies, and also in the study of McDonald et al. (1990) (who did not use PCR) a number of samples tested positive with ELISA and negative with either of the other two methods. These samples may represent recognition of antigens from other *Phytophthora* or *Pythium* species, or cross reactive host antigens, There were also a number of samples that tested negative by ELISA but were positive with PCR and/or isolation (Table 3). These amounted to 9.8% of the samples in the study of Kox et al. (2007) but 26.8% of the samples in the study of Bulluck et al. (2006) despite the fact that in the latter study the same tissue macerate was used for both ELISA and PCR. Failure to detect antigens may be related to the nature of the host tissue and or the pathogen species. McDonald et al. (1990) observed a high incidence of false negatives with *P. cinnamomi* infection of juniper and ascribed this to the naturally dark pigmentation of this host species making it difficult to detect and select lesion tissue for analysis. In the study with on-site detection using the LFD device, Lane et al. (2007) observed that many of the plant species (especially Rhododendron) that gave false negative results produce thick waxy leaves that are difficult to break down to release the antigens. They found that cutting or tearing the plant tissue prior to placing in the extraction bottle improved antigen release and decreased the frequency of false negatives. From the point of view of disease management by quarantine restriction on imports or the transnational movement of plant material, an error rate of 10–26% is of considerable concern as once a species of *Phytophthora* is introduced into a new area it is extremely difficult if not impossible to eradicate. Given the high rate of false negatives obtained with ELISA tests, the reliance on ELISA as an initial screen is questionable. The value of subjecting ELISA positive material to further testing to identify the species is also questionable as the importation of any species of *Phytophthora* constitutes a risk.

DNA detection tests

PCR amplification tests

In recent years DNA has emerged as the method of choice for detection of microbial pathogens. DNA has a number of advantages in that the detection tests are highly specific, unlike proteins DNA structure is not affected by environmental conditions or stage of development, and due to developments in PCR amplification technology, DNA is very easily detected. Virtually all of the DNA detection tests involve PCR amplification in which a pair of oligonucleotide primers flank a region of interest. The DNA then undergoes 30–40 cycles of synthesis resulting in an exponential increase in the number of copies of the flanked region. The amplification products can then be detected by electrophoresis on a gel. PCR detection tests are rapid, sensitive and highly specific (Vincelli and Tisserat 2008). PCR tests have been developed for many species of *Phytophthora* (Tables 4 and 5).

The sensitivity of PCR detection can be increased by the use of nested PCR in which the products of the PCR reaction are used in a second round of PCR amplification either with the same or more usually with a second pair of primers (nested primers) that lie inside the binding sites of the primers used for the first round PCR. Several studies on detection of various species of *Phytophthora* have reported that nested PCR is significantly more sensitive (100–1,000 fold) compared to single round PCR (Table 4).

A more recent innovation in PCR detection is the development of real time PCR in which the amount of product is measured after each cycle of amplification. For further information on the technology of real-time PCR the reader is referred to a number of excellent reviews (Broude 2002; Schena et al. 2004; Marras et al. 2006; Vincelli and Tisserat 2008). Because the size of the amplified region is shorter than in conventional PCR where

Table 4. PCR and nested PCR tests for detection of *Phytophthora* species.

Method	Target species	Target gene	Detection sensitivity (fg DNA)	Amplicon size (bp)	Reference
PCR	*P. cactorum*	SCAR	6,000	450	(Causin et al. 2005)
PCR	*P cinnamomi*	cinnamomin	25	349	(Coelho et al. 1997)
nPCR[1]	*P nicotiana*	ITS	2.5	>737/737[2]	(Grote et al. 2002)
PCR	*P nicotiana*	ITS	2,500	737	(Grote et al. 2002)
nPCR	*P infestans*	ITS	5	1300/613	(Hussain et al. 2005)
PCR	*P infestans*	ITS	500	613	(Hussain et al. 2005)
PCR	*P. ramorum*	*GPA*1	500	248	(Ioos et al. 2006)
PCR	*P ramorum*	TRP	500	527	(Ioos et al. 2006)
PCR	*P fragariae*	*TRP*	500	403	(Ioos et al. 2006)
PCR	*P fragariae*	*RAS*-like	1,000	229	(Ioos et al. 2006)
nPCR	*P. citrophthora* or *P. nicotiana*	*ITS*	25	700/ (120)160	(Ippolito et al. 2002)
PCR	*P. citrophthora*	*ITS*	25,000	700	(Ippolito et al. 2002)
PCR	*P cinnamomi*	Lpv	4	534	(Kong et al. 2003)
nPCR	*P nicotiana*	ParA1	100zsp[3]	378/378	(Lacourt and Duncan 1997)
nPCR	*P. ramorum*	*cox*	2	134	(Martin et al. 2004)
nPCR	Various spp	Ypt1	1,500	470/153-258	(Schena et al. 2008)
PCR	Various spp	Ypt1	>150,000	150-258	(Schena et al. 2008)
PCR	*P. boemheriae*	ITS	10	750	(Shen et al. 2005)
nPCR	*P capsici*	ITS	0.5	700/452 or 595	(Silvar et al. 2005b)
PCR	*P capsici*	ITS	5,000	452 or 595	(Silvar et al. 2005b)
PCR	*P. sojae*	ITS	1	330	(Wang et al. 2006)
PCR	*P. infestans*	ITS	10,000	600	(Wangsomboondee and Ristaino 2002)
PCR	*P. lateralis*	ITS	200zsp[4]	738 bp	(Winton and Hansen, 2001)
nPCR	*P capsici*	ITS	1	>700/ 560	(Zhang et al. 2006)
PCR	*P capsici*	ITS	1,000	560	(Zhang et al. 2006)

[1]nPCR, nested PCR
[2]Relative sizes (bp) of the primary and secondary amplicons.
[3]Sensitivity given as 100 zoospores.
[4]Sensitivity given as 200 zoospores.

the product must be of a size sufficient to be detected by electrophoresis, the amplification is very efficient and hence the sensitivity of detection is very high compared to conventional PCR (Table 5). An attractive feature of real-time PCR is the ability to include several pairs of primers in the same reaction (multiplexing). This allows identification of several species or the inclusion of internal controls in the reaction. However in some cases it has been observed that the primers in multiplex reactions interfere with each other resulting in a lower sensitivity of detection or the generation of additional amplicons compared to reactions with the primers singly (Ippolito et al. 2004; Martin et al. 2004; Tooley et al. 2006).

Real-time PCR has proved to be a very attractive system for plant pathogen diagnostics because it does not require electrophoresis of the products and hence it is amenable to automation. This makes it more economical for diagnostic laboratories and it is now the most widely used format for plant pathogen diagnostics. Another major advantage of real-time PCR is that it avoids the problem of cross contamination of reactions that is inherent in nested PCR.

Primers are decisive for the specificity and sensitivity of PCR detection. When developing a test for a new species, several pairs of primers need to be designed to each end of the target region as not all primers will function equally well. Moving a primer sequence 1 or 2 bases in either the 5' or 3' direction can have a very significant effect on the efficiency of amplification and hence the sensitivity of detection (He et al. 1994). A single base change in the forward primer of the *P. ramorum* assay developed by CSL to prevent cross reaction with *P. lateralis* reduced the sensitivity of detection of *P. ramorum* 50 fold (Hughes et al. 2006b). Kong et al. (2003) also found a 10 fold difference in sensitivity with pairs of primers targeted to different sites in the *Lpv* gene of *P. cinnamomi*, whilst primers designed to different regions of the *Ypt1* gene of *Phytophthora* species exhibited differences in sensitivity of 15–150 fold (Schena et al. 2006; Schena et al. 2008).

Table 5. Real-time PCR tests for detection of *Phytophthora* species.

Method[1]	Format	Target species	Target gene	Detection sensitivity (fg DNA)	Amplicon size (bp)	Reference
rtPCR	Taq-Man	*P. ramorum*	ITS	1	<200	(Bilodeau et al. 2007)[2]
rtPCR	Taq-Man	*P. ramorum*	elictin	10	<200	(Bilodeau et al. 2007)
rtPCR	Taq-Man	*P. ramorum*	β-tubulin	100	<200	(Bilodeau et al. 2007)
rtPCR	SYBR GREEN	*P. ramorum*	β-tubulin	506		(Bilodeau et al. 2007)
rtPCR	Taq-Man	*P. ramorum*	β-tubulin	506		(Bilodeau et al. 2007)
rtPCR[1]	Mol Beacon	*P. ramorum*	β-tubulin	2006		(Bilodeau et al. 2007)
nrtPCR	Taq-Man	*P fragariae*	ITS	0.1		(Bonants et al. 2004)
nrtPCR	Mol Beacon	*P fragariae*	ITS	0.1		(Bonants et al. 2004)
rtPCR	Taq-Man	*P fragariae*	ITS	1,000		(Bonants et al. 2004)
nrtPCR	SYBR GREEN	*P ramorum*	ITS	15	687/73	(Hayden et al. 2006)
nrtPCR	Taq-Man	*P ramorum*	ITS	15	687/73	(Hayden et al. 2006)
rtPCR	Taq-Man	*P ramorum*	ITS	50	687	(Hayden et al. 2006)
rtPCR	Taq-Man	*P ramorum*	ITS	10,000	76	(Hughes et al. 2006)
rtPCR	Scorpion	*P. citrophthora*	ITS	25,000		(Ippolito et al. 2004)
rtPCR	Scorpion	*P. nicotiana*	ITS	25,000		(Ippolito et al. 2004)
rtPCR	Scorpion Multiplex	*P. nicotiana and P. citrophthora*	ITS	250,000 and 2,500,000 resp		(Ippolito et al. 2004)
rtPCR	Taq-Man	*Generic Phytophthora*	ITS	10	44	(Kox et al. 2007)
rtPCR	Taq-Man	*P ramorum*	ITS	NG[3]	73	(Kox et al. 2007)
rtPCR	multiplex TaqMan	*P. citricola P. kernoviae P. quercina P. ramorum*	*Ypt1*	100	68–97	(Schena et al. 2006)
rtPCR	SYBR Green	*P capsici*		10,000		(Silvar et al. 2005a)
rtPCR	Taq-Man	*P ramorum*	ITS	100	76	(Tomlinson et al. 2005)
rtPCR	Taq-man LNA[4]	*P ramorum*	ITS	250	76	(Tomlinson et al. 2007)
rtPCR	Mol beacon	*P ramorum*	ITS	500	76	(Tomlinson et al. 2007)
rtPCR	Scorpion	*P ramorum*	ITS	500	76	(Tomlinson et al. 2007)
rtPCR	Taq-Man	*P. ramorum*	*cox*	1	134	(Tooley et al. 2006)

[1]rtPCR, Real-time PCR; nrtPCR, nested real-time PCR
[2]The sensitivity is approximated from C_T values.
[3]NG, Not Given.
[4]LNA residue at the 3′ end of the forward and reverse primers.

Although PCR is an efficient method for detection of pathogens, results from several studies have shown that it can sometimes result in false negatives. These are represented by samples that are negative in a PCR reaction but positive in culture plate and/or ELISA tests (Tables 1 and 3). These were not caused by inhibition of the PCR reaction as in each case there were controls built into the test to detect false negatives. Mis-identification of the pathogen is a possible explanation, e.g., *P. citricola* like organisms which have been isolated from soil samples in Western Australia also form coralloid hyphae, a feature characteristic of *P. cinnamomi* which can result in the *P. citricola* –isolates being confused with *P. cinnamomi* (Bunny 1996). In a study of isolates from nursery plants in Spain, isolates morphologically similar to *P. cryptogea, P. gonapodyides*, or *P. citricola* were found on the basis of DNA sequence not to belong to these species (Moralejo et al. 2009). Alternatively the negative PCR result could be due to a variant strain containing mutations in the binding sites for either primer. Such mutations have a negative effect on amplification (Vincelli and Tisserat 2008). There is an increasing body of evidence that demonstrates that temporal and geographic distribution of strains significantly impacts the performance of DNA amplification tests (Whiley et al. 2008). The degree of confidence in the results can be increased by designing primers to two independent regions of the genome (Vincelli and Tisserat 2008; Whiley et al. 2008). In interpreting results such as these attention must also be paid to the method used for DNA extraction as this can have a significant effect on the results obtained with PCR (Wangsomboondee and Ristaino 2002).

Across all of the studies the majority of samples that tested positive by PCR also tested positive by culture plate isolation. PCR also detected the pathogen in a number

of samples that tested negative by culture plate isolation (Tables 1 and 3). Hence PCR appears to be a more efficient detection method. However, we also have to recognize that PCR does not differentiate between viable and non-viable pathogen, it simply detects the presence of the pathogen DNA. Therefore the negative results of culture plate isolation may be due to the presence of non-viable or dormant propagules of the pathogen, or to suppression of pathogen growth by fungicides or other chemicals.

Target genes for PCR detection

Specific gene targets

The most commonly used gene targets for the development of species-specific primers are the ribosomal RNA internal transcribed sequences (ITS) (Table 2). This is because they can be amplified using universal primers without any prior sequence knowledge, and because they contain regions of low intraspecific and high interspecific variability which are useful for design of species specific primers (Bruns et al. 1991). The rRNA genes are also present in multiple copies, 100s to 1,000s depending on the species making the test more sensitive compared to detection of single copy genes. A comparison of the sensitivities of tests based on different genes (Table 5) shows that those based on ITS regions are more sensitive, although the sensitivities of the different ITS tests spans a very wide range (0.1–25,000fg) perhaps reflecting the influence of primer design.

Another multicopy gene that has been used is the mitochondrial cytochrome oxidase (cox) gene. Since there are several hundred mitochondria in the vegetative cell, these genes are present in high copy number and hence should be useful for developing highly sensitive detection tests. They also contain variable regions that are useful for the design of specific primers. Martin et al. (2004) designed a PCR test for detection of P. *ramorum* based on the *cox* genes. This test had a detection level of 2 fg DNA which is similar to the levels of sensitivity reported for nested PCR tests based on the ITS genes (Table 4). Although useful, the mitochondrial genes have the limitation that they are uniparentally inherited and may not be able to detect hybrid species.

Although the ITS region is the most commonly used it is not always able to separate closely related species of *Phytophthora* e.g., *P. Lateralis,* and *P. ramorum* (Hughes et al. 2006b), or *P. quercina* and *P. cambivora* (Schubert et al. 1999). Schena et al. (2008) described the use of the *Ypt1* gene for differentiation of *Phytophthora* species. Variable intron sequences were used to develop species-specific primers to fifteen species of *Phytophthora*. However, as with the ITS sequences some closely related species e.g., *P. cactorum/P. idaei*, and *P. iliciss/P. nemerosa* could not be differentiated. The sensitivity of detection was in the range 1,500–15,000 fg DNA (Table 5). In tests with naturally infested soil and water, positive detection was achieved only with nested PCR. However, this low level of sensitivity appears to be due to primer design as primers designed to different regions of the *Ypt1* gene were able to detect down to 100fg DNA without using nested PCR (Schena and Cooke 2006). A test for detection of *P. cinnamomi* based on the *Lpv* storage protein was described by Kong et al. (2003) (Table 4). Results obtained with this test correlated well with results of pathogen isolation tests. Of 18 diseased plants tested, 10 were positive with the PCR test and the pathogen was isolated from 9 of these. Tests based on a number of other single copy genes have been developed (Tables 4 and 5). In general, they are not as sensitive as those based on multicopy ITS or mitochondrial genes.

An alternative approach to developing PCR tests based on a specific gene, is to identify a species specific sequence and use this to develop PCR primers. Species-specific sequences can be identified by comparing arbitrary amplification patterns (RAPD-PCR or AFLP) for the target species and closely related species. Bands occurring only in the target species are recovered, and sequenced to enable the development of PCR primers (Dobrowolski and O'Brien 1993; Brisbane et al. 1995; Chiocchetti et al. 2001; O'Brien 2008). Very often such bands are found to be species specific. This is however a more laborious approach to developing species specific primers as bands have first to be identified, recovered and sequenced. Moreover, what appears to be a single band on a gel often consists of multiple co-migrating DNA sequences each of which has to be cloned and evaluated for specificity (O'Brien, unpublished) (Smith et al. 1997). A more expedient approach is to use sequences available from public databases to design species specific primers. Although ITS is the most common *Phytophthora* sequences available and hence the most widely used (see above), other sequences are available for many species.

A major challenge in detection of *Phytophthora* is the emergence of hybrid species. Such species are being detected with increasing frequency (Ersek and Nagy 2008). Most often these species are initially discovered by means of their novel pathogenicity characters rather than by the use of molecular detection methods. Hybrid species such as *Phytophthora alni* would escape detection by the use of detection tests based on the ITS region since the ITS region in *P. alni* appears to have undergone hybridization and to be different than that of either parent (Ioos et al. 2005). They might not be detected using tests based on mitochondrial markers (Martin et al. 2004) as mitochondria are uniparentally inherited. The development of tests using primer pairs targeted to different regions of the genome, or the use of generic *Phytophthora* primers are ways to address this problem.

Detecting multiple species

A number of techniques have been described for detection of multiple species of *Phytophthora* within a sample. One such approach is PCR-ELISA in which digoxygenin-11-UTP (DIG) is incorporated into the amplicon during the amplification phase (Bailey et al. 2002). The amplicons are hybridized to an oligonucleotide capture probe immobilized in the well of a microtiter plate. The amount of DIG retained in the well is determined by an ELISA type reaction (Bailey et al. 2002). Capture probes for different species of *Phytophthora* can be immobilized in different wells, and hence multiple species can be detected simultaneously by this technique. In the original study with mixtures of DNA extracted from isolates the technique could clearly differentiate between eight species of *Phytophthora* and two species of *Pythium* (Bailey et al. 2002). In a subsequent study, Bonants et al. (2004) found the sensitivity PCR-ELISA for detection of *P. fragariae* to be comparable to that of TaqMan PCR.

Drenth et al. (2006) used RFLPs in amplified ITS sequences to differentiate 27 species of *Phytophthora*. However, some related species (*P. infestans* and *P. mirabilis*, and *P. erythroseptica* and *P. cryptogea*) could not be differentiated with this system. The detection sensitivity was 2 pg DNA extracted from laboratory isolates.

Systems that contain an array of probes each for a different target species are capable of differentiating multiple species (Vincelli and Tisserat 2008). DNA extracted from the sample is labelled and hybridized to the array. Since the probe for each species is in a defined location, the presence of label on a spot indicates the presence of that target species in the sample. Anderson et al. (2006) explored the use of microarrays for differentiation of species of *Phytophthora*. The array consisted of several oligonucleotide probes to each of eight species of *Phytophthora* as well as generic *Phytophthora* probes. Although some probes showed cross hybridization to non-target species all eight species could be differentiated. All species gave a positive reaction with the generic *Phytophthora* probes. This study showed the value in having multiple probes to each species. Although potentially useful, microarray technology is not yet at the stage where it can be used as a routine screening technique.

Some of the drawbacks of the microarray, such as the need for specialized equipment and variation between spots are not encountered with macroarray technology. These are based on the attachment of probes to nylon membranes (Vincelli and Tisserat 2008). Although of lower density than the microarray, their capacity (1,000 probes on an array the size of a microtiter plate) is greater than that needed for routine pathogen screening. Zhang et al. (2008) described the use of a macroarray for the detection of a range of pathogens that commonly infect solanaceous species. The array contained a number of probes for each target species as well as single base mismatched probes, universal probes and negative probes. Except for species of the *Fusarium solani* species complex, isolates of 23 target species including a number of *Phytophthora* species were unambiguously detected without cross hybridization to closely related non-target species. Single nucleotide polymorphisms could be clearly differentiated. In sensitivity tests the array could detect 0.04 pg DNA.

Another technology that has received considerable attention for identification of microbial species is MALDI-TOF mass spectroscopy (MALDI-TOF MS) (Leushner and Chiu 2000, Kim et al. 2005, Jackson et al. 2007). In this technique species specific primers annealed to template DNA are extended by a single nucleotide, dissociated from the template and separated by mass. Since each of the four nucleotides has a different mass, four products are obtained for each primer, and since multiple primers can be annealed to the template the resolving power of the technique is very high. Attractive features of this technique are that it is very high throughput, cost effective, and amenable to automation. Although the application of MALDI-TOF MS to diagnosis of plant pathogens is in its infancy, preliminary studies have demonstrated that it can differentiate species of *Phytophthora* in DNA extracted from soil (Siricord and O'Brien 2008).

In general tests for detection of multiple species are more complex and hence more expensive and of lower throughput compared to single species PCR detection tests. In testing plant material it is generally not necessary to use tests for multiple species as typically we would expect only a small number of species to be present. Inclusion of a reaction with generic *Phytophthora* primers would be a more appropriate method for detection of non-target species. Tests for multiple species are most appropriately used for detection of *Phytophthora* in milieu where there are likely to be any number of different species, e.g., soil and water. However, even in these instances it would be simpler and more economical to use generic *Phytophthora* primers.

Analysis of environmental samples

Detection of *Phytophthora* in plant tissue samples by PCR

In virtually all studies the sensitivity of PCR detection tests is determined with DNA extracted from laboratory isolates. However, typically *Phytophthora* detection tests are used with DNA extracted from plant tissue or from soil which amplifies much less efficiently due to the presence of PCR inhibitory substances that co-extract with the DNA. Martin et al. (2004) reported that plant DNA decreased the sensitivity of their *P. ramorum* detection assay by 100–1,000 fold depending on the plant species. Similar inhibitory effects of plant DNA on PCR

amplification were reported by other researchers (Grote et al. 2002; Hayden et al. 2006; Bilodeau et al. 2007). The degree of inhibition also depends on the tissue from which the DNA is extracted. Belbahri et al. (2007) found that the sensitivity of detection of *P. ramorum* was five-fold lower when Rhododendron leaf DNA was added to the reaction, but 100 fold lower when stem DNA was added. The difference is probably due to differences in the concentration of PCR inhibitors in the tissues.

Traditional methods for extraction of plant DNA are unsuitable for high throughput diagnostic laboratories because they are complex, time consuming, and/or involve the use of organic solvents. Such methods have been superseded by more rapid simpler commercially available automated protocols which produce DNA that although not chemically pure, is of sufficient purity for use in PCR reactions (Kox et al. 2007). However given the large degree of chemical variation that exists both between and within plant species, and even between different tissues of the same plant it is imperative that all samples are tested for chemical inhibition using positive control primers. These could be primers to the plant ITS or mitochondrial *cox* sequences (Hughes et al. 2006b). Strategies that can be taken to reduce the effects of inhibitors include the addition of BSA or T4g32 proteins or the addition of DMSO or glycerol to the PCR reaction (Ma and Michailides 2007). Dilution of the DNA prior to PCR is also an effective strategy although it reduces the sensitivity of detection.

The type of DNA polymerase is also important in considering the effects of PCR inhibitors. Different DNA polymerases show different levels of susceptibility to inhibitors. Al-Soud and Radstrom (1998) reported a 5000 fold difference in the sensitivity of DNA polymerases to PCR inhibitors in blood. In our studies we have found that *Taq*1 DNA polymerase was more sensitive to PCR inhibitors in soil than either *Taq*F1* or *Tth* + polymerases (Williams et al. 2009).

Detection of *Phytophthora* in soil samples by PCR

Many species of *Phytophthora* are soil borne pathogens and spread through the movement of infested soil, or by water flow through infested soil (Shearer and Tippett 1989). A key element in the management of such diseases is the ability to detect the pathogen in soil and water. However, DNA extracted from soil contains substances such as humic acids, lignins, carbohydrates, resins, and so on which are very inhibitory to PCR amplification (Tien et al. 1999; Robe et al. 2003). The amounts of inhibitory substances will vary widely with soil type, vegetation type, and composition of the soil microflora. As the microflora varies even over small distances (1 m scale) (Scala and Kerkhof 2000), the efficiency of PCR amplification is likely to vary widely even over small distances. It is therefore critical that an internal standard is used for PCR analysis of soil samples. Strategies used to reduce the effects of PCR inhibitors in plant DNA (previous section) can also be used with soil DNA.

As with the extraction of DNA from plant tissue, the best methods for extraction of soil DNA are the commercially available kits followed by dilution of the DNA before PCR. Another factor that has to be taken into account when interpreting the results of PCR analysis of soil samples is the longevity of DNA in soil. Does a PCR positive result indicate the presence of a viable pathogen, or are we simply detecting residual DNA from dead mycelium? Although naked DNA added to soil is degraded rapidly (England et al. 1998), DNA enclosed within mycelium, or plant tissue appears to be more persistent. Gebhard and Smalla (1999) found that when litter from transgenic plants was tested for the transgene, specific signals could be detected for up to 6 months whereas naked DNA was completely degraded within days. Herdina et al. (2004) showed that although heat treated mycelium of *Gaumannomyces tritici* buried in soil the DNA was 98.6% degraded within 2 d, infected heat treated organic debris was detectable for up to 4 weeks. Hussain et al. (2005) recorded positive detection of *P. infestans* for up to twelve months from soil in which infested leaf tissue had been buried and for up to 24 months from soil containing leaf tissue infected with both mating types. The difference is ascribed to the formation of sexual oospores in the doubly infected material. However Anderson (2006) showed that infective propagules of *P. cinnamomi* do not persist for long periods in soil. After incubation of infested soil for 12 weeks at 25°C the pathogen could not be detected by baiting, and although it could be detected by PCR the detection efficiency was significantly reduced. When the soil was incubated at 30°C for 12 weeks the pathogen could not be detected by either baiting or PCR.

For detection in soil, where there is no obvious lesion tissue to sample from, soil baiting has the advantage that it enables large amounts of soil (0.5–1 kg) to be tested whereas DNA extraction protocols are all based on extraction of small (1–10 g) samples. Consequently, with sampling for DNA extraction and analysis by PCR we may simply miss the pathogen. Baiting also has the advantage that it only detects viable pathogen. Despite the advantages, baiting is too slow and low throughput to be useful and can be subject to a high degree of false negatives. Nechwatal et al. (2001) suggested that testing the bait water by PCR would have a number of advantages: (1) It overcomes the problem of sample size in relation to testing soil, (2) since we are testing water, problems with soil PCR inhibitory substances that co-extract with the DNA would be reduced, and (3) it also overcomes the problem that zoospores do not always infect the bait tissue leading to false negatives (Wilson et al. 2000). However, as discussed above in the section on soil baiting, viable pathogen may be present but only produce zoospores after washing and

drying of the soil (Jeffers and Aldwinkle 1987; Davison and Tay 2005). These would not be detected by testing the bait water unless this additional step was routinely included in the baiting procedure. Dormant forms of the pathogen such as chlamydospores or oospores would also not be detected.

Priorities for future research

Globalization of business has created a situation where plants are sourced from suppliers far from the retail outlets thus increasing the volume of plants being transported. This facilitates the spread of *Phytophthora* and can lead to the appearance of new genotypes with devastating consequences. The importation of new strains of *Phytophthora infestans* into the United States from Mexico in 1992 led to a complete change in the genetic structure of the *P. infestans* population in the United States (Goodwin 1997). Increased transport may also increase the chances of hybrid species formation (Brasier 2000).

A major driving force in the development and adoption of molecular techniques in plant pathogen detection is the requirement by commerce for quick answers. Commerce moves quickly and cannot wait for days or week for answers from diagnostic laboratories, they require answers within hours. The necessities of commerce are driving the development of on-site tests that can fulfill this requirement. An on-site PCR test for *P. ramorum* has been developed and trialed and found to compare very well with the laboratory procedure (Tomlinson et al. 2005; Hughes et al. 2006). There are a number of non-PCR amplification DNA technologies (nucleic acid based amplification; exponential nucleic acid amplification; helicase dependent isothermal amplification; and rolling circle amplification) being developed that open up possibilities for development of onsite tests without the need for amplification equipment (reviewed in (Boonham et al. 2008). A recent study demonstrated that as little as 10 pg *P. ramorum* DNA could be detected by isothermal amplification of nucleic acid (Tomlinson et al. 2007). The detection was a simple color change in the reaction.

Underpinning an effective containment and pathogen management strategy is a thorough understanding of the pathogen genetics, taxonomy and biology. We need to know how the pathogen population changes and the frequency with which these changes occur as mutations in primer binding sites can lead to false positives and false negatives (Vincelli and Tisserat 2008). The type of propagules we are detecting can have an important effect on the result. What conditions induce or repress the formation of oospores and or chlamydospores and can these propagules be detected as efficiently as mycelium? We need to understand the taxonomy of *Phytophthora* so that we can ensure the specificity of the detection test, e.g., tests for detection of *P. ramorum* cross react with the very closely related *P. lateralis* (Hughes et al. 2006a). These aspects of the biology of *Phytophthora* are priorities for future research.

A major challenge in preventing the spread of *Phytophthora* is detection of the pathogen in asymptomatic tissue. With the increased volume of plant germplasm being transported across national boundaries each year it is obviously impossible for a country to test every plant that crosses its borders so a more strategic approach needs to be adopted. This might involve testing plants that come from high-risk areas such as testing plants from the US West Coast for *P. ramorum*, or testing particular species of plants e.g., testing Rhododendron for *P. kernoviae*. Surkov et al. (2008) developed a multinomial logistic (MNL) regression model that attempts to predict the likelihood of a pest/pathogen infestation in shipments of imported plants. They found that significant risk factors were: (a) the geographic region of the exporting country; (b) the size of the shipment; (c) the plant species; (d) year and season of importation. To factor these risks into import decisions it is crucial that quarantine operators have access to the latest information on outbreaks of *Phytophthora* diseases in different parts of the world. Initiatives such as the recently announced *Phytophthora* database (Park et al. 2008) have an important role to play in this regard.

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