THE USE OF NESTED PRIMERS IN THE POLYMERASE CHAIN REACTION FOR THE DETECTION OF *PHYTOPHTHORA FRAGARIAE* AND *P. CACTORUM* IN STRAWBERRY

I. Lacourt [1], P.J.M. Bonants [2], M.P. Van Gent-Pelzer [2], D.E.L. Cooke [1], M. Hagenaar-De Weerdt [2], L. Surplus [3] and J.M. Duncan [1]

[1] Scottish Crop Research Institute, Invergowrie, DD2 5DA, UK
[2] Research Institute for Plant Protection (IPO-DLO), PO Box 9060, 6700 GW Wageningen, The Netherlands
[3] Mylnefield Research Services Ltd, Invergowrie, DD2 5DA, UK

Additional keywords: *Fragaria x ananassa*, disease, red stele, crown rot, molecular diagnostics

Summary

Phytophthora fragariae var. *fragariae* Hickman, which causes red core disease of strawberry, is a quarantine organism on which a nil tolerance is placed. Detection of the fungus is by a root tip bait test which is highly specific and sensitive but time-consuming (5-6 weeks), has to be done at 12°C and can require some taxonomic experience. Even with the test, detecting red core can still be difficult, especially with early primary infection of propagation beds or infected symptomless resistant cultivars. Moreover, *P. cactorum*, a closely related fungus, causes crown rot of strawberry. It is not a quarantine organism, typical tolerances of 1% infection are allowed, and can confuse diagnosis. Techniques based to the Polymerase Chain Reaction (PCR) are promising alternatives.

Various parts of the ribosomal RNA gene repeat (rDNA) have been used to design primers with different specificities: specific PCR primers that can differentiate between *P. fragariae* and *P. cactorum* and others designed to detect a wider range of Peronosporales, to which *Phytophthora* belongs. In nested PCR, combinations of these primers can detect both pathogens with high specificity and sensitivity. Competitive PCR has also been developed to allow quantification of *P. cactorum* infection in strawberry crowns. With these techniques, *P. fragariae* was detected in strawberry plants one day after inoculation whereas the symptoms were only visible after one week. Moreover, very low numbers of zoospores could be detected in water samples. The test has been evaluated on samples provided by the inspection services of Scotland and The Netherlands.

1. Introduction

Phytophthora fragariae, an important soilborne pathogen, causes severe root-rot symptoms on strawberry (red core) and raspberry plants (root rot). The two diseases are caused by different varieties of the fungus, var. *fragariae* and var. *rubi* respectively

Proc. Third Int. Strawberry Symp.
Eds. H.A.Th. van der Scheer, F. Lieten, J. Dijkstra
Acta Hort. 439 Vol. 2. ISHS 1997

(Wilcox *et al.*, 1993). Physiologic races occur in both diseases (Duncan and Kennedy, 1995) and they are major limitations on crop growth, requiring the application of expensive fungicides with potential for associated environmental problems. Spread worldwide of both diseases has been *via* the movement of infected rootstock (Duncan, 1993). *P. fragariae* is therefore a quarantine organism in many countries, with the requirement that propagation stocks entered for certification must be "disease free". Zoospores spread the disease in water and infect the host after encystment and also are therefore an important target for detection. They are the most common propagule in water: numbers can reach as high as 400 per litre in recirculated irrigation water (MacDonald *et al.*, 1994).

A bait test of strawberry root tips has been used for fifteen years to detect the disease in planting material (Duncan *et al.*, 1985). In the test, root tips (1-2 cm long) collected from young runner plants growing in the field are mixed with compost in which strawberry cultivars highly susceptible to all races are then planted. The plants are left for five weeks in a glasshouse, under conditions suitable for infection by the pathogen, to allow symptoms to develop. Eventually, the pathogen can be isolated from the lesions which have formed, and identified. This procedure gives good results but is time- and space-consuming. Moreover, correct identification requires good knowledge of the disease on plants and of the morphology of *Phytophthora* species in culture, especially as other *Phytophthora* spp. can occur on strawberry. In recent years *P. cactorum* has been an important pathogen on strawberry across much of Europe causing crown rot; tolerances typically of *c.* 1% are allowed for this disease in most certification schemes.

Recently, new techniques have been used to lessen or eliminate the difficulties of detection and diagnosis. Diagnostic tools, based on polyclonal antibodies, allow sensitive but generally not species-specific detection of several pathogens (Olsson, 1995). The Polymerase Chain Reaction (PCR) can exponentially amplify DNA sequences of a fungus by *in vitro* DNA synthesis from very low levels to levels easily detectable by non- radioactive methods. Primers for PCR to detect a single pathogen or many members of a group of related pathogens can be designed and produced more quickly and at a lower cost than comparable serological reagents. Moreover, nested PCR, using two sets of primers in two successive PCR amplifications, has greatly improved the level of sensitivity of the technique (MacManus and Jones, 1995).

An increasing number of PCR-based diagnostics are using sequences from the nuclear ribosomal RNA gene repeat, more commonly referred to as ribosomal DNA (rDNA) as sources of primers. There is an abundance of publications on rDNA, on sequence variation and mutation rates, and many copies of the repeat are present in each nucleus, improving the chances of detection. Spacer regions of the repeat unit which are not thought to play a functional role (Nues *et al.*, 1994) are less conserved than those parts encoding ribosomal RNA and show useful levels of interspecific variation in fungi (Lee and Taylor, 1992, Zambino and Szabo, 1993, Sherriff *et al.*, 1994). Therefore, for the purposes of detection, primers can be designed within the genes or spacer regions to give appropriate levels of specificity.

In this paper, we described how several sets of primers have been designed and used in two rounds of nested PCR. One set is only specific for fungal genera, including *Phytophthora* and *Pythium*, that belong to the order Peronosporales within the Oomycetes. Other sets of primers, designed from the sequence of probe pPS3003 (Stammler *et al.*, 1993) or ITS sequences within rDNA are specific for *P. fragariae;* in

the latter case specific primers for *P. cactorum* have also been made (Cooke *et al.*, 1995). Nested PCR using combinations of these rDNA-based primer sets detected and distinguished between both pathogens *in planta*, and detected zoospores in water. In the case of detection in strawberry plants, the efficiency of nested PCR has also been compared to conventional single round PCR with only one set of primers. Quantitative PCR (Lamar *et al.*, 1995) has also been used to estimate the amount of fungal biomass in necrotic plant tissue.

2. Materials and methods

2.1. Fungal isolates and cultural conditions

A large collection of isolates of *P. fragariae, P. cactorum*, *P. idaei* and other *Phytophthora* species were maintained on oatmeal agar sloped cultures at 4°C. Mycelium, grown on French Bean agar medium at 20°C in the dark, was peeled off and used to inoculate 20 mL of a defined sucrose/asparagine/mineral salts medium, at 20°C in the dark. After one week, the mycelium was harvested by filtration through filter paper (Whatman), washed with distilled water and stored frozen (-20°C). Zoospores of *P. fragariae* were produced by placing agar plugs of mycelium in a water extract of soilless compost for three days at 14°C in the dark with the extract being changed every day. On the last day the extract was replaced with distilled water and after incubation overnight at 4°C and four hours at room temperature, the zoospores were collected.

2.2. Inoculation of plants, microscopic examination and collection of samples

Strawberry plants, cv. Alexandria, were grown from seed in pots for 6 weeks in a growth chamber (temperature 15°C, 16 hours light). Immediately before inoculation, water was poured into the pot to soak the soil. The plants were then inoculated with a suspension of 25,000 zoospores in 50 mL of water and at various times intervals thereafter, 2 hours, 16 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days and 12 days, plants were removed from pots, their roots were washed in the tap water and two cm long root fragments were cut and examined under the microscope or used directly for DNA extraction. For histological study with fluorescent staining, roots tissue was prepared and observed as described by Williamson *et al.* (1995).

2.3. DNA extraction

2.3.1. method at SCRI

Fungal DNA was extracted using a Nucleon II extraction kit (Scotlab Ltd, Glasgow) according to the manufacturer's recommendations. Between 150 and 250 mg of compressed wet-weight mycelium were extracted in a protocol scaled down for use in 1.5 mL Eppendorf tubes; this yielded 30 to 50 mg of DNA. Plant DNA was extracted from two or three 2 cm long root fragments as described by Lacourt and Duncan (in press).

2.3.2. method at IPO-DLO

DNA was prepared from zoospores by mixing 50 mL of a solution containing zoospores with 200 mL of Instagene (6% solution, Biorad) according to the manufacturer recommendations. The supernatant containing the DNA was used in the PCR experiments. DNA extracted from water samples from bait tests was cleaned with the Wizard clean-up kit (Promega), according to the manufacturer recommendations.

2.4. PCR reactions

All the primers were designed around parts of the ribosomal RNA gene repeat (rDNA). Both laboratories initially designed their own primers (Table 1) but as the work progressed primers were exchanged and used in various combinations and for various purposes.

Table 1. PCR primers used in this study, their sequence, origin, specificity and use.

Primer	Sense	Sequence	Detects	Source
P1	forward	5'-CCGTTACTAGGGGAATCCTT	*P. fragariae*	pPS3003
P2	reverse	5'-TTCATTTTCGGATAGAACCG	*P. fragariae*	pPS3003
DC6	forward	5'-GAGGGACTTTTGGGTAATCA	Peronosporales	18S rDNA
ITS4	reverse	5'-TCCTCCGCTTATTGATATGC	Eukaryotes	28S rDNA
B1	forward	5'-GATGCCACCCGCAGCACACC	*P.fragariae*	pPS3003
B2	reverse	5'-TGTGTGTCGGGCCCTATCAT	*P. fragariae*	pPS3003
B5	reverse	5'-TGAGATGCCACCCGCAGC	*P. fragariae*	pPS3003
DC1	forward	5'-ACTTAGTTGGGGGCCTGTGT	*P. fragariae*	ITS1 rDNA
DC5	reverse	5'-CGCCGACTGGCCACACAG	*P. fragariae*	ITS2 rDNA
ADF1	forward	5'-TACTGTGGGGACGAAAGTCCT	*P. cactorum*	
			P. idaei	ITS1 rDNA
ADR1	reverse	5'-CCGATTCAAAAGCCAAGCAACT	*P. cactorum*	
			P. idaei	ITS2 rDNA

2.4.1. IPO-DLO: Nested PCR detecting zoospores

Primers P1 and P2 (Table 1) had been used already to detect *P. fragariae* in infected strawberry and raspberry roots (Stammler and Seemüller, 1993). Other primers, including B1, B2 and B5, based on sequences internal to P1 and P2 were developed at IPO-DLO. Nested PCR with primers P1 and P2 in the first round and primers DC1 and B5 were used to detect zoospores of *P. fragariae* (section 3.2.1.) and the fungus in infected roots.

2.4.2. SCRI: Nested primers

Primer DC6 was derived by comparing 18S rDNA sequences from oomycetes,

plants, other fungi and organisms. It is a downstream primer, specific for members of the order Peronosporales including *Pythium*, *Phytophthora* and the downy mildews. Together with the reverse universal primer ITS4 (White *et al.*, 1990), whose sequence is derived from the beginning of the 28S rDNA gene, it produces a product containing both ITS1 and ITS2 sequences. These two primers were used in the first round of nested PCR.

Specific primers DC1 and DC5 were designed respectively from the ITS1 and ITS2 sequences of *Phytophthora fragariae* after comparing them with sequences from nearly twenty other *Phytophthora* species. The primers were designed around as few as three base pair differences at their 3' ends, sufficient for specific DNA amplification. DC1 and DC5 were used either in direct PCR for *P. fragariae* or in the second round of nested PCR after primers DC1 and ITS4.

Specific primers ADF1 and ADR1 were designed in a similar fashion but to detect *P. cactorum*. They do not distinguish between this species and the very closely related *P. idaei* which occurs commonly on raspberry. However, there is a single base pair sequence difference between the products from these two species that generates a unique restriction site with *Bst* EII, a DNA restriction enzyme: the product of *P. idaei* is cut into two pieces whereas the product from *P. cactorum* is unaffected.

2.4.3. Conditions

Reaction mixtures varied according the primers and their use, as did the cycling times and temperatures. The methods given here were used at SCRI but the protocols at IPO-DLO were very similar. Generally primers were used at 1 mM with 0.2 mM of the four dNTPs (Adenine, Cytosine, Thymidine and Guanine deoxyribonucleotides), 1 mg mL^{-1} Bovine Serum Albumin (BSA), 0.05 units of *Taq* polymerase (Gibco BRL) per microlitre of reaction and the PCR buffer supplied with the enzyme (20 mM Tris HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$). Test DNA (0.5 mL) was added at the end in the reaction mix. In the case of the nested PCR, the amplification product obtained after the first round PCR was diluted one hundred times in sterile water and 0.5 mL was added to the second round PCR. Depending on requirements, the final volume of PCR reactions could be 25, 50 or 100 mL.

The thermocycler parameters for direct and nested PCR were as follows. DNA was denatured at 94°C for 3 min. For direct PCR with DC1/DC5, the tubes were subjected to 25 cycles of denaturation 94°C, 30 s., annealing 65°C, 15 s. and synthesis, 72°C 1 min. 30 s. The reaction was finished by leaving the tubes for 10 min. at 72°C. For nested PCR in the first round with primers DC6 and ITS4, DNA was denatured at 94°C for 3 min, followed by 30 cycles of denaturation 94°C, 30 s., annealing 55°C, 15 s. and synthesis, 72°C for 1 min. 30 s. The reaction was finished by leaving the tubes for 10 min. at 72 °C. In the second round with DC1 and DC5, DNA was denatured at 94°C for 3 min. followed by 23 cycles of denaturation 94°C, 30 s., annealing 65°C, 15 s. and synthesis, 72°C 1 min. 30 s. The reaction was finished by leaving the tubes for 10 min. at 72°C.

The results of the PCR reaction were assayed by electrophoresis of the PCR products on a TAE 1X (40 mM Tris-acetate, 1 mM EDTA) agarose gel, staining with ethidium Bromide (0.5 mg/mL) and looking for the presence/absence of appropriate bands using an UV transluminator.

2.5. Quantitative PCR for *P. cactorum*

Competitive PCR for quantifying *P. cactorum* DNA exploited the restriction site difference between the products from *P. cactorum* and *P. idaei*. Known amounts of DNA from *P. idaei* in the plasmid PCRscript were added with primers ADF1 and ADR1 to the reaction mixture and after the reaction was complete, 1 unit of enzyme *Bst* EII was added to each tube. The reaction was stopped after 1 hour at 65 °C and products run on gels.

3. Results

3.1 Primers

DC6 and ITS4 gave a product with DNA from all *Phytophthora* species tested, including *P. fragariae*, single isolates of *Pythium ultimum* and *Pythium sylvaticum*, and tissue from a range of plant species infected with the downy mildews, *Peronospora viciae*, *Peronospora sparsa*, *Peronospora parasitica* or *Albugo candida*. In each case, a band of *c.* 1500 bp. was obtained. No amplification was obtained with isolates of *Achlya* spp, *Saprolegnia* spp and *Thraustotheca* spp., which belong to other orders of the Oomycetes or with healthy plant material.

Specific primers DC1 and DC5 amplified DNA (a single band 550 bp) from ten isolates of *P. fragariae* var. *fragariae* and four of *P. fragariae* var. *rubi* of different physiological races and/or geographical origins. In contrast, it did not amplify DNA from 16 other species: *P. cactorum, P. cambivora*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, *P. gonapodyides, P. idaei*, *P. ilicis, P. megasperma*, *P. nicotianae*, *P. palmivora* and *P. syringae*. Initially, there was a faint band with *P. syringae* but a slight modification of the conditions eliminated this artefact. No amplification was obtained with DNA from *P. cactorum.* Primers P1, P2, B1, B2 and B5 were all specific for *P. fragariae*.

Primers ADF1 and ADF2 only amplified DNA from *P. cactorum* and *P. idaei* and from none of the other species listed above, including *P. fragariae.*

3.2. Detecting *Phytophthora fragariae*

3.2.1. Zoospores (IPO-DLO)

Using the primers P1 and P2 in the first round of nested PCR and DC1 and B5 in the second, twenty zoospores of *P. fragariae* were easily detectable. Likewise, *P. fragariae* was readily detected in water samples provided by the NAKB, the Dutch Inspection Service, that had been collected from root tip bait tests ten days after the start of the tests.

3.2.2. *In planta* (SCRI)

In roots from the time-scale experiment, symptoms of infection with *P. fragariae* were only visible after six or seven days, roots became flaccid and necrotic. After eight days, the volume of the whole root system on infected plants was noticeably reduced in

comparison to the controls and the pathogen was diagnosed readily by the presence of oospores around the stele of the roots.

In roots stained with aniline blue, mycelium started to develop within a day of inoculation, mainly in the root tips. Differentiating sporangia, oogonia and antheridia were detectable after four days and numerous oospores were observed along the vascular tissues after seven days. Roots were completely disorganised after twelve days.

In a comparison of direct and nested PCR on the same root material, no amplification was obtained by either method with DNA from roots from the controls. By two days, only 30% of the samples from inoculated plants gave a product with direct PCR but by four days this had risen to 100% and although it declined on days 5 and 6 (60-70%), it rose again to 100% by the end of the experiment. In contrast all samples from 2 days onwards were positive with nested PCR.

Nested PCR was also used with the DNA samples extracted two hours, 16 hours and one day after inoculation. *P. fragariae* was detected in all samples taken one day after inoculation but only in some roots after 16 hours and in none after two hours.

3.3. Detection of *P. fragariae* and *P. cactorum* in root samples from the field

Six samples of strawberry root tips were collected by the Horticulture Inspectorate of the Scottish Office Agriculture, Environment and Fisheries Department Inspection Office from strawberry stocks entered for official certification in Scotland for testing in the root tip bait test. Sub-samples of approximately 20-30 grams from each of the six samples were provided blind, *i.e.* under code numbers, to SCRI. Three rotted or brown roots were selected from each sample and DNA was extracted from two 2 cm pieces from each root to give 36 DNA samples in total. A first round of PCR was made on the DNA samples using the Peronosporales-specific primers, the products from which were then diluted in water and used in a second round PCR, either with *P. fragariae* or *P. cactorum*-specific primers. No *P. fragariae* was detected in any of the six samples, a result confirmed by bait tests on the same strawberry root samples (Dr Ali, personal communication). However, *P. cactorum* was detected by nested PCR in two of the six DNA extracts from four samples and in one of the six extracts from another.

The same technique has detected *P. fragariae* var. *rubi* on raspberry samples collected from the field as well as *P. idaei.* This latter species was distinguished in PCR from *P. cactorum* by exposing the products of the reaction to *Bst* EII.

3.4. Quantification of PCR for *P. cactorum*

Adding competing target DNA distinguishable from the PCR product of *P. cactorum* by digestion with a restriction enzyme allowed some estimation of the amount of DNA from *P. cactorum.* In agarose gels, the signals from the latter were increasingly stronger with smaller amounts of the competitor DNA. However, the amounts of DNA of *P. cactorum* as estimated by nested PCR have not yet been related to particular infection levels in strawberry plants nor to the infection levels permitted in certification schemes.

4. Discussion

The results obtained with nested PCR for *Phytophthora fragariae* and *P. cactorum*, show that it has clear potential for detecting these two species in infected strawberry plants from the field and in drainage water in glasshouse or field. Nested PCR definitely improved sensitivity over direct PCR, judging from the results obtained with zoospores and in the time course experiment. In the latter case it detected *P. fragariae* on plants without any visible symptoms. Nested PCR may also improve specificity through the enrichment in the product from the first PCR reaction with the target sequence *vis-a-vis* background DNA.

Both sets of primers used in nested PCR for *P. fragariae* gave satisfactory results. The PI and P2 primers used in the first round of nested PCR at IPO-DLO are themselves specific primers, so that a signal in the first round is a good indication that *P. fragariae* is present. In contrast the DC6 and ITS4 primers used at SCRI will amplify DNA from most fungi belonging to the Peronosporales, including *Pythium*. Thus a signal could be expected more often in the first round of PCR when these primers are used. However, one advantage of using such primers is that the product from the first round can be tested using several specific primers to detect more than one *Phytophthora* species if present or to identify what species it is, if it is not *P. fragaria*; this indeed was done with the strawberry samples collected from the Scottish stocks entered for certification and showed that while there was no red core present, *P. cactorum* was! Easier and improved sequencing techniques or restriction digests also could be used to determine the nature of the signal when specific primers fail to identify the species involved

At present the products of PCR are visualised on agarose gels. More sensitive and easier systems are under development, *e.g.* the team at IPO-DLO have developed an ELISA-PCR in which the concentration of product in the final reaction is assessed in an ELISA plate reader (results not shown here). Likewise, the system could be made quantitative by the addition of a competing target DNA such as has been done for *P. cactorum*. More problematic is the question of sampling and size of samples to be taken from fields and plants. In the four field samples where *P. cactorum* was detected, only one or two DNA extracts from each batch were positive: clearly not all roots were infected with the pathogen. In contrast to bait testing in which large batches of root tips can be tested, only small part of roots presently can be tested by PCR. Several procedures might overcome this problem. Batches of roots could be incubated under conditions favourable for disease development and mixed at regular intervals throughout the incubation to ensure a good distribution of diseased material in the sample before testing. Alternatively, they could be irrigated with water and the drainage water could be tested for the presence of the fungus. The feasibility of this idea is strengthened by the results obtained with very small numbers of zoospores in nested PCR.

Those involved in checking the health of strawberry plants with regards to Phytophthora diseases can look forward to highly sensitive and discriminatory PCR-based tests that increasingly will be more user friendly and routinely applicable.

Patent protection of primers DC1, DC5, DC6, ADF1 and ADR1 has been secured.

5. References

Cooke D.E.L., D.M. Kennedy, D.C. Guy, J. Russell, S.E. Unkles S.E. and J.M. Duncan. 1995. Relatedness of Group I species of *Phytophthora* as assessed by RAPDs and sequences of ribosomal DNA. Mycological Research 100:297-303.

Duncan J. M. 1993. Data sheets on Quarantine Organisms: *Phytophthora fragariae*. Pp. 599-608 in: Quarantine Pests for Europe (I.M. Smith, D.G. McNamara, P.R. Scott, K.M. Harris, eds). CAB International, Wallingford, UK.

Duncan J.M., W. Fordyce, P.C. Harper and P.A. Rankin, 1985. Eliminating red core (*Phytophthora fragariae*) from Scottish certified stock strawberries. Research & Development in Agriculture 3:43-46.

Duncan J.M. and D.M. Kennedy, 1995. Effect of temperature and host genotype on the production of inoculum by *Phytophthora fragariae* var. *fragariae* from the roots of infected strawberry plants. Plant Pathology 44:10-21.

Lacourt, I. and J.M. Duncan. Specific detection of *Phytophthora nicotianae* using the polymerase chain reaction and primers based on the DNA sequence of its elicitin gene ParA1. European Journal of Plant Pathology (In press).

Lamar, R.T., S.A. Van den Wymelenberg, P. Stewart, D.M. Dietrich and D. Cullen, 1995. Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonised soil. Applied Environmental Microbiology 61:2122-2126.

Lee, S.B. and J.W. Taylor, 1992. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. Journal of Molecular Biology and Evolution 9:636-653.

MacDonald, J.D., M.S. Ali-Shtayeh, J. Kabashima and J. Stites, 1994. Occurrence of *Phytophthora* species in recirculated nursery irrigation effluents. Plant Disease 78:607-611.

MacManus, P.S. and A.L. Jones, 1995. Detection of *Erwinia carotovora* by nested PCR and PCR-dot-blot and reverse-blot hybridization. Phytopathology 85:618-623.

Milholland, R.D. and M.E. Daykin, 1993. Colonization of roots of strawberry cultivars with different level of susceptibility to *Phytophthora fragariae*. Phytopathology 83:538-542.

Nues, R.W., J.M.J. Rientjes, C.AS.F.M. Van der Sande, F.Z. Shuraila, C. Sluiter, J. Venema, R.J. Planta and H.A. Raué, 1994. Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA. Nucleic Acids Research 22:912-919.

Olsson, C.H.B., 1995. Diagnosis of *Phytophthora* infections in raspberry and strawberry plants by ELISA tests. Journal of Phytopathology 143:307-301.

Sherriff, C., J.W. Mitzi, G.M. Arnold, J. Lafay, Y. Brygoo and J.A. Bailey, 1994. Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. Experimental Mycology 18:121-138.

Stammler, G. and E. Seemüller, 1993. Specific and sensitive detection of *Phytophthora fragariae* var. *rubi* in raspberry roots by PCR amplification. Journal of Plant Disease Protection 100:394-400.

Stammler, G., E. Seemüller and J.M. Duncan, 1993. Analysis of RFLPs in nuclear and mitochondrial DNA and the taxonomy of *Phytophthora fragariae*. Mycological Research 97:150-156.

White, T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 in: PCR protocols: A guide to Methods and Applications (M.A. Ignis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds.). Academic Press, San Diego, USA.

Wilcox, W.F., P.H. Scott, P.B. Hamm, D.M. Kennedy, J.M. Duncan, C.M. Brasier and E.M. Hansen, 1993. Identity of a *Phytophthora* species attacking raspberry in Europe and North America. Mycological Research 97:817-831.

Williamson, B., W.A. Breese and R.C. Shattock, 1995. A histological study of downy mildew (*Peronospora rubi*) infection of leaves, flowers and developing fruits of Tummelberry and other *Rubus* spp. Mycological Research 99:1311-1316.

Zambino, P.J. and L.J. Szabo, 1993. Phylogenetic relationships of selected cereal and grass rusts based on rDNA sequence analysis. Mycologia 85:401-414.