Project Title:	Detection of Phytophthora Diseases in Horticultural Planting Stocks by the Polymerase Chain Reaction (PCR)
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Project leader:	Jim Duncan
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Coordinator:	Peter Thompson
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Practical section for growers

Background and objectives

Phytophthora spp. cause serious losses of value and quality in many important horticultural crops including soft fruit, hardy ornamentals and glasshouse crops: The principal problem is that the fungus is spread in and on diseased nursery stocks or in contaminated irrigation water or nutrient solution. Detecting contamination and infection by *Phytophthora* in nurseries or in water supplies is difficult, time-consuming and, with present technologies, unreliable. Solutions to this problem are central to the future development of a healthy horticultural industry within the UK and trade in nursery stocks both within the UK and as exports to Europe.

New technologies, especially those based on molecular genetics, could provide a solution to the above problems. All forms of life, except some viruses, contain DNA and this is unique for each species or individual animal, plant or fungus *etc.* This uniqueness can be used to distinguish between species and varieties of *Phytophthora* and to detect them when present at low and otherwise undetectable levels in plants or water.

The polymerase chain reaction (PCR) is a fast, discriminatory, sensitive, non-radioactive and relatively cheap technique which can amplify very small amounts of a target DNA, which is characteristic of *Phytophthora*, to easily detectable amounts, even if that DNA is mixed with much larger amounts of DNA from plants such as strawberry or tomato. Thus PCR has the potential to detect very low amounts of *Phytophthora* that would otherwise go unnoticed.

The objective of the present project was to develop:

1) PCR technology for detecting the presence of very small amounts of *Phytophthora* in nursery stocks of fruit, hardy ornamental and glasshouse crops and in water.

2) Practical tests which could be used by producers, inspectors and purchasers of nursery stocks to test the status of the health of plants.

Summary of results

The first task of the project was to find suitable target DNA that would be unique to the *Phytophthora* species of interest. This was found in genomic rDNA, parts of which provided suitable 'signatures' that could be used in PCR to detect individual *Phytophthora* species or in one case, all species of *Phytophthora* and their close allies. These signatures are used as the starting point for the PCR reaction and are commonly referred to as primers. Specific primers were developed for P. *fragariae* (both varieties: var. *fragariae* causes red core of strawberry; and var. *rubi* causes raspberry root rot), P. *idaei,* P. *cinnamomi* (root rot of hardy ornamentals), P. *cambivora,* P. *cryptogea* and *P. nicotianae* (root rot of tomato, peppers, glasshouse flowers *etc.*) .In each case the primers detected and identified the appropriate species and no other, as well as detecting very small amounts of these fungi in symptom-less plants.

A further refinement of the test called 'Nested PCR' increased sensitivity and flexibility. Results on a wide range of diseases could be obtained within 2 days and very low levels of fungus were detected in water.

The success of the project can be judged by the following list of achievements of PCR-based diagnostics:

- Pure cultures of *Phytophthora* can be identified routinely in less than a day using specific primers. This previously took more than a month. For example the identity of *Phytophthora* attacking *Difjenbachia* plants was confirmed as *P. nicotianae* (in collaboration with Dr Tim O'Neill of ADAS as part of his HDC-funded project on root rot of *Difjenbachia),*

- Suspect hyphae growing from plant material on isolation plates can be identified directly without detailed morphological examination,

- Specific primers have been developed for *P. fragariae* var, *fragariae* and *rubi; P. idaei,' P. cambivora,' P. cinnamomi,' P. cryptogea,' P.nicotianae,*

- Nested PCR detected:
  - *P. fragariae* var. *fragariae* in infected but symptom less roots and in roots from field-grown plants,
  - *P. cactorum* in a strawberry stocks entered for certification in Scotland,
  - *P. fragariae* var. *rubi* in roots from field-grown plants from infested sites, and in 'spiked' raspberry samples included in a survey of raspberry stocks entered for certification Scotland. Detection was as least as sensitive as the existing bait test.
  - Natural infection by *P. idaei* and an unidentified *Phytophthora* in raspberry samples in the same Scottish survey. These species would normally have gone undetected. Ten zoospores of *P. fragariae* var, *fragariae* in water samples,
  - *P. fragariae* var .*fragariae* (red core) in water samples collected from bait tests within ten days of establishment of the tests,
  - *P. cryptogea* in tomato roots from a commercial crop and in the rock-wool substrate in which the plants were growing,
  - *P. nicotianae* in infected tomato roots,
  - Mixed infections with *P. cryptogea* and *P. cambivora* of tree roots,
  - Low levels of infection of raspberry leaves by the downy mildew, *Peronospora,* The ability to detect downy mildews and other pathogens related to *Phytophthora* was an unforeseen bonus of the project.

Action Points for Growers

There is no specific action recommended to growers as for the foreseeable future PCR testing will be done in official Plant Health or advisory laboratories. As the tests become more widely available they will be used more and more routinely by such organisations to test samples of plants and water for growers. Tests also may be applied routinely to filters and traps installed in irrigation lines and nutrient solution recycling plants, thereby providing easy access points for routine testing and quality control in glasshouses.

Science section

Introduction

Phytophthora spp. cause serious losses of value and quality in many important horticultural crops including strawberry, raspberry, apple and hardy ornamentals. The principal problem is that the pathogens are spread in and on diseased nursery stocks. Detecting contamination and infection by *Phytophthora* in nurseries is difficult, time-consuming and, with present technologies, unreliable. Solutions to this problem are central to the future development of a healthy horticultural industry within the UK and trade in nursery stocks both within UK and as exports to Europe.

The solution must lie in the development of new technologies, especially those based on molecular genetics, in which the UK has technological lead over most other European countries. Nucleic acid technology has revealed enough variation in mitochondrial, ribosomal and genomic DNA, to be able to distinguish between species, varieties and *formae speciales* of *Phytophthora.* Probes (cDNA) have been used to detect *P. parasitica* and *P. citrophthora* in enrichment cultures from soil or water but probes are relatively insensitive and require the use radioactive isotopes. In contrast, the polymerase chain reaction (PCR) is a fast, discriminatory, sensitive, non-radioactive and cheap technique which can amplify selected fragments of DNA from a background of non-specific DNA of heterologous origin. It is being applied increasingly to soil- and water-borne microorganisms, and has already been used to distinguish between *Phytophthora* species in pure culture using primers for specific gene sequences or RFLPs. Primers based on RFLPs characteristic of the *Phytophthora* species causing raspberry root rot have been used to detect the causal fungus in artificially inoculated raspberries in glasshouse studies. It could be used therefore to detect *Phytophthora* in naturally infected plants as well as soil and water, where it could provide producers and inspectors with early warning of disease problems long before symptom development.
The main aim of the present project was to develop

1) PCR technology for detecting the presence of very small amounts of *Phytophthora* in nursery stocks of fruit crops and hardy ornamentals

2) Practical tests which could be used by producers, inspectors and purchasers of nursery stocks to test their plant health status.

Materials and Methods

RAPDs

In the initial phases of the work, the use of Randomly Amplified Polymorphisms (RAPDs) as a technique for distinguishing among *Phytophthora* species was investigated. RAPDs proved useful for separating strains of the same species and closely related species but generally lacked the reliability needed for diagnostics. After a comprehensive review of progress at the end of Year 1, a different approach was adopted.

Ribosomal RNA gene repeats

Using what are known as the 'universal' primers, the internally transcribed spacer regions, ITSl and ITS2, of the genomic ribosomal RNA gene repeat (rDNA) were amplified. The universal primers are located in the 18S, 5.8S and 28S of rDNA and amplify across ITSl and ITS2, which because they do not code for any part of the final structure of the ribosomes are more variable in sequence

than other parts of rDNA. The level of variation encountered is useful for distinguishing among species within many genera of plants and animals and proved equally useful in the case of *Phytophthora*.

PCR products containing ITSl and ITS2 were sequenced, initially manually but later using an automatic sequencer, and the sequences were aligned and compared. Short variable regions of sequence within ITSl and ITS2 were chosen and 20 bp oligonucleotide primers were designed to be specific for individual species. Because two 'primers are needed for amplification and there were sequence variations in both ITSl and' ITS2, a series of primers were designed for both ITSl and ITS2. It was then possible to adopt a 'mix and match' approach to using the forward and reverse primers. The level of discrimination required could be altered either by using different pairs of primers or by changing only one of the pair at either end. This gave considerable flexibility and allowed the development of suitable primer combinations for *P. fragariae* (the two varieties cannot be separated from one another but that is not strictly relevant), *P. idaei, P. cinnamomi, P. cambivora, P. cryptogea* and *P. nicotianae.* In each case the primers amplify DNA from the appropriate species and no others with the exception of the closely related *P. cactorum* and *P. idaei* which are amplified by the same primer set but are then distinguished from one another by a treatment with a digest enzyme that cleaves the product of one but not the other, thereby distinguishing between them.

Amounts of pure DNA of *Phytophthora* that could be detected were around 100 femtograms and the sensitivity of the primers could only be described as excellent, and this before later improvements in the technique such as the use of nested PCR (see below). However, even with such levels of sensitivity, single-round PCR with the specific primers was not sensitive enough to detect reliably *Phytophthora fragariae* var. *fragariae* in lightly infected strawberry roots.

Nested PCR

Accordingly another approach was developed, namely nested PCR. In nested PCR, there are two rounds of PCR using primers in the second round that are internal to the primers used in the original round, *i.e.* they amplify a subsidiary product from the product of the first round using the first round product as a starting point for the reaction. This has two advantages: (1) two rounds of amplification greatly increases sensitivity, and (2) the product of the first round can be subjected to different treatments in the second round to allow the detection of a range of species. The main disadvantages are increased cost (two rounds require twice the chemicals that one round needs) and longer time to carry out a test, but these are not serious problems, *e.g.* diagnosis takes a day instead of half a day!

Thus a 'generic' approach to detecting any *Phytophthora* species was developed using nested PCR. By comparing the 18S sequences of rDNA from *Phytophthora* and other genera of fungi belonging to the order Peronosporales, with other fungi and plants, a new primer was designed. Known as DC6, this primer is not highly specific for any particular *Phytophthora* species, or even *Phytophthora vis-a-vis pythium,* but it is specific for the Peronosporales, an order within the Oomycota to which *Phytophthora* belongs. When used in conjunction with a 'universal ' primer, it amplifies a product from DNA of any member of the Peronosporales but not from DNA of other fungi or plants. Thus it amplifies DNA from *Phytophthora, pythium* and downy mildews such as *Peronospora* and *Albugo.* As importantly, the product of the reaction contains both the ITSl and ITS2 regions of rDNA, and a second round of PCR can be made on the product using the specific primers for individual *Phytophthora* species described above. A first test can be made on DNA from any suspect plant material and if a positive reaction is obtained then a subsequent test or series of tests can be made using the product from the first reaction as a starting point, to determine precisely which species were present in the material.

It is possible to search for more than one species in a single sample of plant material and confirm the presence or absence of each without ambiguity.

Nested PCR has also proved to be much more sensitive than single-round PCR. As few as ten zoospores of *P. fragariae* var *.fragariae* have been detected and the fungus has been found in roots that were symptom less but infected with the fungus within two days of inoculation. DNA extraction and PCR protocols have been optimised to allow disease detection in plant material. The addition of bovine serum albumin, for example, radically improves PCR efficiency. Nested PCR has been developed to the stage of routine testing for several *phytophthora* diseases (final milestone), including red core of strawberry and raspberry root rot. A full list of achievements is given below. Some of the work has been done in collaboration with Dr Peter Bonants of the IPO-DLO, Wageningen in the Netherlands who unknown to us had started a parallel project. Nevertheless, intellectual property rights have been applied for through Mylnefield Research Services Ltd which realised the potential of the technology at an early stage. It also provided additional resources to speed certain aspects of the work that would allow it to incorporate the technology into their systems for producing clean planting material under SPUR, specifically a test for *P. cactorum.*

Results

The major results are as follows:

- Pure cultures of *Phytophthora* can be identified routinely in less than a day using specific primers. This previously took more than a month. For example the identity of *Phytophthora* attacking *Diffenbachia* plants was confirmed as *P. nicotianae* (in collaboration with Dr Tim O'Neill of ADAS as part of his HDC-funded project on root rot of *Diffenbachia).*

- Suspect hyphae growing from plant material on isolation plates can be identified directly without detailed morphological examination.

- Specific primers have been developed for *P. fragariae* var. *fragariae* and *rubi*, *P. idaei; P. cambivora; P. cinnamomi; P. cryptogea; P. nicotianae.*

- Nested PCR detected:
  - *P. fragariae* var *.fragariae* in infected but symptomless roots and in roots from field-grown plants.
  - *P. fragariae* var. *rubi* in roots from field-grown plants from infested sites, and in 'spiked' raspberry samples included in a survey of raspberry stocks entered for certification Scotland. Detection was as least as sensitive as the existing bait test.
  - Natural infection by *P. idaei* and an unidentified *Phytophthora* in raspberry samples in the same Scottish survey. These species would normally have gone undetected. Ten zoospores of *P. fragariae* var. *fragariae* in water samples.
  - *P. fragariae* var. *fragariae* (red core) in water samples collected from bait tests within ten days of establishment of the tests.
  - *P. cryptogea* in tomato roots from a commercial crop and in the rock-wool substrate in which the plants were growing.
  - *P. nicotianae* in infected tomato roots.
  - mixed infections with *P. cryptogea* and *P. cambivora* on tree roots.
  - Low levels of infection of raspberry leaves by the downy mildew, *Peronospora.* The ability to detect downy mildews and other pathogens related to *Phytophthora* was an unforeseen bonus of the project.

Conclusions

The tests developed to date are immediately applicable to a wide range of *phytophthora* diseases affecting soft fruit, hardy ornamental nursery and glasshouse crops. Indeed, a number of UK bodies are already applying them to certification schemes (SASA) or planning to do so (CSL). The generic approach developed in this link could be applicable, without much further work, to an even wider range of important diseases caused by *Phytophthora*, as well as diseases caused by *pythium* and a host of downy mildews. In addition the work has shown that *Phytophthora* can be detected in water samples. However, time has prevented attempts to apply the technique directly to soil or develop even further more user friendly formats such as PCR-ELISA which would allow results to be read and quantified using an ELISA plate reader, a common piece of apparatus.

PCR-based tests have proved their potential in helping to ensure the health of planting material. They should find increasing use and acceptance by plant pathologists giving advice or providing extension services to growers.