Project title:	Detection and decontamination of *Phytophthora* spp., including those of statutory significance, from commercial HONS nurseries.
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Project leader:	Dr Philip Jennings Central Science Laboratory Sand Hutton York YO41 1LZ
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Key Workers:	CSL: Gilli Humphries, Sam McDonough STC: Dr Martin McPherson, Ms Cathryn Lambourne, Ms Debbie Liddel, Ms Iwona Burdon
Location:	CSL, York Stockbridge Technology Centre
Project Co-ordinator:	Mr Danny Elliott HONS Nursery Business Advisors 2 Albemarle Road York YO23 1FB
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Contents

Grower Summary - HNS 134

Detection and decontamination of *Phytophthora* spp., including those of statutory significance, from commercial HONS nurseries.

Headline

- A successful baiting method has been developed for the detection of *Phytophthora* species on HONS nurseries.
- Initial testing of slow sand filters indicated they effectively removed zoospores of a broad range of *Phytophthora* species from contaminated water.

Background and expected deliverables

Significant annual economic losses to HONS are attributed to infection by various *Phytophthora* species, especially those causing root rot symptoms. *Phytophthora ramorum*, a newly described species, is currently the most significant quarantine pathogen in the UK. The disease, known as sudden oak death in the USA, has affected a wide range of ornamentals in the UK including *Viburnum*, *Rhododendron*, *Pieris*, *Camellia*, *Kalmia* and *Syringa* spp. and has also been found on a number of tree species. To date, there have been over 420 confirmed outbreaks of *P. ramorum* on nurseries in England and Wales. Emergency UK and EC measures have been introduced with the specific aim to prevent spread of the disease. The legislation requires destruction of all plants within a 2 m radius of a diseased plant and holding all susceptible plants within a 10 m radius, plus any remaining plants from the same consignment as the diseased plants, for a period of 3 months without use of fungicides active against oomycete species (includes species of *Phytophthora* and *Pythia*), for further assessment. These measures are having a major impact on the HONS industry, resulting in the destruction of large numbers of plants.

This project aims to evaluate techniques for improved detection of *Phytophthora* species on nurseries, to investigate the effectiveness of slow sand filtration for the removal of different *Phytophthora* species, including those of quarantine significance,

from water sources and test the effectiveness of disinfectant/chemical treatments for the decontamination of irrigation equipment, standing areas, Danish trolleys and other equipment. The effectiveness and applicability of the refined technologies will be compared under commercial conditions.

The expected deliverables from this project are:

- A validation of baiting techniques for detection of a broad range of *Phytophthora* spp., of significance to nursery stock, from water sources, Danish trolleys, soil/compost and other potential risk sites on nurseries.
- An increased speed and accuracy of *Phytophthora* detection and identification utilising currently available techniques (PCR and ELISA).
- An evaluation of the effectiveness of slow sand filtration for the removal of different *Phytophthora* species, including those of quarantine significance, from water sources on the nursery.
- Determination of the most effective disinfectant/chemical treatments for the decontamination of irrigation equipment, standing areas and other equipment found on nurseries.
- A comparison of the effectiveness and applicability of refined disinfection technologies under commercial conditions.

Summary of the project and main conclusions

Validation of baiting methods

Two baiting methods (autoclaved rhododendron leaves and a selective agar) were compared to determine how effective they were in detecting different spore types (sporangia or zoospores) of a range of *Phytophthora* species from water. All the *Phytophthora* species tested, regardless of the spore type, were detected using the rhododendron leaf bait, however the level of detection varied depending both on the species present and the individual spore type tested. The rhododendron leaf bait detected contamination of water by *P. ramorum* and *P. cryptogea* most effectively, with detection at levels as low as 100 zoospores/L water. The leaf bait was least effective for *P. ilicis*, with the limit of detection occurring at 1000 sporangia/L water. Where both zoospores and sporangia spore suspensions were tested for individual

Phytophthora species, the rhododendron bait appeared to be 10 to 100 times more sensitive for sporangial suspensions over zoospore suspensions. This result is not surprising, as sporangia release zoospores in water thus increasing the actual spore concentration e.g. *P. ramorum* sporangia contain approximately 30 zoospores, so we would expect the bait to appear at least 10 times more sensitive to sporangia than zoospores.

The selective agar bait was less effective than the rhododendron leaf bait for detection of the *Phytophthora* species tested to date. *P. ramorum* was not detected at any of the zoospore concentrations used, but was detected at the two highest concentrations of sporangial suspension (10000 and 1000 sporangia/L). *P. ilicis* and *P. cryptogea* were also detected by the bait but only at the highest spore concentrations used. *P. kernoviae* and *P. cactorum* were not detected.

The two baiting methods will also be tested against *P. cinnamomi* and *P. nicotianae* once reliable methods for the production of sporangia and zoospores of these species have been developed.

Detection

Three methods for detecting *Phytophthora* species from bait material were tested, direct plating onto agar, a genus specific lateral flow device (LFD) and TaqMan PCR analysis. The results for direct plating of bait material have already been discussed. Direct plating is the method traditionally used; it has the advantage that all the species present will be detected (although expertise in their identification is required), but has the disadvantage that it takes 5-7 days before a result can be obtained.

The LFDs were tested on bait material placed in water contaminated with sporangia for all the *Phytophthora* species examined except *P. cryptogea*, where they were tested on material taken from zoospore contaminated water. The LFD detected *Phytophthora* in leaf material for all the species tested, however the LFD was less sensitive than the direct plating method. In general, the detection limit using the LFD was 1000 sporangia/L water, whereas using the direct plating method the limit was between 100 and 10 sporangia/L water depending on the species involved.

Using the LFD for the detection of *Phytophthora* from rhododendron leaf material was more rapid (results in minutes) and the results were easier to interpret than the direct plating method, however it did not give a result to species level and was less sensitive than the direct plating method.

When the LFD was used with the selective agar bait positive reactions for the presence of *Phytophthora* species were obtained for the water control and all spore concentrations. The false positive reactions were a result of antibodies in the LFD cross-reacting with the agar in the bait itself, and as a result this method of detection could not be used with the agar bait.

TaqMan PCR was used to detect *P. ramorum* and *P. kernoviae* in autoclaved rhododendron leaf baits taken from water spiked with either zoospore or sporangial suspensions. The use of TaqMan PCR gave results equivalent to the direct plating method for both species examined, with lower levels of detection for *P. kernoviae* compared to *P. ramorum*, and higher levels of detection where water was spiked with a sporangial suspension compared to a zoospore suspension.

Currently, TaqMan PCR seems to be the most robust detection method, both in terms of sensitivity and ease of species identification. Primers for detecting the other *Phytophthora* species will be tested as they become available.

Effectiveness of slow sand filters

Two sets of slow sand filters (SSF) have been constructed, one set for quarantine *Phytophthora* species at CSL and one set for indigenous species at STC. A flow rate of approximately 400 ml min^{-1} was established for all the SSFs constructed. Following filter priming, SSF headwaters were inoculated with *Phytophthora* species on a monthly basis. To date, the *P. ramorum* and *P. kernoviae* SSF have been tested twice on the 31/1/2006 and 28/2/2006, and the *P. cactorum* SSF on 02/02/2006 and 14/03/2006. No *Phytophthora* species have been detected from water sampled at the SSF outlet following contamination of the SSF headwater. Filters will also be tested against other *Phytophthora* species including *P. ilicis, P. cinnamomi*, *P. cryptogea* and *P. nicotianae*.

Sources of Phytophthora contamination on nurseries

Six HONS nurseries distributed throughout the UK were chosen, by the HDC grower co-ordinator based on previous history of *Phytophthora* spp. on the nursery and their willingness to co-operate in the project, to monitor areas likely to be contaminated by *Phytophthora* species e.g. soil/compost, water, Danish trolleys, mypex, conveyor belts etc., in order to determine the potential extent of contamination and allow judgements to be made on where disinfection would be appropriate. Samples collected from all six nurseries have been processed. Whilst all the results are currently not yet available, initial analysis indicates only low levels of *Phytophthora* species were being detected; the majority of these arising from 'soil' samples. All the samples were collected during a single visit to individual nurseries in early spring 2006 and it may be that a higher recovery of *Phytophthora* may occur at different times of the year.

Financial benefits

It is too early to predict the likely financial outcome from this project. However, *Phytophthora* spp., are aggressive pathogens and can cause significant economic losses, especially in wet seasons. The occurrence of the new quarantine species, *P. ramorum* and *P. kernoviae*, has added another dimension to the potential for economic damage and therefore any actions that can be taken to minimise risk is potentially very valuable. Already the project has provided an early indication of the potential for reducing the risk of water-borne transmission of a range of *Phytophthora* spp. through slow sand filtration and it is hoped that further confidence in this approach will be gathered as the project progresses.

Action Points for Growers

- Continue to check plants for suspicious symptoms of *Phytophthora* infection both on the roots tissues and the foliage & stems.
- Where suspicious symptoms are found submit a sample of the material to a reputable diagnostic laboratory for identification purposes.
- If buying in plants regularly establish a temporary 'quarantine' or 'holding' area well away from other susceptible plants. Check the plants before more widespread release onto the site.

- Consider the risk of water-borne dissemination of *Phytophthora* species on your nursery. If there is a risk of leaf debris or other plant material entering reservoirs , holding tanks etc then consider routine water monitoring for the pathogen.
- Because of the known risk of dissemination of *Phytophthora* species via water ensure all holding tanks are covered to prevent contamination and where appropriate consider some form of disinfection treatment such as slow sand filtration prior to use (see HDC Grower Guide).
- Because *Phytophthora* species prefer wet conditions take measures to minimise standing water around the site by improving drainage.
- Maintain a high level of nursery hygiene and use disinfectants where appropriate to further reduce the risk of pathogen dissemination.

Future work

Work in the second year of the project will focus on the effectiveness of disinfectant/chemical treatments for the decontamination of irrigation equipment, standing areas and other equipment as appropriate.

Evaluate the effectiveness of slow sand filtration for the removal of different *Phytophthora* species, including those of quarantine significance, from water sources on the nursery will continue.

Science Section - HNS 134

Detection and decontamination of *Phytophthora* spp., including those of statutory significance, from commercial HONS nurseries.

Introduction

Significant annual economic losses in HONS are attributed to infection by various *Phytophthora* species. *Phytophthora ramorum* (Werres *et al*. 2001), a newly described species, is currently the most significant quarantine pathogen in the UK. The disease, known as sudden oak death in the USA, has affected a wide range of ornamentals in the UK including *Viburnum*, *Rhododendron*, *Pieris*, *Camellia*, *Kalmia* and *Syringa* spp. and has also been found on a number of tree species. To date, there have been over 420 confirmed outbreaks of *P. ramorum* on nurseries in England and Wales. Emergency UK and EC measures have been introduced with the specific aim to prevent spread of the disease. The legislation requires destruction of all plants within a 2 m radius of a diseased plant and holding all susceptible plants within a 10 m radius, plus any remaining plants from the same consignment as the diseased plants, for a period of 3 months without application of fungicides active against oomycetes, for further assessment. These measures are having a major impact on the HONS industry, resulting in the destruction of large numbers of plants.

This project aims to evaluate techniques for improved detection of *Phytophthora* species on nurseries, to investigate the effectiveness of slow sand filtration for the removal of different *Phytophthora* species, including those of quarantine significance, from water sources and test the effectiveness of disinfectant/chemical treatments for the decontamination of irrigation equipment, standing areas, Danish trolleys and other equipment. The effectiveness and applicability of the refined technologies will be compared under commercial conditions.

Materials and Methods

1. Baiting Phytophthora *species from water*

The effectiveness of two bait types was tested for the detection of *Phytophthora* species from water:

i. autoclaved rhododendron leaves
ii. 'fishing bait' containing selective agar

Baits were tested against seven *Phytophthora* species (two quarantine species: *P. ramorum* and *P. kernoviae*; and five indigenous species: *P. cactorum*, *P. ilicis*, *P. cryptogea*, *P. cinnamomi* and *P. nicotianae*). Where possible, baits were tested against both zoospore and sporangial suspensions.

1.1. Bait production

1.1.1. Rhododendron leaf bait

For each test, 30 young healthy leaves were pinched from a rhododendron 'Cunninghams White' plant, cut into four, and autoclaved at 110°C for 10 min. Baits were constructed by wrapping eight autoclaved leaf sections together with a piece of polystyrene packing and two small pieces of sterile gravel (≈6 g) in a piece of muslin [approx 9cm^2 (Figure 1a)]. The addition of polystyrene and gravel ensured that the bait floated just below the surface of the water. The contents of the muslin bag were secured with string (Figure 1b); the string also allowed easy retrieval of the bait.

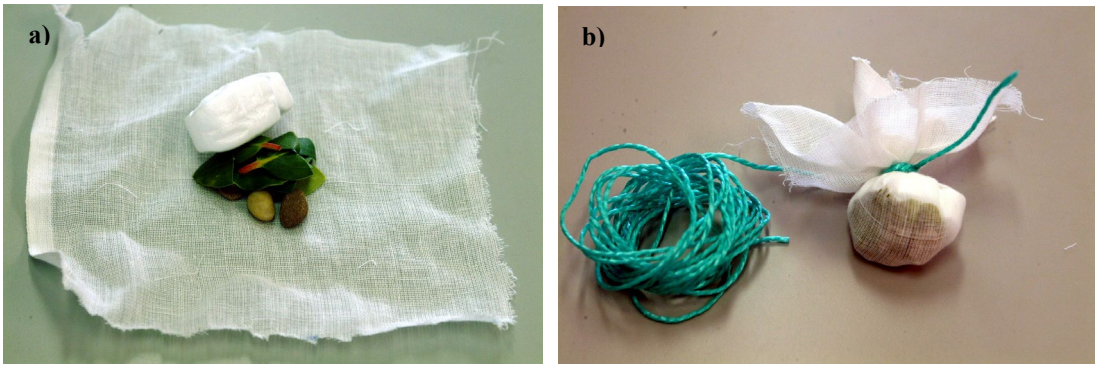

Figure 1. Construction of rhododendron bait a) showing bait contents and b) completed bait.

1.1.2. ‘Fishing bait’ containing selective agar

Plastic bait holders (Figure 2) were bought from a local fishing tackle shop and the attached lead weight removed. Eight 1 cm^2 pieces of a Pythium/Phytophthora selective agar (based on cornmeal agar) and two pieces of polystyrene packing were added to each bait holder. Again the polystyrene was added to the bait so it would float just below the surface of the water.

Figure 2. Plastic ‘fishing bait’ containing selective agar and polystyrene float.

1.2. Phytophthora inoculum production

1.2.1. *Phytophthora ramorum*, *P. kernoviae*, *P. cactorum* and *P. ilicis*

Plugs of *P. ramorum* (CSL ref cc47), *P. kernoviae* (CSL ref cc95), *P. cactorum* (CSL ref 2151) and *P. ilicis* (CSL ref 2195) were taken from the CSL culture collection and grown on 10% V-8 agar (Appendix I) at 20°C, under day light bulbs (12h light/12h dark regime) until the colonies reached the edge of the agar plates. The agar plates were flooded with 5 mL of sterile distilled water (SDW) and the sporangia removed from the agar surface using a sterile plastic rod. Fresh 10% V-8 agar plates were inoculated with 100 µL of the resulting sporangial suspension and incubated under the same temperature and light regime, as previously described, for 3 days.

Different methods were employed depending on whether sporangia or zoospores were required for the baiting test. For testing baits against sporangial suspensions, the three-day-old plates were flooded with 10 mL SDW, sporangia removed from the agar surface using sterile plastic rods and sporangial counts carried out using a haemocytometer.

Where zoospores were required, the three-day-old plates were flooded with 15-20 mL SDW, chilled at -20°C for 5 min and then returned to 20°C for 1 h. Plates were then checked for zoospore release and the spore suspension filtered through Whatman No 113V filters (retention size >30 μm) to remove any spent or full sporangia. Zoospore counts were carried out using a haemocytometer.

1.2.2. *P. cryptogea*

Plugs of *P. cryptogea* (CSL ref 1708) were taken from the CSL culture collection and grown on 10% V-8 agar at 20°C under day light bulbs (12h light/12h dark regime) for five days. Plugs (5 mm) from the leading edge of the five-day-old colonies were placed in Petri dishes containing 20 mL of a 50/50 (v/v) mix of SDW and sterile pond water containing grass leaves (previously boiled for 5 min). Plates were incubated at 20°C under day light bulbs (12h light/12h dark regime) for 5 days or until sporangia were produced on agar plug and leaf surfaces. Zoospore release was initiated as described in section 1.2.1.

1.2.3. *P. cinnamomi* and *P. nicotianae*

Methods for sporangia and zoospore production of *P. cinnamomi* (CSL ref 2085) and *P. nicotianae* (CSL ref 2084) are still being investigated.

1.3. Bait testing

Bait tests were set up in 1 L plastic microwave tubs. For each bait type, four replicate tubs were set up containing 500 mL water with either 0.01, 0.1, 1 or 10 spores mL^{-1} (5, 50, 500 or 5000 spores total). Tubs containing 500 mL distilled water only were used as controls. One bait was placed into each tub and left at room temperature for two days. Baits were removed after two days and the rhododendron leaf sections/selective agar pieces tested in one of three ways to detect the presence of *Phytophthora* species:

i. Direct plating onto Phytophthora selective agar
ii. Phytophthora (genus specific) lateral flow device
iii. TaqMan PCR (where primers are already available)

1.4. Comparison of methods used for the detection of *Phytophthora* species from bait material.

1.4.1. Direct plating onto Phytophthora selective agar

For all baits, four leaf sections or four pieces of selective agar were selected at random and plated directly onto $PARP_5H$ agar (Appendix I). After seven days incubation at room temperature the $PARP_5H$ plates were examined for the presence of *Phytophthora* species. For each spore concentration and bait type, the number of leaf sections/agar pieces showing growth of a *Phytophthora* species was recorded and the percentage baits infected calculated.

1.4.2. Phytophthora (genus specific) lateral flow device (LFD)

For all baits, two leaf sections or two pieces of selective agar were selected at random for testing with the Phytophthora (genus specific) CSL Pocket Diagnostic lateral flow test. Extraction from leaves/agar and the test itself were carried out according to the manufacturers instructions. Following results from the first experiment, the LDF was not used for testing for the presence of *Phytophthora* species on the 'fishing bait'.

1.4.3. TaqMan PCR

For all rhododendron leaf baits, two leaf sections were selected at random for testing with TaqMan PCR. Only baits tested against *P. ramorum* or *P. kernoviae* were examined, as at the time of testing primers and probes were not available for the other *Phytophthora* species.

1.4.3.1. DNA extraction from leaf material

Leaves were ground in a plastic mesh bag with 1.5 mL of C1 grinding buffer (NucleoSpin plant kit) under a mat bed grinder, and 300 µL of the sample pipetted into a labelled 2 mL centrifuge tube using a wide bore tip. Chloroform (100 µL) was added to each centrifuge tube, vortexed for 10 s and the phases separated by centrifugation for 5 min at full speed, the top aqueous layer was pipetted into a new 2 mL centrifuge tube and incubated at 65°C for 30 min. C4 buffer (300 µL) and ethanol (200 µL) were added to the heated aqueous phase samples, vortexed for 30 sec and the mixture passed through a labelled NucleoSpin plant column. CW buffer (400 µL) and C5 buffer (total 900 µL) were passed through the column before eluting DNA

from the column using 200 µL of elution buffer. DNA could then either be stored at 4°C for immediate use or frozen at -20°C for use later.

Testing for the presence of *P. ramorum* or *P. kernoviae* DNA was carried using a Smart Cycler ABI Prism 7900HT and appropriate primers and probes.

2. Slow sand filters (SSFs)

In total, six SSFs were constructed, two for use with *P. ramorum* and *P. kernoviae* [held in the glasshouse at CSL (Figure 3)] and four for use with the indigenous *Phytophthora* species: *P. cactorum*, *P. ilicis, P. cinnamomi*, *P. cryptogea* and *P. nicotianae* [held outdoors at STC (Figure 3)].

Figure 3. Slow sand filters held at CSL (left) and STC (right)

2.1. Construction of SSFs

A schematic of the SSF set up is shown in Figure 4. All filters were constructed in plastic barrels (height = 80 cm, diameter = 60 cm) supported on blocks for ease of sampling. The gravel drainage system at the bottom of the filter consisted of a 10 cm layer of course gravel (16-23 mm) supporting a 5 cm layer of fine gravel (4-6 mm). A perforated drainage pipe connected to the filter outlet was supported within the course gravel layer. Above the fine gravel was a 15 cm layer of course sand (particle size 1-1.4 mm), which supported the filter bed consisting of a 50 cm layer of fine sand (particle size 0.2-1 mm). Filters and header tanks were filled with pond water and the filtered water collected in the collection tank and returned to the header tank using a submersible pump. The flow rate of the filters was adjusted using the isolation valve connected to the outlet of the filter.

Filters were run for a minimum of 4 weeks before commencement of any testing; this period of priming allowed the establishment of the schmutzdecke (the biologically active layer) at the surface of the filter bed.

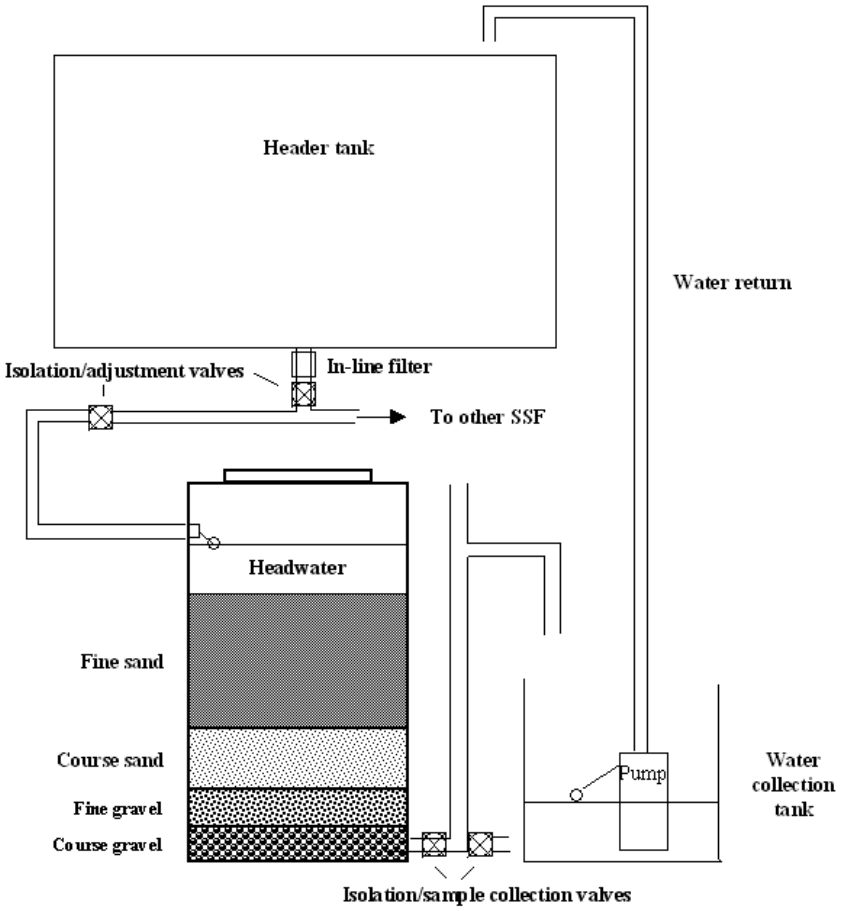

Figure 4. Schematic of slow sand filter construction.

2.2. Removal of *Phytophthora* species from water using SSF

The effectiveness of the SSFs for the removal of *Phytophthora* species was tested through the addition of phytophthora spores to the filter headwater. Initial tests were carried out on *P. ramorum*, *P. kernoviae* and *P. cactorum* zoospores added to the headwater at a concentration of 10^4 zoospores/litre of headwater for *P. ramorum* and *P. kernoviae*, and 10^6 zoospores/litre of headwater for *P. cactorum*.

Water samples (250 mL) were taken from the headwater immediately following zoospore addition, 1 L samples were taken from the SSF outlet two hours after zoospore addition and then hourly for the next six hours. All water samples were

filtered through Durapore® membrane filters (5μm) and the filter papers inverted onto $PARP_5H$. Plates were incubated at room temperature for seven days and any growth of *Phytophthora* species recorded. Rhododendron leaf baits were placed in the water collection tank to detect any failures in the filters between testing of the SSF. Baits were changed every 7-14 days and plated onto $PARP_5H$ agar.

3. Detection of Phytophthora *species present on HONS nurseries*

Six HONS nurseries were chosen to determine the extent and areas likely to be contaminated by *Phytophthora* species in order to determine the extent of contamination by *Phytophthora* species and allow judgements to be made on where disinfection was most likely to be required. Grower questionnaires were sent out pre-sampling to determine the growing practices used at each nursery (see Appendix II).

Nurseries were visited in the spring of 2006. The type of samples taken included:

i. Water samples from reservoirs, water tanks etc.
ii. Swabs of Danish trolleys, mypex, pathways, conveyor belts, equipment etc.
iii. Soil/compost samples

Presence of *Phytophthora* species was determined in water samples by filtration through 5 μm filters, and in the swabs/soil samples by baiting with rhododendron leaves.

Results and Discussion

1. Baiting Phytophthora *species from water*

1.1. Comparison of baiting methods

Two baiting methods (autoclaved rhododendron leaves and a selective agar) were compared to determine how effective they were in detecting different spore types of a range of *Phytophthora* species from water. Rhododendron leaf sections are routinely used by Defra to determine the presence of quarantine *Phytophthora* species in soil and water samples This project validated the use of autoclaved Rhododendron leaf tissues to avoid the potential concern and risk of such host plant material acting as a carrier of *Phytophthora* pathogens onto the nursery during the baiting process. All the *Phytophthora* species tested, regardless of the spore type (sporangia or zoospores), were detected using the rhododendron leaf bait (Figure 4), however the level of detection varied depending on the species present and the spore type tested. The rhododendron leaf bait detected contamination of water by *P. ramorum* and *P. cryptogea* most effectively, with detection as low as 100 zoospores/L water. The leaf bait was least effective for *P. ilicis*, with the limit of detection occurring at 1000 sporangia/L water. Where both zoospores and sporangia spore suspensions were tested for individual *Phytophthora* species, the rhododendron bait appeared to be 10 to 100 times more sensitive for sporangial suspensions. This result was not surprising, as on introduction to water sporangia release zoospores thus increasing the actual spore concentration e.g. *P. ramorum* sporangia contain approximately 30 zoospores, so we would expect the bait to appear at least 10 times more sensitive to sporangia than zoospores.

The selective agar bait was less effective than the rhododendron leaf bait for detection of the *Phytophthora* species tested to date (Figure 5). *P. ramorum* was not detected at any of the zoospore concentrations used, but was detected at the two highest concentrations of sporangial suspension. *P. ilicis* and *P. cryptogea* were also detected by the bait, but only at the highest spore concentrations used *P. kernoviae* and *P. cactorum* were not detected.

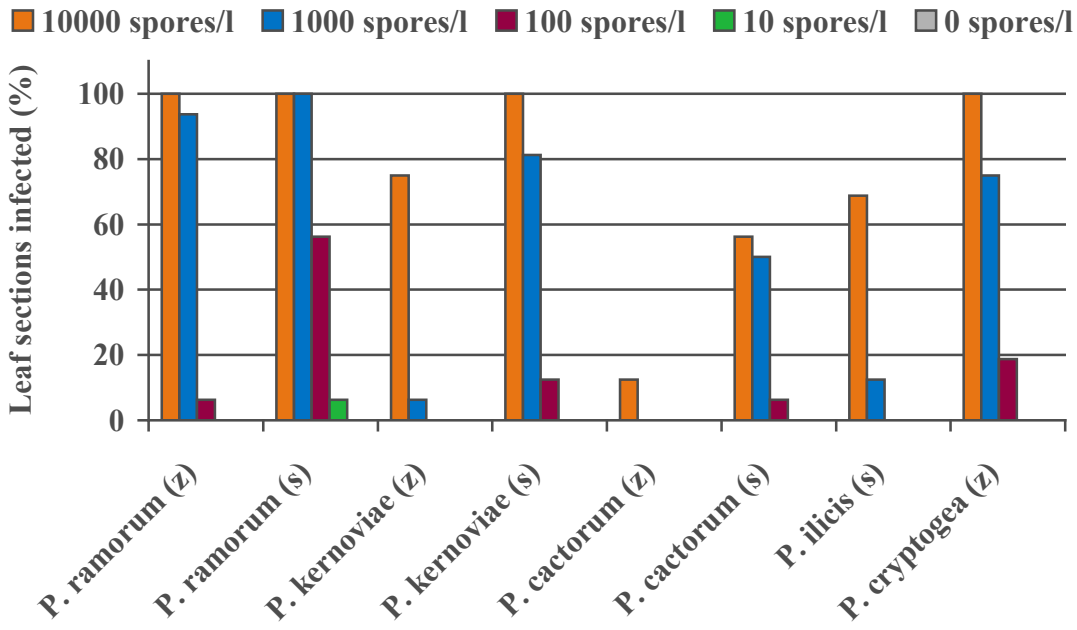

Figure 4. Detection of *Phytophthora* species from water using a rhododendron leaf baits. Phytophthora isolation from leaf sections was on $PARP_5H$ agar. Zoospore suspensions (z); sporangial suspensions (s).

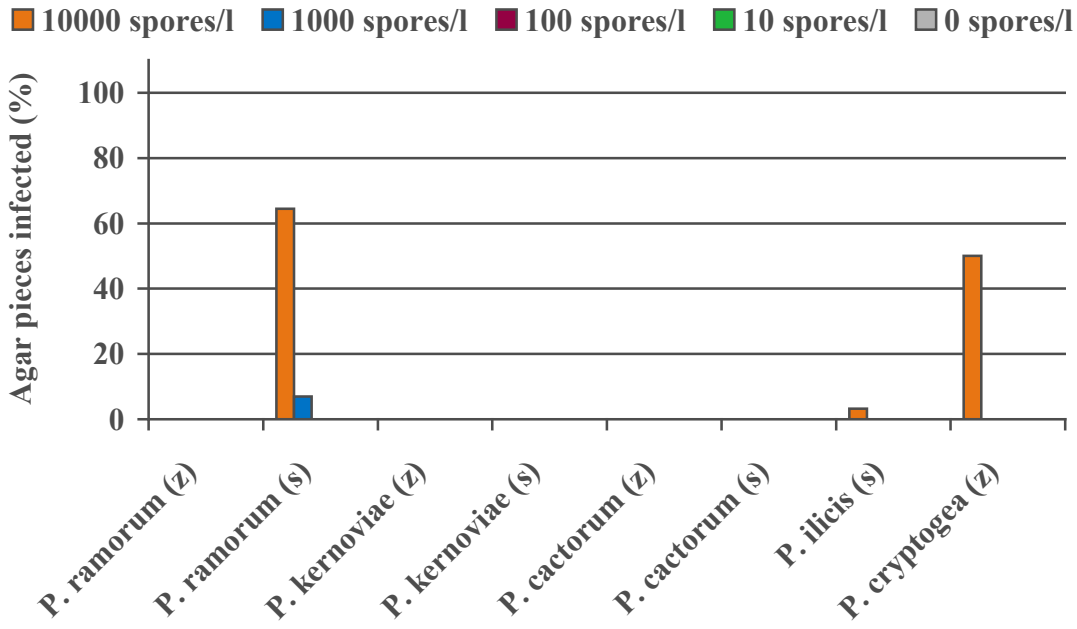

Figure 5. Detection of *Phytophthora* species from water using the 'fishing bait' (isolation from leaf sections on $PARP_5H$ agar). (z) indicates use of zoospore suspensions; (s) indicates use of sporangial suspensions.

The two baiting methods will be tested against *P. cinnamomi* and *P. nicotianae* once reliable methods for the production of sporangia and zoospores of these species have been developed.

1.2. Comparison of methods used for the detection of *Phytophthora* species from bait material.

Three methods for detecting *Phytophthora* species from bait material were tested: direct plating onto agar, a genus-specific lateral flow device (LFD) and PCR analysis. The results for direct plating of bait material are shown in Figures 4 and 5. Direct plating is the method traditionally used; it has the advantage that all the species present will be detected (although expertise in their identification is required), but has the disadvantage that it takes 5-7 days before a result can be obtained.

The LFDs were tested on bait material placed in water that had been inoculated with sporangia for all the *Phytophthora* species except *P. cryptogea*, where they were tested against material taken from zoospore contaminated water. The LFD detected phytophthora in leaf material for all the species tested (Figure 6); however the LFD was less sensitive than the direct plating method. In general, the detection limit using the LFD was 1000 sporangia/L water, whereas using the direct plating method the limit was between 100 and 10 sporangia/L water depending on the species involved.

When the LFD was used with the selective agar bait positive reactions for the presence of *Phytophthora* species were obtained for the water control and all spore concentrations. This indicated that the LFD was cross-reacting with the agar bait itself and as a result could not be used in conjunction with the agar bait.

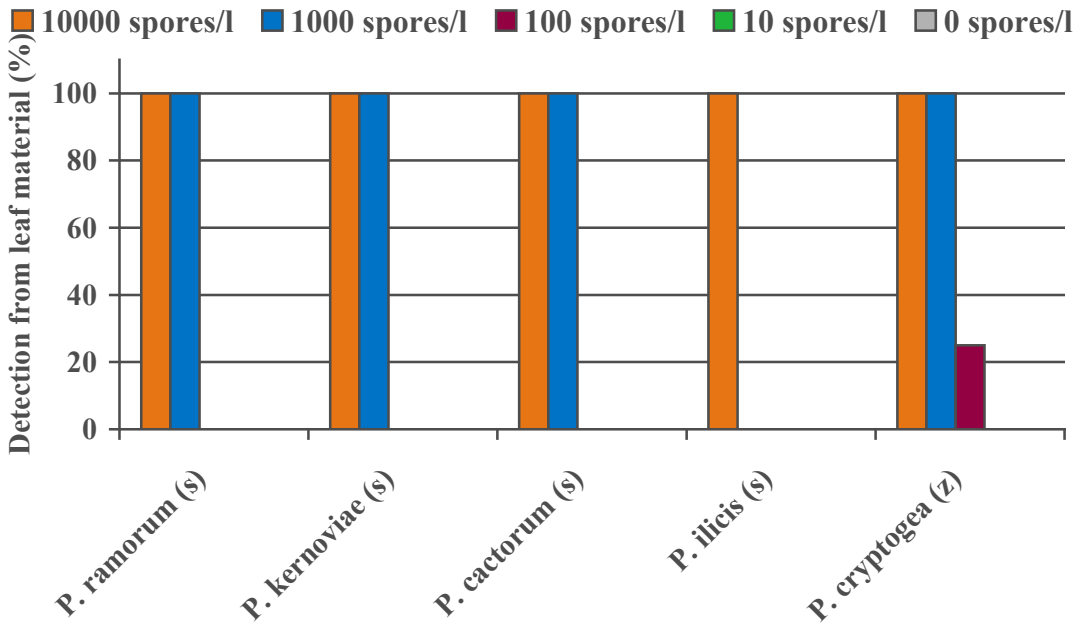

Figure 6. Detection of *Phytophthora* species from rhododendron leaf baits using the phytophthora genus LFD. Zoospore suspensions indicated by (z); sporangial suspensions indicated by (s).

Using the LFD for the detection of phytophthora from rhododendron leaf material was more rapid (results in minutes) and the results were easier to interpret than the direct plating method; however it did not give a result to species level and was less sensitive than the direct plating method.

TaqMan PCR was used to detect *P. ramorum* and *P. kernoviae* in autoclaved rhododendron leaf baits taken from water spiked with either zoospore or sporangial suspensions. The use of TaqMan PCR (Figure 7) gave results equivalent to the direct plating method for both species examined, with lower levels of detection for *P. kernoviae* compared to *P. ramorum*, and higher levels of detection where water was spiked with a sporangial suspension compared to a zoospore suspension.

Currently, TaqMan PCR seems to be the most robust detection method, both in terms of sensitivity and ease of species identification. Primers for detecting the other *Phytophthora* species will be tested as they become available.

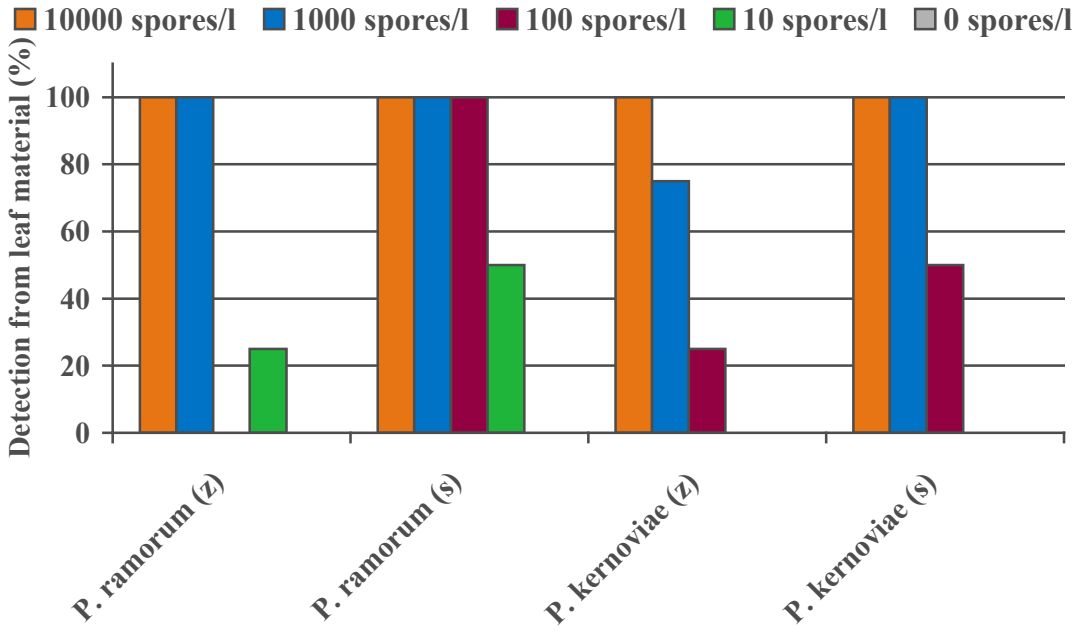

Figure 7. Detection of *P .ramorum and P. kernoviae* from autoclaved rhododendron leaf baits using TaqMan PCR primers. Zoospore suspensions indicated by (z); sporangial suspensions indicated by (s).

2. *Slow sand filtration for the removal of Phytophthora species from water*

A flow rate of approximately 400 ml min^{-1} was established for all the SSFs. Following filter priming, SSF headwaters were inoculated with the appropriate *Phytophthora* species on a monthly basis. To date, the *P. ramorum* and *P. kernoviae* SSFs have been tested twice on the 31/1/2006 and 28/2/2006, and the *P. cactorum* SSF on 02/02/2006 and 14/03/2006. No *Phytophthora* species have been detected from water sampled at the SSF outlet following contamination of the SSF headwater (Table 1).

3. *Determination of* Phytophthora *species present on HONS nurseries*

Samples collected from the six nurseries have been processed. Not all the results are currently available, however initial analysis indicates only low levels of *Phytophthora* species were detected; the majority of these have come from 'soil' samples (Table 2).

Conclusions

- Of the two water bait methods tested, the autoclaved rhododendron leaf was the most effective at recovering a range of *Phytophthora* species from water and was more sensitive than the selective agar, for a wider range of *Phytophthora* spp.
- The Rhododendron leaf bait did not appear to be selective and detected *Phytophthora* pathogens which occur on hosts other than Rhododendron spp.
- In terms of *Phytophthora* detection from the baits, both direct plating and TaqMan were more effective than the LFD.
- Whilst the Taqman PCR offers the potential of high sample throughput and therefore potentially a lower-cost option for the majority of smaller samples, the direct plating remains the most cost-effective option.
- Initial results from the testing of the slow sand filters (SSF) indicates that they are effective at removing zoospores of a broad range of *Phytophthora* species, including those of quarantine significance, from contaminated water.
- Initial results from sampling on nurseries indicate that *Phytophthora* spp. were not particularly widely disseminated around the nursery or were present at levels below the current detection limits or were dormant and in an inactive state at the time of sampling.

Future work

Work in the second year of the project will focus on the effectiveness of disinfectant/chemical treatments for the decontamination of irrigation equipment, standing areas and other equipment as appropriate.

Evaluate the effectiveness of slow sand filtration for the removal of different *Phytophthora* species, including those of quarantine significance, from water sources on the nursery will continue.

Table 1. Effect of slow sand filtration for the removal of *Phytophthora* species from contaminated water

Phytophthora species	Date	External Temp (°C)	Spore type	Spore concentration (spores/litre headwater)	Sample time (Hours after introduction of *Phytophthora* to SSF headwater)							
					0*	2	3	4	5	6	7	8
P. ramorum	31/01/06	18	Zoospores	$1x10^6$	✓	x	x	x	x	x	x	x
	28/02/06	18	Zoospores	$1x10^5$	✓	x	x	x	x	x	x	x
P. kernoviae	31/01/06	18	Zoospores	$1x10^4$	✓	x	x	x	x	x	x	x
	28/02/06	18	Zoospores	$1x10^5$	✓	x	x	x	x	x	x	x
P. cactorum	02/02/06	-1 to 5	Zoospores	$1x10^4$	✓	x	x	x	x	x	x	x
	14/03/06				✓	x	x	x	x	x	x	x

*sample taken from SSF headwater

Table 2 Recovery of Phytophthora species from soil, water and swabs samples taken from four HONS nurseries

Nursery 1

Sample number	Sample type	Sample description	Phytophthora present
1	Water	Reservoir	None
2	Soil		None
3	Swab	Pot holders in house H	None
4	Swab	Danish trolleys in house H	None
5	Swab	Puddles in house 9	None
6	Swab	Muddy path H9	None
7	Soil	Loose compost from floor of house 9	None
8	Swab	Mypex in house 9	None
9	Soil	Soil debris from mypex just inside old house 10	None
10	Swab	Conveyor belt in house A	None
11	Soil	Excess compost on potting line in house A	None
12	Swab	Danish trolleys in house A	None
13	Swab	Fork lift in house A	None
14	Swab	Prongs on folk lift in house A	None
15	Water	Silver tank	None
16	Swab	Path in house 2	None
17	Swab	Mypex in house 2	None

Nursery 2

Sample number	Sample type	Sample description	Phytophthora present
1	Swab	Path in propagator	None
2	Swab	Polythene in propagator	None
3	Soil	Capillary matting in propagator	None
4	Swab	Danish trolley in propagator	None
5	Swab	Mypex - 22 bay house	None
6	Soil	Earth from path - 22 bay house	None
7	Swab	Orange trolley	None
8	Swab	Central pathway – 24 bay house	None
9	Swab	Mypex - 24 bay house	None
10	Soil	Soil from door way - 24 bay house	*P. citrophthora*
11	Swab	Mypex	None
12	Swab	Mypex in shaded area	None
13	Swab	Pot holders near shaded area	None
14	Swab	Danish trolleys at back of shed	None
15	Swab	Tractor wheel	None
16	Soil	Spent compost	None
17	Swab	Potting bench	None
18	Soil	Soil/mud from shed doorway	None

Table 2. Recovery of Phytophthora species from soil, water and swabs samples taken from four HONS nurseries (continued)

Nursery 3

Sample number	Sample type	Sample description	Phytophthora present
1	Water		None
2	Swab	Path in propagation house 2	None
3	Soil	Sand from prop house 2	None
4	Swab	Danish trolley	None
5	Swab	Dirty module trays	None
6	Swab	Wheels of mini tractor	None
7	Swab	Spray tank wheels	None
8	Soil	Soil from dirty yard	None
9	Soil	Compost from outside pile	None
10	Water		None
11	Swab	Flat bed trolley	None
12	Swab	Mypex – standing out area	None
13	Water		None
14	Soil	Gravel	None
15	Swab	Mypex in tunnel 3	None
16	Soil	Soil from mypex	None
17	Soil	Compost from potting bench	None
18	Swab	Potting bench in tunnel 2	None

Nursery 4

Sample number	Sample type	Sample description	Phytophthora present
1	Swab	Propagation area – concrete floor	None
2	Soil	Sand from propagator floor	None
3	Water		None
4	Soil	Soil from mypex	None
5	Swab	Path in house 6	None
6	Soil	Top dressing in potting up house	None
7	Swab	Wood trolley	None
8	Soil	Compost from potting area floor	None
9	Soil	Debris from Danish trolley	None
10	Swab	Trolley near potting area	None
11	Soil	Soil from around infected plant	None
12	Soil	Debris from mypex near reservoir	None
13	Water		None
14	Swab	Mypex, capillary sand bed by reservoir	None
15	Soil	Sand from capillary sand bed by reservoir	None
16	Swab	Pot holders – tunnel near capillary sand bed	None
17	Swab	Fork lift wheels	None
18	Swab	Tap area near potting shed	None
19	Swab	Empty seedling tray	None
20	Swab	Danish trolley	None

Table 2. Recovery of Phytophthora species from soil, water and swabs samples taken from four HONS nurseries (continued)

Nursery 5

Sample number	Sample type	Sample description	Phytophthora present
1	Water		None
2	Swab	Green trolley	None
3	Swab	Path T12	None
4	Soil	Standing area T12	None
5	Swab	Danish trolley outside T12	None
6	Swab	Path T5	None
7	Soil	Standing area T5	?
8	Swab	Roller bench - despatch shed	None
9	Swab	Red trays - despatch shed	None
10	Swab	Tractor wheel	None
11	Soil	Compost from outside bunker	None
12	Swab	Danish trolley	None
13	Swab	Conveyor belt wooden boards	None
14	Swab	Metal pricking out bench	None
15	Soil	Floor in pricking out area	None
16	Swab	Mypex bed 17	None
17	Swab	Mypex in doorway H33	None
18	Swab	Green trolley in H33	None
19	Swab	H40 - viburnum plastic trays	None
20	Soil	Compost from potting shed	None
21	Swab	Conveyor belt - potting shed	None

Nursery 6

Sample number	Sample type	Sample description	Phytophthora present
1	Swab	Danish trolley in propagation house	None
2	Swab	Path inside propagation house	None
3	Swab	Polythene under plants in prop house	None
4	Swab	Mypex – floor of prop house	None
5	Swab	Mypex – outside standing area	None
6	Swab	Old mypex in new house	None
7	Swab	Danish trolley (by gate)	None
8	Swab	Wheels of green mini tractor	None
9	Swab	Path in glasshouse by potting shed	None
10	Water		None
11	Soil	Debris from path	?
12	Swab	Mypex – multispan house	None
13	Soil	Compost from potting table	None
14	Swab	Compost conveyor	None
15	Swab	Potting machine	None

? indicates *Phytophthora* species being identified

References

Jeffers SN, Martin SB, 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* 70, 1038-1043.

Werres SR, Marwitz R, Man in't Veld W A, Cock A W A M, de. Bonants P J M, Weerdt M, de Themann K, Ilieva E, Baayen RP, 2001. *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research* **105**, 1155-1165.

Appendix I

10 % V-8 agar

V8 juice	200 mL
$CaCO_3$	2 g
Agar Nº3	40 g
0.1M KOH	50 mL (0.280 g in 50 mL distilled water)
Distilled water	1750 mL

Autoclave at 121˚C for 15 min.

PARP$_5$H agar (Jeffers and Martin, 1986)

Cornmeal Agar (CMA)	17 g/L

All amendments were either suspended or dissolved in 10 ml SDW and added to CMA after it had been autoclaved and cooled to 50˚C in a water bath.

Pimaricin	5 mg
Sodium ampicillin	250 mg
Rifampicin	10 mg dissolved in 1ml DMSO
PCNB	100 mg
Hymexazol	50 mgL^{-1}

Appendix II

Detection and decontamination of *Phytophthora* species from commercial HONS Nurseries (HNS 134)

HDC sponsored study being carried out by CSL and STC

GROWER QUESTIONNAIRE

Contact Name ……..……

Nursery Address ……..

…….

Postcode ……. Tel No. ……

TYPE OF NURSERY

Production Methods	Approx. Acreage Outdoor	Approx. acreage protected
Container		
Field		N/A
Young plants		

Markets	% by value
Retail sector	
Landscape sector	
Other nurseries	

OWN CROPS - Origin of plant material

From your own young plants	Please tick	Approximate %
From seed		
From your own stock beds		
From your growing crop		
From landscape or garden plantings		
From bought-in young plants		
UK liner suppliers		
Imported		

Type of young plants	Approximate %
Pot liner or cell	
Field grown	

Source country of young plants							
Country	NL	F	B	D	IRL	NZ	Other
% total							

TRADED PLANTS – Origin of plant material

Source country of traded plants							
Country	NL	F	B	D	IRL	NZ	Other
% total							

IRRIGATION – Container growers

Type of irrigation	Please tick	Type of base (*see below)
Overhead		
Drip		
Capillary sand		N/A
Capillary mat		N/A
Ebb and flow		N/A
Other		

*base types mypex on soil, mypex with gravel, mypex with sand, other

IRRIGATION – Source

Source	Please tick
Borehole	
Mains	
Surface source	
Recirculation	

IRRIGATION – Storage

Storage type	Please tick
Not stored	
Covered tank	
Uncovered tank	
Covered reservoir	
Uncovered reservoir	

IRRIGATION – Hygiene

Water treatment	Please tick
None	
UV irradiation	
Slow sand filtered	
Chemical treatment	

Check for pathogens in water	Please tick
Never	
On occasion in past	
Never	

Have you experienced any problems with plants that you have attributed to, or had confirmed as, being caused by a *Phytophthora* disease e.g. root rots, stem base infections? Please give the host plant, species it was seen on and extent of the problem.

……

……

……

……

……

……

Would you be happy for us to visit the nursery during early spring 2006 to collect water, soil/compost and other growing media/standing area materials e.g. sand/gravel?

☐ Yes ☐ No

PLEASE NOTE THAT DETAILS PROVIDED AND, ANY RESULTS GENERATED FROM SAMPLES COLLECTED, WILL BE KEPT CONFIDENTIAL AND WILL NOT BE PASSED TO

Please return this form by 3rd March to :

Cathryn Lambourne
Stockbridge Technology Centre Ltd
Cawood
Selby
N. Yorks YO8 3TZ

A reply-paid envelope is provided for your use.

THANK YOU FOR YOUR PARTICIPATION