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Grower Summary - HNS 123

Control of *Phytophthora ramorum* in nursery stocks (COPRINS)

Headlines

- Tests using containerised rhododendron showed that the most effective fungicides were SL 567A, Amistar and Sonata, all of which completely inhibited growth of *P. ramorum* when applied as a protectant foliar spray either 4 or 7 days pre-infection. On viburnum, SL 567A again completely inhibited growth when applied either 4 or 7 days pre-infection, whereas Amistar and Sonata were less effective.
- A number of fungicides also showed good eradicant activity including SL 567A, Standon Etridiazole, Tanos, Amistar and Sonata. However, control was not 100% effective using these single applications.
- Despite the fact that SL 567A (metalaxyl-M) was the most effective fungicide for control of *P. ramorum*, use of this fungicide cannot be recommended due to the significant risk of the rapid development of resistance. Co-formulations and mixtures of metalaxyl-M with other active ingredients, including those shown to be effective in this study (e.g. Amistar and Sonata), need to be investigated to develop a protocol for durable fungicidal control of *P. ramorum*. .
- Jeyes Fluid at a dilution of 30 mL L^{-1} eradicated *P. ramorum* from contaminated gravel:sand:soil substrates after 10 minutes exposure. Panacide M at a dilution of 17 mL L^{-1} was effective after an exposure period of 30 minutes.

Background and expected deliverables

Phytophthora ramorum is a newly described species which has been recently introduced to both the USA and Europe. In Europe it is currently the most significant quarantine pathogen. The disease, known as sudden oak death (SOD) in the USA, has affected a wide range of ornamentals in the UK including *Viburnum, Rhododendron, Pieris, Camellia, Kalmia, Syringa* and has also been found on a number of tree

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species. Trade of HONS constitute the most important means for the introduction and spread of the disease, to date (15 April 2004), there have been approximately 328 confirmed outbreaks of *P. ramorum* in England and Wales, of which 288 have been in nurseries (Source: Defra website (www.defra.gov.uk)). Emergency UK and EC measures have been introduced with the specific aim to prevent spread of the disease. These measures are primarily a response to the potential threat the pathogen poses in the wider environment, especially towards European tree species and other at risk ecosystems such as heathland and moorland habitats. 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 for further assessment. This is currently having a major impact on the HONS industry, resulting in large numbers of plants having to be destroyed. The policy for eradication/containment of this disease is continually under review, as more information on the epidemiology of the pathogen becomes available. This project aims to explore the possible role of chemical control treatments in future strategies for control of the disease.

The expected deliverables from this project are:

- An evaluation of the efficacy of existing active substances used to control diseases in hardy ornamental nursery stocks.
- An evaluation of new substances and alternative chemicals.
- Determination of the most effective disinfectants, which may be appropriate for sterilising standing areas and capillary matting etc.
- Provision of a management guide to minimise disease spread via movement of plants, on implements or by operators and visitors.

Summary of the project and main conclusions

Thirteen fungicides: Filex, Cuprokylt FL, Lindrex, Invader, Sipcam C50, Tanos, Shirlan, Sonata, Curzate M68, SL 567A, Standon Etridiazole 35, Amistar and Aliette were selected for investigation into their efficacy against *P. ramorum*. All had proven activity against *Phytophthora* species and either had full approval for use on HONS,

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or could be used under the Revised Long Term Arrangements for Extension of Use (2002). Fungicides were initially tested for *in vitro* activity against mycelium and spores of two isolates of *P. ramorum* from the UK. Agar plate tests showed that SL 567A, Invader, Standon Etridiazole 35 and Sonata were the most effective fungicides against mycelial growth of *P. ramorum*. Tests on activity of the fungicides against spore germination showed that SL 567A, Standon Etridiazole 35, Sonata, Amistar and Tanos were most effective. In both tests SL 567A (metalaxyl-M) was by far the most effective of all the fungicides tested.

Tests were also carried out to investigate the efficacy of fungicides against *P. ramorum* infections on rhododendron and viburnum. Assays using detached leaves, to test the protectant activity of the fungicides, showed that SL 567A, Invader, Curzate M68 and Sonata all completely inhibited growth of *P. ramorum* on both rhododendron and viburnum when applied pre-infection.

Seven fungicides: SL 567A, Invader, Curzate M68, Sonata, Standon Etridiazole 35, Amistar and Tanos were tested for protectant and eradicant activity to containerised rhododendron and viburnum. All fungicides were applied as foliar sprays at the manufacturer's recommended rate. On rhododendron, SL 567A, Amistar and Sonata completely inhibited symptom development when applied as protectant treatments either 4 or 7 days prior to infection. However, on viburnum only SL 567A was completely effective at all protectant timings. Sonata was effective when applied 4 days prior to infection but efficacy was very reduced if the treatment was applied 3 days earlier. Fungicides were generally less effective when applied as eradicants. The most effective was SL 567A completely inhibiting disease development when applied 4 days after infection. None of the fungicides completely controlled disease development on viburnum when applied after the same time period.

Tests to indicate the viability of the pathogen following fungicide application showed that the pathogen could not be recovered from rhododendron leaves after either protectant or eradicant treatments of Amistar or SL 567A. In general, levels of viability were much lower after protectant applications. In contrast, on viburnum, levels of viability were lower after application of eradicant treatments with the pathogen surviving only after treatment with Amistar. Protectant treatments with SL

567A and Sonata also resulted in zero recovery of the pathogen on viburnum. In some cases the effect of fungicide on the two hosts were very different and it is possible that there was an interaction between the fungicide and the plants defence systems. Lesions on viburnum were always discrete and tended not to spread, indicating a lower level of susceptibility to *P. ramorum* compared to rhododendron.

Observations on disease spread down the stem on rhododendron showed that protectant treatments were more effective than eradicants in preventing spread to the stem, with all fungicides except Tanos and Invader effective as protectants but only SL 567A effective as an eradicant.

Tests on disinfectants showed that Panacide-M and Jeyes Fluid used at the manufacturers recommended rates were very effective in sterilising substrates contaminated with *P. ramorum* after an exposure period of 10-30 minutes. This confirmed earlier work carried out by Lane (unpublished), which demonstrated that Panacide-M or Antec FFS were very effective in disinfecting a range of substrates, including weed suppressant fabric, limestone chippings and wood, after an exposure period of at least 10 minutes. These disinfectants were also tested as a sterilant for secateurs during pruning, but the disease did not establish infections in host plants following pruning with contaminated secateurs, possibly due to levels of inoculum being too low.

Overall, the project has demonstrated that, subject to further investigation, chemical treatments could play a major role in future control and containment strategies for *P. ramorum.* However, before growers could adopt the results of the *in planta* work there would need to be changes in current UK eradication policy for *P. ramorum* and further experimental work on co-formulations and mixtures of fungicides shown to be active in this study. Results of work on disinfectants and on guidance within the management strategy can be implemented immediately.

Financial benefits

There are no financial benefits to be gained by growers from the work on disease control in the short term. However, effective use of disinfectants and observance of

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the management guidelines could significantly improve the chances of long-term eradication of *P. ramorum* from nurseries. Thus ensuring considerable savings by avoiding further re-infection and the subsequent destruction of plants. Total financial losses from destruction of plants and costs of decontamination as a result of outbreaks of *P. ramorum* in individual nurseries have ranged up to in excess of £250,000.

Action points for growers

- There are probably two main routes for the introduction of the disease onto nurseries - bringing in infected planting material and via contaminated soil or growing media. It is therefore, important to keep informed of the susceptible host species, as they become known and to observe good hygiene practice.
- Only buy plants which have been correctly passported.
- In high risk situations establish a quarantine area for susceptible containerised stock and place imported plants under quarantine for at least two weeks.
- Consider requiring that plants are not treated with a fungicide for at least six weeks prior to delivery as fungicides may mask infection.
- Inspect plants regularly for symptoms of ramorum dieback.
- Ensure all personnel working on the nursery are familiar with the disease and the reasons why precautionary measures are required.
- Use hygiene precautions, e.g. disinfection of tools, boots and leggings. Maintain disinfection baths if infection occurs. Use Jeyes Fluid, Panacide-M or Antec FFS.
- Overhead sprinkler irrigation can spread the disease very effectively. Drip irrigate if possible. If overhead irrigation must be used, then apply the water at a time when the foliage will dry quickly and minimise the amount of water applied.

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Science Section - HNS 123

Control of *Phytophthora ramorum* in nursery stocks (COPRINS)

Introduction

Phytophthora ramorum (Werres *et al*. 2001) (the cause of sudden oak death in the USA) has become a serious problem in southwest coastal regions of the USA, killing large numbers of tan oaks (*Lithocarpus densiflorus*) and *Quercus* species (e.g. coast live oak, black oak, shreve oak, canyon live oak). Many other forest trees, shrubs and ornamental species are also affected and the pathogen is known to have a wide and growing host range. To date (15 April 2004) there have been 328 confirmed outbreaks of *P. ramorum* in England and Wales, of which approximately 40 have been in managed gardens/woodland situations (Source: Defra website (www.defra.gov.uk)). The pathogen attacks the foliage and twigs of some hosts but only the bark of others. *P. ramorum* is present in parts of Europe (e.g. The Netherlands, Germany, Belgium, Denmark, Sweden, Spain, Poland and the UK), and prior to November 2003 had only been found on certain ornamental genera in nurseries and a number of large managed gardens (e.g. *Viburnum, Rhododendron, Pieris, Camellia, Kalmia, Syringa* and single findings on pot-grown *Taxus baccata* (yew) and *Hamamelis virginiana* (witch hazel). In early November 2003, the disease was confirmed in a Southern Red Oak (*Quercus falcata*) within a large garden in the south-east of England while the Netherlands reported a finding on a Northern Red Oak (*Quercus rubra*). In the south west of England, a number of infected trees were subsequently identified, again within previously infected managed gardens, including *Fagus sylvatica* (beech), *Aesculus hippocastanum* (horse chestnut), *Castanea sativa* (sweet chestnut) and *Quercus ilex* (Holm oak).

HONS constitute the most important means of introduction and spread of the disease through trade. There is also evidence of spread from infected plants on nurseries into the wider local environment. Spread of *P. ramorum* in outbreaks located in garden

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and woodland sites in the UK has been highly correlated with infections on foliage of under-storey hosts, especially *Rhododendron.* These leaf hosts are considered to be the key sources of inoculum (sporangia for infection and chlamydospores for survival) that initiate and maintain epidemics. Sporangia are considered to be primarily dispersed by rain or wind-driven rain; there is as yet no evidence for aerial dispersal in the absence of rain. Rizzo and Garbelotto (2003) suggest that rain splash or rain generated aerosols are the most likely means of dispersal of the pathogen and foliar and twig hosts serve as ready sources of rain-dispersed inoculum. Tjosvold *et al*., (2003) isolated *P. ramorum* from soil taken from hikers' boots where they had walked through infected woodland, indicating a major source of spread of the disease. It has been found that infested soils can be an important source of inoculum as spores in soil can spread to above ground leaves via infection of green leaf litter (Davidson *et al*., 2002). The possible transmission by insects (McPherson *et al*., 2002) and vertebrates (Blomquist *et al*., 2002) has yet to be demonstrated but must be considered a risk. The epidemiology of *P. ramorum* has been investigated under a number of Defra projects, which indicate that light, temperature, humidity and nutrient status are all important for sporulation and that the sporangia and chlamydospores are relatively robust and are able to survive considerable extremes of temperature and pH. No evidence has been found for latency in infection with symptoms on most hosts appearing after three days under optimum conditions. However, incubation temperature has been shown to affect the interval between infection and symptom expression, with cooler conditions delaying expression by up to 7 days. Monitoring of natural outbreaks has shown that *P. ramorum* can survive the UK winter in soil and leaf litter and there is some evidence for infections remaining in cut stumps. Spore trapping using aerial traps has not demonstrated movement of spores in the air but rain trapping indicates that levels of inoculum may be increasing as the spring develops. Baiting of watercourses has shown extensive contamination of the outbreak sites in ponds and streams, although in some cases there have been no new infections for over 12 months. Results from soil and leaf litter sampling and observational studies of vertebrate activity are indicating a possible role for vectors such as rabbits, deer and humans in pathogen movement.

P. ramorum is the subject of Emergency UK and EC measures which aim to prevent the spread of European isolates and the introduction of non-European isolates. This is

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primarily due to the potential threat the pathogen poses to European tree species, but other ecosystems are also potentially at risk and there is concern about heathland and moorland habitats. 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 for further assessment. This is currently having a major impact on the HONS industry, resulting in large numbers of plants having to be destroyed. The policy for eradication/containment of this disease is continually under review as more information on the epidemiology of the pathogen becomes available, and this project aims to explore the possible role of chemical control treatments in future strategies for control of the disease.

A number of active substances are currently used for the control of *Phytophthora* spp. in HONS, particularly *P. cinnamomi* of conifers, i.e. etridiazole as a drench and soil incorporation, fosetyl-aluminium as a ground spray, tolclofos-methyl as a drench, ground spray or soil incorporation, propamocarb hydrochloride as a drench or soil incorporation, furalaxyl as a drench or soil incorporation and prochloraz as a dip, drench or ground spray. Phosphonate compounds have been evaluated in the USA with some success (Schmidt *et al*., 2002) and in Australia for phytophthora root rot control in avocados (Whiley *et al*., 1992). A range of additional active ingredients, which have proven activity against *Phytophthora* species, could be used for control of *P. ramorum* under the Revised Long Term Arrangements for Extension of Use (2002). These include fungicides registered for use against *P. infestans*, which include active ingredients such as famoxadone, fenamidone, azoxystrobin, fluazinam and dimethomorph. It is well documented that *P. infestans* rapidly developed resistance to the phenylamide fungicides e.g. metalaxyl and that other fungi have developed resistance to the strobilurins e.g. azoxystrobin. The risk of resistance developing in *P. ramorum* is high and as a result fungicide control strategies must be developed to minimise this risk through use of mixtures. Fungicides used in this project and details of mode of action and resistance risk are shown in Table 1.

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Table 1. Fungicides selected for study: modes of action and resistance risk of fungicides with proven activity against *Phytophthora* spp.

Common name of active ingredient	Product name (example)	Approval status on HONS	Mode of action and mobility	Resistance risk
Propamocarb hydrochloride	Filex	On-label	Cell membrane permeability. Systemic	No resistance has been detected.
Copper sulphate Copper oxychloride Cupric ammonium carbonate	Cuprokylt FL Lindrex	On-label	Multisite. Protectant, non-systemic	Have been used since the early 1900s with no loss in performance.
Dimethomorph	in Invader	LTAEU	Cell wall synthesis. Locally systemic.	No resistance detected
Cymoxanil	Sipcam C 50 in Tanos	LTAEU	Prevents several cellular processes, including respiration, production of amino acids and cell wall permeability. Locally systemic. Curative and protectant.	Resistance described in other pathogens
Fluazinam	Shirlan	LTAEU	Multisite (stops cellular energy production). Protectant, non-systemic	Multisite inhibitor. No resistance detected.
Mancozeb	in Invader in Sonata in Curzate M68	LTAEU	Multisite. Protectant, non-systemic	Multisite inhibitor. No reduced sensitivity detected.
Metalaxyl-M	SL 567A	LTAEU	Interferes with synthesis of ribosomal RNA. Systemic. Prevents zoospore penetration.	A major resistance problem suddenly developed in 1980, with complete loss of *P. infestans* control.
Famoxadone	in Tanos	LTAEU	Inhibits fungal respiration at Qo site. Locally systemic. Protectant and anti-germination activity.	A number of other plant pathogens have developed resistance to this chemistry. No resistance detected in *P. infestans*.
Fenamidone	in Sonata	LTAEU		
Azoxystrobin	Amistar	LTAEU*		
Fosetyl-aluminium	Aliette	On-label	Highly systemic. Direct fungitoxic effect, enhancement of plant defences	Multi site inhibitor. No resistance detected
Etridiazole	Standon Etridiazole 35	On-label	Protectant, non-systemic	No resistance detected

*protected crops only
LTAEU – Revised Long Term Arrangements for Extension of Use (2002).

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Minimisation of transfer and spread of the disease via equipment or vectors such as rabbits, insects and humans is also an important means of controlling the disease and the project has examined the use of disinfectants in reducing spread via these routes.

The overall objective of the project was to carry out a preliminary investigation into current and potential fungicides and media sterilants for the control of *P. ramorum* on HONS and to formulate results into management advice on control for the benefit of producers. The project has linked well with a comprehensive Defra-funded programme to investigate the basic ætiology and epidemiology of the disease. The project aims to benefit growers directly, providing guidance on specific control measures for the nursery other than by destruction of stock, and management advice to reduce the risk of spread of the disease via implements and workers/visitors.

Materials and Methods

Isolates used and inoculum production

Two isolates of *P. ramorum* were used throughout this study.

Isolate Code	Organism	Host species	Country of origin
1560	*Phytophthora ramorum*	*Viburnum tinus* cv Eve Price	UK
1604	*Phytophthora ramorum*	*Rhododendron grandiflora* cv Cunninghams White	UK

Fungicides

A total of thirteen fungicides were assessed for efficacy against *P. ramorum* using a range of tests. These fungicides are detailed in Table 2.

Table 2. Fungicides: active ingredients and recommended application rates

Fungicide	Active ingredient	Application rate
Amistar	Azoxystrobin (250 g L^{-1})	5 mL L^{-1}
Shirlan	Fluazinam (500 g L^{-1})	1.5 mL L^{-1}
Aliette	Fosetyl-aluminium (800 g kg^{-1})	8.4 g 10 L^{-1}
SL 567A	Metalaxyl-M (480 g L^{-1})	3 mL 10 L^{-1}
Cuprokylt FL	Copper oxychloride (270 g L^{-1})	20 mL L^{-1}
Lindrex	Copper sulphate (150 g L^{-1})	5 mL L^{-1}
Standon Etridiazole 35	Etridaiazole (350 g kg^{-1})	1.5 g L^{-1}
Invader	Dimethomorph (75 g kg^{-1}) + Mancozeb (667 g kg^{-1})	1 g L^{-1}
Sipcam C50	Cymoxanil (500 g $kg^{-1)}$	1.2 g L^{-1}
Curzate M68	Cymoxanil (45 g kg^{-1}) + Mancozeb (680 g kg^{-1})	10 g L^{-1}
Tanos	Famoxadone (250 g kg^{-1}) + Cymoxanil (250 g kg^{-1})	3.5 g $10L^{-1}$
Filex	Propamocarb hydrochloride (800 g kg^{-1})	1 g L^{-1}
Sonata	Fenamidone (100 g kg^{-1}) + Mancozeb (500 g kg^{-1})	7.5 g L^{-1}

1. Efficacy of fungicides *in vitro*

The effect of fungicides on mycelial extension and sporangial germination of two *P. ramorum* isolates was examined *in vitro*. Agar plate assays were used to test the effect of fungicides on mycelial growth and an optical densitometry technique was used to determine effects on zoospore/sporangial germination.

Agar plate assays

Fungicide efficacy was assessed using two isolates of *P. ramorum* (1604 and 1560). A primary screen was carried out to test all thirteen chemicals at concentrations of 0, 1, 10,

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100 and 1000 ppm. A V-8 agar base medium (see Appendix) was ammended with fungicide to give a final fungicide concentration series as above. Three replicate plates for each fungicide concentration and controls (0 ppm) were inoculated with a 5 mm agar plug taken from the leading edge of a 7 day old culture of each isolate. Plates were sealed with parafilm and incubated at 20˚C for 7 days. Colony diameters were measured and the most effective fungicides selected for further testing to investigate dose responses.

The most effective chemicals from the primary screen were tested using a lower range of concentrations, from 0.05 ppm to 200 ppm. Methods were as described above. Colony diameters were measured after 7 days incubation and the EC_{50} values calculated from the dose response curves. The EC_{50} was defined as the fungicide concentration at which growth of the fungus was inhibited by 50% compared to growth on the untreated controls.

Photometric assays

The effect of fungicides on zoospore and sporangial germination of two *P. ramorum* isolates was determined using a photometric technique adapted from Pijls *et al*. (1994), which uses optical densitometry to measure the amount of spore germination. The fungicides used are detailed in Table 2 and were tested at concentrations of 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ppm with three replicates and controls.

Dilutions of each fungicide were prepared in a glucose-peptone growth medium (GPM) (Appendix I). 100 µL of each test concentration was pippetted into wells in flat-bottomed microtitre plates (96 well) using three replicates for each concentration. 100 µL of unamended GPM was used as the control concentration.

Sporangia of *P. ramorum* isolates 1604 and 1560 were produced by inoculating ten plates of V8 agar for each isolate and incubating at 20˚C under daylight bulbs (12 h light/12 h dark) for 7 days. Sporangia were harvested from each plate in 5 mL of GPM and sporangial suspension for each isolate combined. To facilitate the release of zoospores, the sporangial suspensions were placed at 5˚C for 1 h and then at 20˚C for 1.5 h. Zoospores and ungerminated sporangia were counted and the spore suspension adjusted to 10^4 spores mL^{-1}. 150 µL of the spore suspension was pippetted in each treatment well and 150 µL GPM was pippetted into control wells.

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Absorbances were read across all wells on the plates at 405 nm immediately after the addition of spores and then after 12 h incubation at 20˚C. The absorbance readings were used to calculate the % inhibition compared to the control. Dose response curves were plotted and the EC_{50} values determined.

2. Efficacy of fungicides *in vivo*

The activity of fungicide *in vivo* was investigated using both detached leaf and whole plant assays.

Detached leaf assay

The detached leaf assay was carried out using rhododendron and viburnum leaves. All thirteen fungicides were tested and were applied according to the manufacturer's recommended rate (Table 2). For each fungicide treatment, eight rhododendron and eight viburnum leaves, all of equivalent age, were sprayed to run off and allowed to air dry. Leaves were then wounded with a scalpel blade (cut approx 5 mm long) and eight leaves per fungicide treatment (four rhododendron and four viburnum) were inoculated with isolate 1560. The remaining leaves in the treatment were inoculated with isolate 1604. Leaves were inoculated with a 5 mm agar plug, taken from the leading edge of a 7 day old culture, which was placed over the wounded area. Inoculated and uninoculated controls (agar plug only) were also set up. All control and treated leaves were incubated in a moist chamber at 20˚C.

The length and breadth of leaf lesions were measured after 10 days' incubation and EC_{50} values calculated from plotting the dose response curves.

In planta fungicide testing

Thirty rhododendron 'Cunninghams White' and 30 *Viburnum tinus* plants were placed in the quarantine CE room four weeks prior to the start of the experiment to ensure plants were fully acclimatised. Conditions of CE room were 18˚C, 80 % humidity and a 12 h day/night light regime. By the start of the experiment fresh shoots and new leaves had developed indicating the plants were no longer in a dormant state.

Seven fungicides were included in the study based on their effectiveness in the *in vitro* tests. These were SL 567A, Amistar, Standon Etridiazole 35, Invader, Curzate M68, Tanos

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and Sonata. Fungicides were applied as foliar sprays at 7 days pre-, 4 days pre-, 4 days post- or 7 days post-inoculation. Fungicides were applied at the rates indicated in Table 2 in a water volume equivalent to 200 L ha^{-1}.

Two isolates of *P. ramorum* were used in this study, 1604 isolated from a rhododendron plant and 1560 isolated from a viburnum plant. Sporangia were harvested from 7 day old cultures grown on V8 agar at 20˚C under a 12 h day/night white light regime. The resultant sporangial suspensions were incubated at 5˚C for one hour and then at 20˚C for 1.5 h to induce zoospore release. Total counts for ungerminated sporangia and zoospores were carried out and the concentration adjusted to give a spore count of 10^5 spores mL^{-1}. Just prior to inoculation, 3 leaves of equivalent age were selected on each plant and each wounded ten times with a dissection needle to mimic insect damage. Plants were inoculated within one hour of the spore count to ensure zoospores were still motile. Plants were inoculated with 8 mL of the spore suspension (sufficient to give even coverage of the plant). Rhododendron plants were inoculated with isolate 1604 and viburnum plants with isolate 1560. Following inoculation, plants were bagged overnight to raise humidity and aid leaf infection.

A record was kept of symptom development on the control plants and a full assessment carried out on all plants 10 days after inoculation. The % leaf area affected by symptoms was recorded for the three wounded leaves. A measurement of lesion development down the stem was also carried out. Data were expressed as % control compared to the untreated plants.

After the ten-day assessment, viability of *P. ramorum* within the inoculated leaves was assessed by plating the lesions from three leaves per treatment onto P_5ARPH agar (Appendix 1). Plates were incubated at 20˚C and assessed for growth of *P. ramorum* after 7 days.

3. Efficacy of disinfectants against *P. ramorum*

In initial tests, two substrates were used to test the effectiveness of disinfectants, a pea gravel:sand:soil mix (4:2:1) and concrete disks. In later tests, only the gravel:sand:soil mix

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was used. These substrates were used to simulate the types of standing area found in a nursery situation.

Concrete disks were prepared in 9 cm Petri-dishes using gravel:sand:cement (4:2:1) mixed with water. Three concrete disks were prepared for each disinfectant treatment, placed in an inverted clear plastic 'sandwich' box and autoclaved on 3 consecutive days at 121˚C for 15 minutes. Samples of the gravel:sand:soil mix (300 mL) were also set up in lidded clear plastic 'sandwich' boxes and then sterilised as described above.

Boxes containing the gravel:sand:soil mix were inoculated with 15 mL of a 10^5 sporangia/zoospore mix of *P. ramorum* (isolate 1604). Each concrete disk was inoculated with 1 mL of the same spore mix. Both substrates were incubated for 7 days at 20ºC, to allow mycelial growth and production of chlamydospores. Three replicates were set up for each treatment, and for both substrates an equivalent volume of sterile distilled water replaced the spore mix in uninoculated controls.

The disinfectants tested were Panacide-M, Virkon S, Hortisept and Jet 5 (details in Table 3). Treatments were applied to the gravel:sand:soil mix until the mix was wetted throughout (approx. 20 ml) whereas for the concrete disks, 1 mL of chemical was applied to the surface of the disks. Sterile distilled water was used for the untreated controls.

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Table 3. Disinfectant and rate used in tests to eradicate *P. ramorum* from different substrates.

Trade name	Dilution rate	Current recommendations for treatment (substrates)
Panacide-M	1:60	Tool disinfectant
	1:500	Footbath
Virkon S	1:100	Fungicidal activity
Hortisept	1:125	Surface disinfectant
Jet 5	1:125	Surface disinfectant
Dettol	25 mL:1 L	Household/nurseries
	44mL:120 L	In the bath
Jeyes fluid	30 mL:1 L	Cleaning concrete paths
	35 mL:5 L	Cleaning poly-tunnels, glasshouses, plant pots, seed boxes etc.

Samples were taken after 48 h exposure to the treatment. Three replicate 4 g samples were taken from each gravel:sand:soil treatment and plated directly onto P_5ARPH agar. Concrete disks were plated face down onto P_5ARPH agar. Plates were assessed for growth of *P. ramorum* after 7 days incubation at room temperature.

In a second experiment, the effect of length of exposure to Panacide-M, Jeyes Fluid and Dettol was examined using the gravel:sand:soil mix as the test substrate. Samples were taken as described above after 0, 10, 30 min, 1, 2, 4, 24 and 48 h exposure.

4. Control of transfer of *P. ramorum* through pruning.

The potential for transfer of *P. ramorum* via pruning equipment was assessed using rhododendron plants infected with *P. ramorum* isolate 1604.

The leading edge of *P. ramorum* lesions on three rhododendron stems was cut with a pair of surface-sterilised secateurs. The 'contaminated' secateurs were then used to cut a rhododendron leaf into thirds (with the first cut towards the petiole end). The secateurs were surface sterilised and the process repeated for a further two leaves. Control leaves cut

were using secateurs, which had previously been used to cut through uninfected rhododendron stems.

The procedure was then repeated using a sterilisation step, involving dipping the secateurs into 100 % IMS solution for 30 sec or a 1:60 Panacide-M solution for 2 min, before cutting the fresh rhododendron leaf.

All cut leaves were incubated in a moist chamber at 20˚C. Lesion development was assessed after 10 days' incubation.

Results and Discussion

1. Efficacy of fungicides *in vitro*

(a) Agar plate tests

A preliminary study carried out to test the efficacy of thirteen fungicides against growth of mycelium on agar showed that Tanos, Cuprokylt FL, Sipcam C50, Lindrex, Aliette and Filex had poor activity against mycelial growth of *P. ramorum*, with EC_{50} values exceeding 10 ppm. Seven chemicals, SL 567A, Standon Etridiazole 35, Sonata, Amistar, Invader, Shirlan and Curzate M68, were selected for further study to investigate dose responses.

Tests on selected chemicals at a range of concentrations showed no significant difference in sensitivity between the two isolates tested (Figures 1 & 2). SL 567A was the most effective chemical with an EC_{50} value of less than 0.01 ppm. Invader, Standon Etridiazole 35, Sonata and Curzate M68 were also effective with EC_{50} values of less than 2 ppm.

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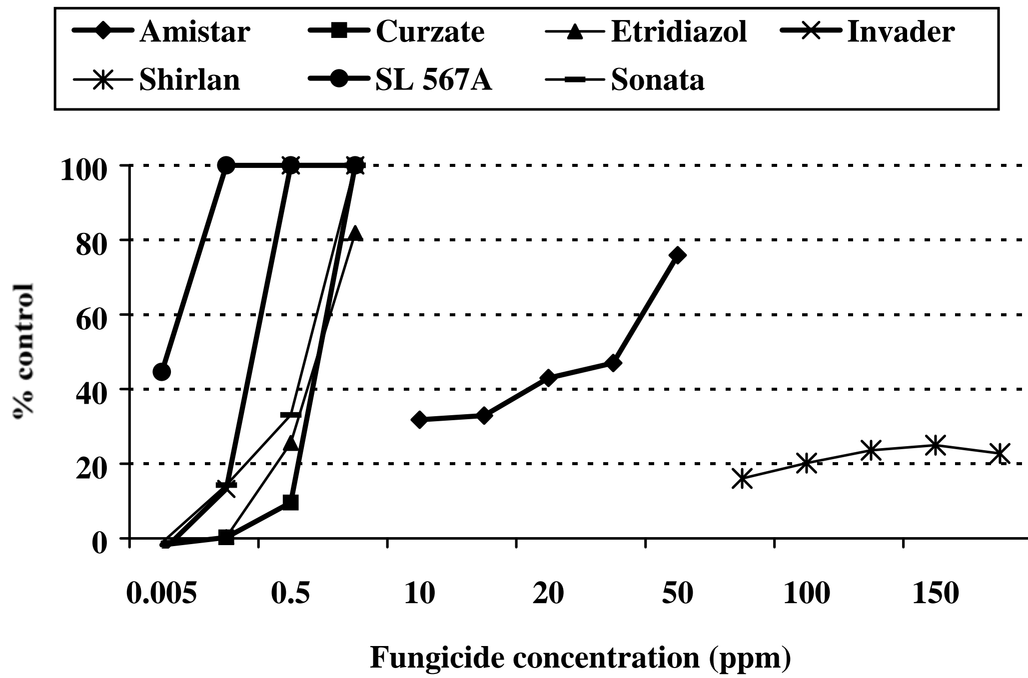

Figure 1. Sensitivity of isolate 1560 to fungicides in agar plate tests.

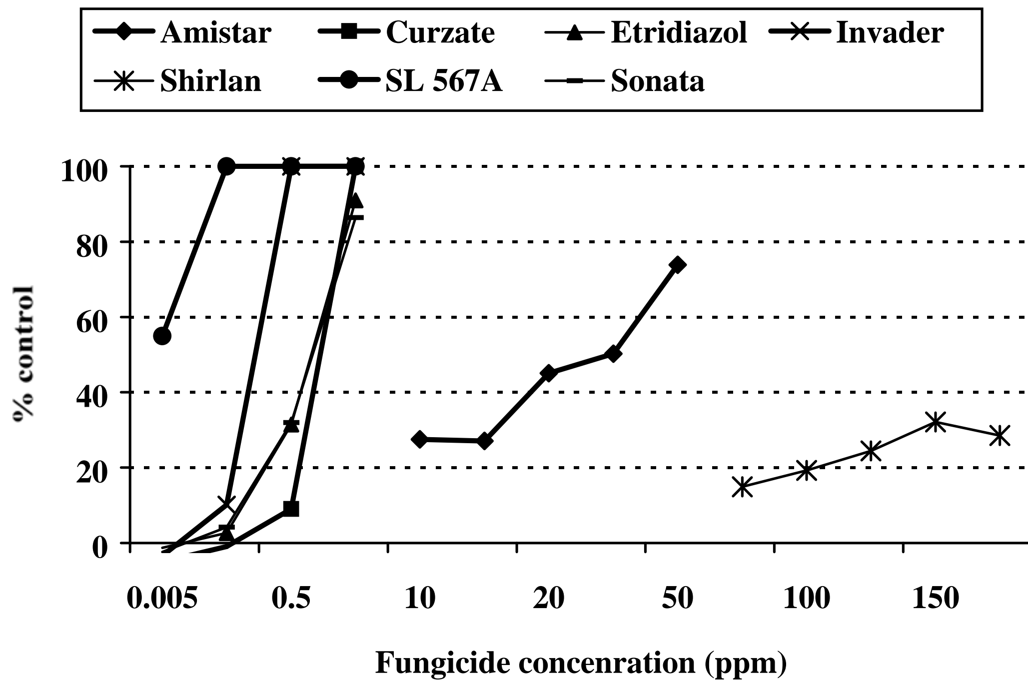

Figure 2. Sensitivity of isolate 1604 to fungicides in agar plate tests

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(b) Photometric tests

The photometric test was used to determine the efficacy of fungicides against spore germination of *P. ramorum*. Tests were carried out using all thirteen fungicides and the results are shown in Figures 3 & 4. There were more differences between the two isolates in sensitivity to fungicides in this test, particularly to Curzate M68 and Amistar. The most effective chemical was SL 567A followed by Standon Etridiazole 35 and Sonata. Fungicides such as Shirlan and Amistar, which were relatively ineffective against mycelial growth, were far more effective against spore germination.

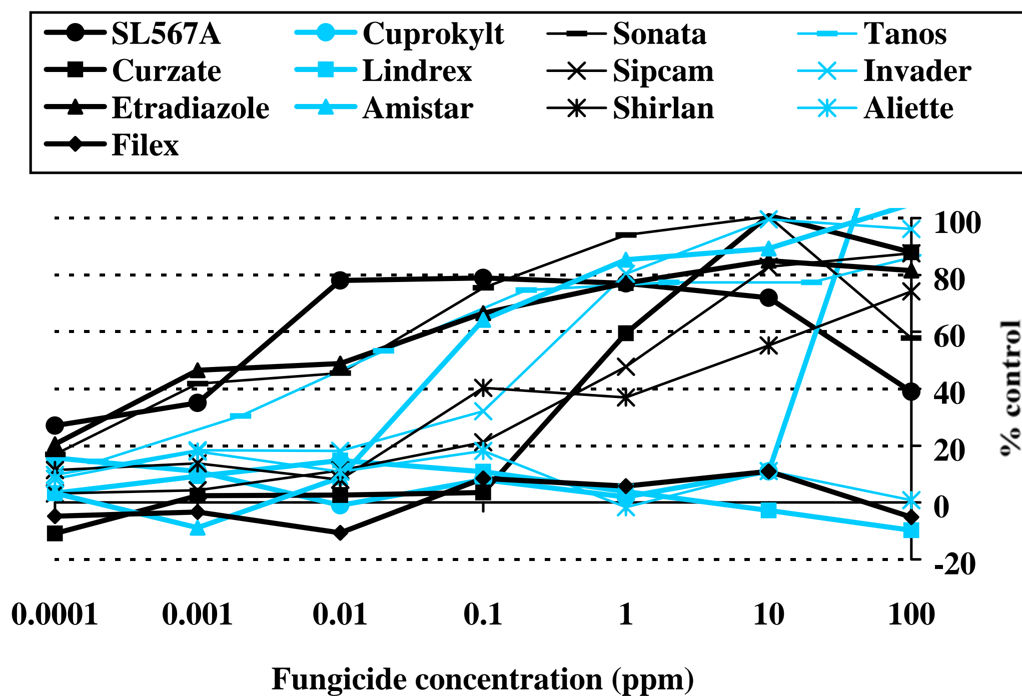

Figure 3. Sensitivity of isolate 1560 to fungicides in photometric tests

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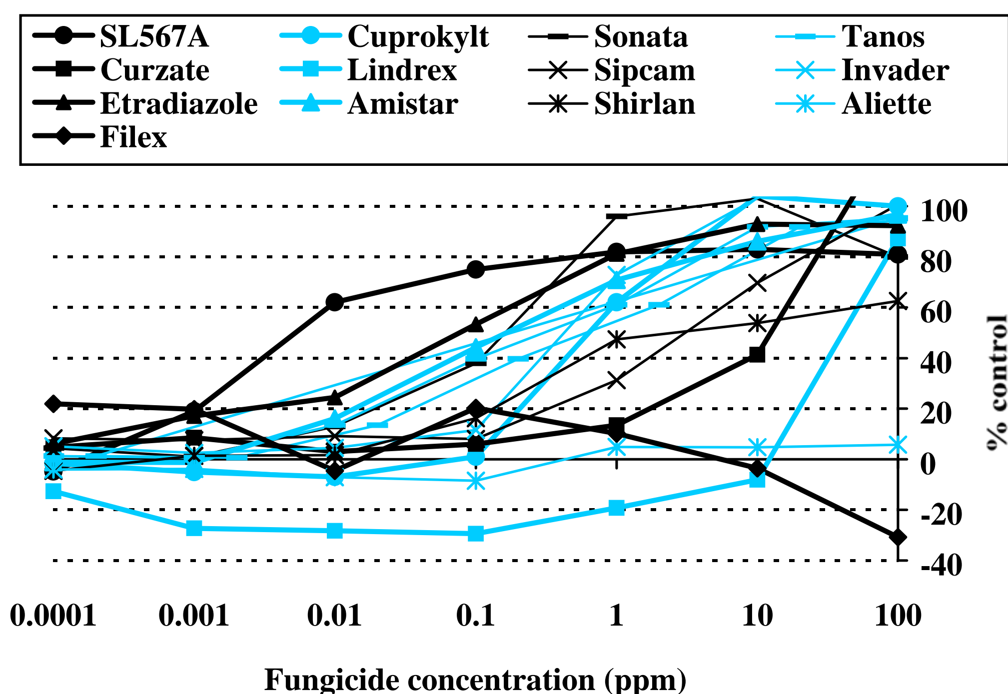

Figure 4. Sensitivity of isolate 1604 to fungicides in photometric tests

Comparison of EC_{50} values from agar plate and photometric tests showed that SL 567A was the most effective fungicide against both mycelial growth and spore germination and that Sonata, Invader and Standon Etridiazole 35 also showed broad activity (Table 4).

Table 4. EC_{50} values for two isolates of *P. ramorum* tested for sensitivity using agar plate and photometric tests

	1604		1560	
Fungicide	Agar plate test	Photometric test	Agar plate test	Photometric test
SL 567A	<0.01	0.005	<0.01	0.0024
Invader	0.14	0.31	0.12	0.25
Etridiazole 35	1.0	0.075	1.9	0.013
Sonata	1.0	0.170	0.95	0.170
Curzate M68	1.5	13.5	1.9	0.6
Amistar	25.0	0.170	38.0	0.05
Shirlan	>100	1.5	>100	5.5

2. Efficacy of fungicides *in vivo*

(i) Detached leaf assays

(a) Rhododendron

Detached leaf assays using agar discs to infect previously wounded leaves were used to test the protectant activity of selected fungicides *in vivo*. The two isolates tested showed significant differences in sensitivity to a number of the fungicides tested. The most effective chemicals were SL 567A, Invader, Curzate M68 and Sonata, which completely inhibited development of disease symptoms (Figures 5 & 6). With the exception of Standon Etridiazole 35, these are the same chemicals which were most effective in the agar plate tests. Some chemical treatments caused stimulation of disease symptom development but the effect was not consistent across individual chemicals or isolates.

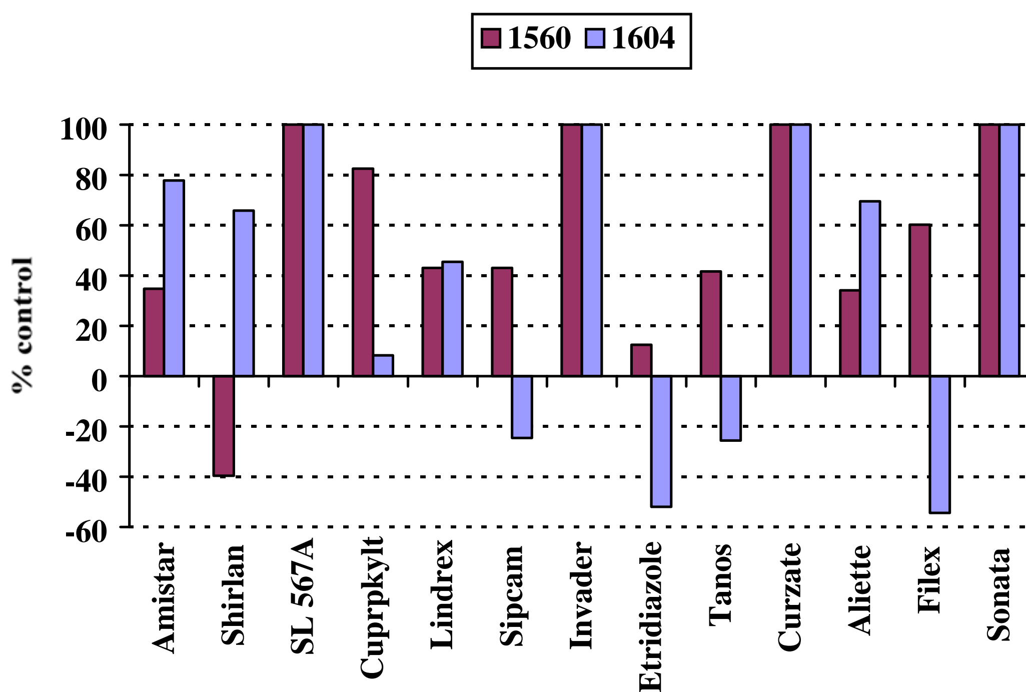

Figure 5. Sensitivity of *P. ramorum* isolates to fungicide in detached rhododendron leaf assays

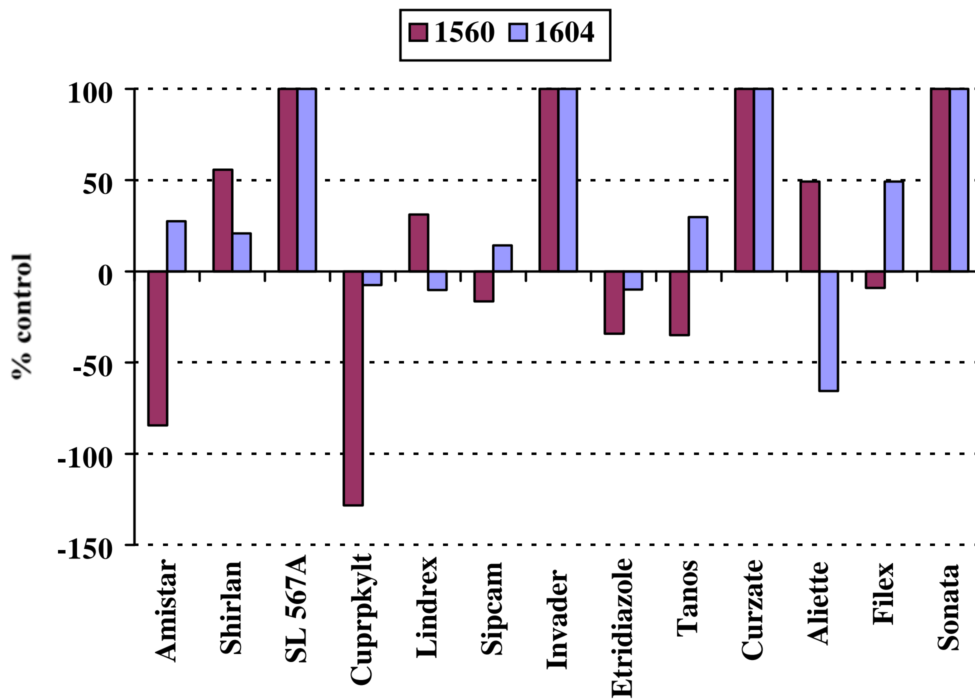

Figure 6. Sensitivity of *P. ramorum* isolates to fungicide in detached viburnum leaf assays

<u>(ii) Efficacy of selected fungicides *in planta* – leaf infections</u>

Inoculations of rhododendron and viburnum resulted in widespread disease symptoms on the control treatments affecting leaves, stems (rhododendron only) and flowers (rhododendron only).

(a) Protectant activity

Techniques used in this experiment tested the protectant activity of fungicides against infections caused by splash/water-borne zoospore inoculum on containerised plants. The most effective fungicides on rhododendron were Amistar, SL 567A and Sonata (Figure 7), completely inhibiting symptom development when applied either 7 or 4 days before infection. Tanos and Curzate M68 were most effective when applied 7 days before infection whereas Standon Etridiazole 35 and Invader were more effective when applied 4 days prior to infection.

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On viburnum, SL 567A was the most effective fungicide tested (Figure 8), again completely inhibiting symptom development when applied either 7 or 4 days prior to infection. Invader and Sonata were very effective when applied 4 days pre-infection but showed very reduced activity when applied 7 days pre-infection.

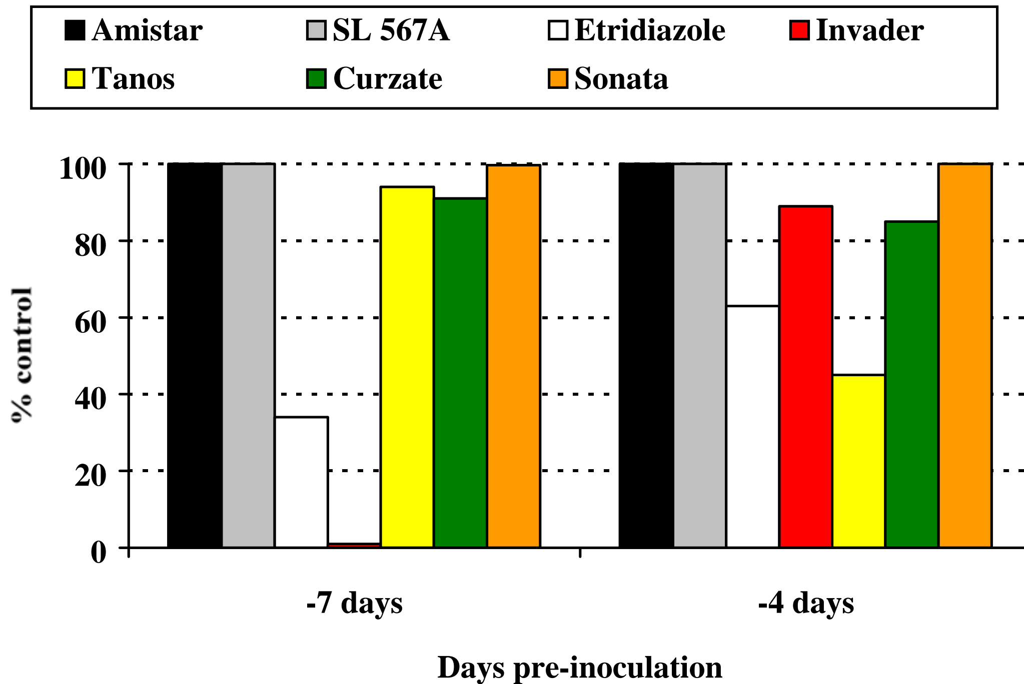

Figure 7. Effect of fungicides applied as protectant treatments on rhododendron

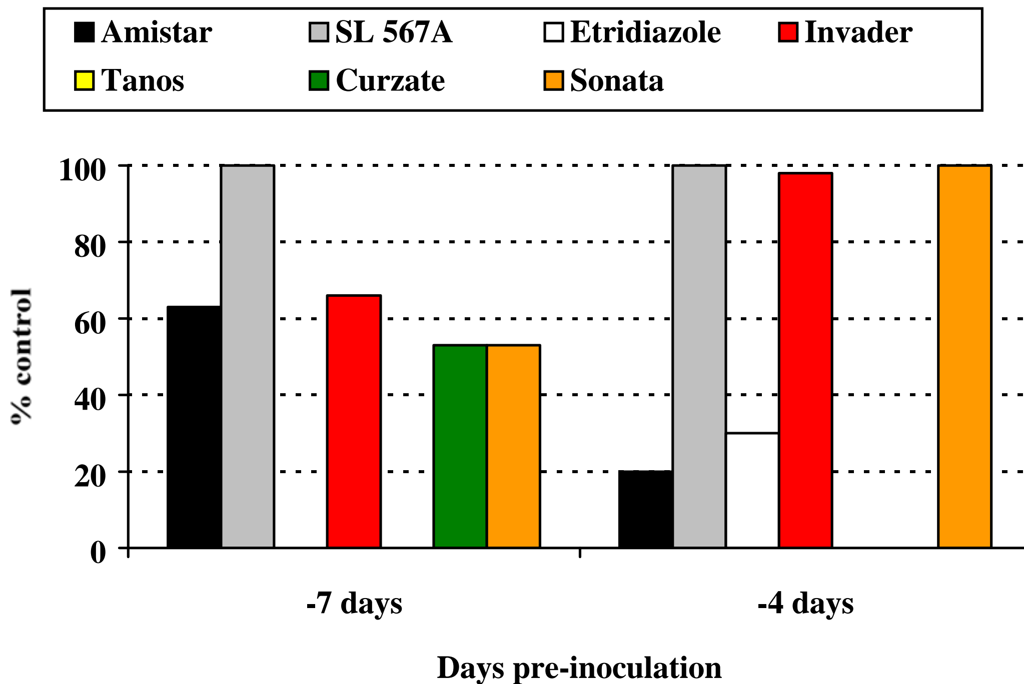

Figure 8. Effect of fungicides applied as protectant treatments on viburnum

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(b) Eradicant activity

Effectiveness of the fungicide treatments varied significantly depending on the host plant tested. On rhododendron, the most effective fungicides were SL 567A (applied 4 days after infection), Standon Etridiazole 35 and Sonata (both applied 7 days after infection) (Figure 9). On viburnum, Amistar and SL 567A, applied 4 days after infection were most effective. However, levels of control were only 83% and 76% respectively (Figure 10).

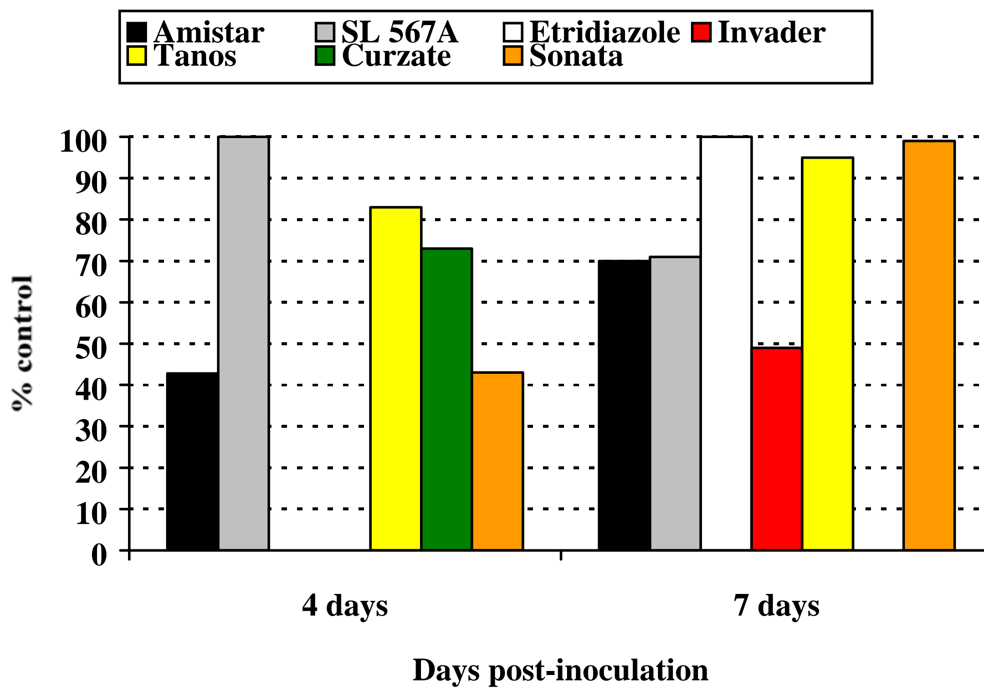

Figure 9. Effect of fungicides applied as eradicant treatments on rhododendron

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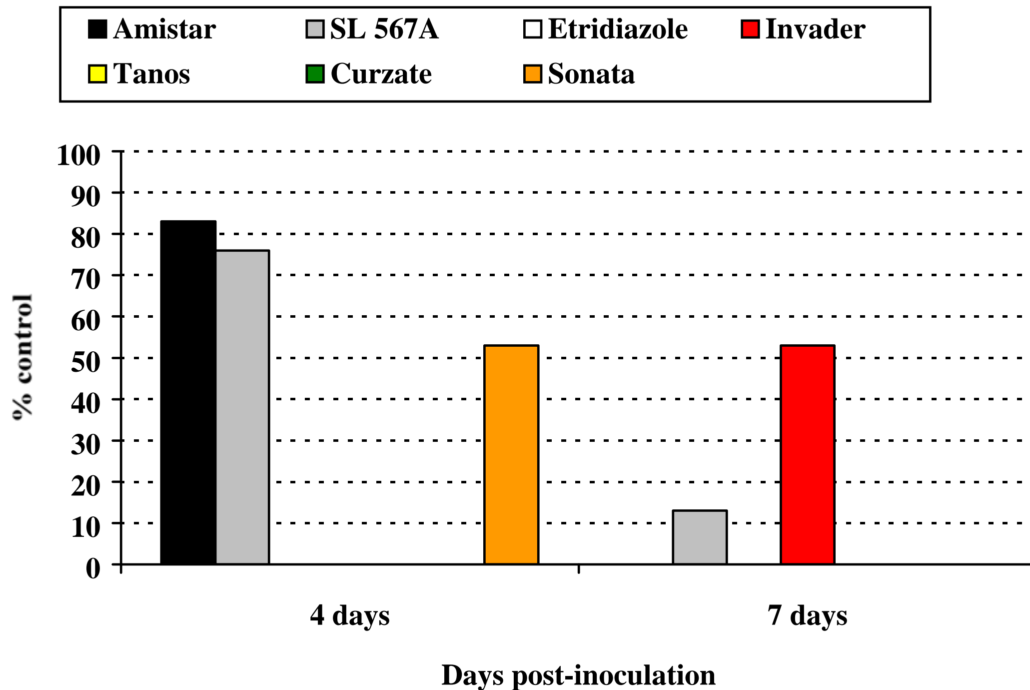

Figure 10. Effect of fungicides applied as eradicant treatments on viburnum

Efficacy of selected fungicides *in planta* – stem infections

It was observed on the *in planta* tests that disease symptoms on viburnum tended to remain as relatively discrete lesions whilst symptoms on rhododendron tended to spread and move down the stem. Assessments were carried on the effectiveness of chemicals applied as either protectant or eradicant treatments on stem infections on rhododendron. Results showed that protectant treatments were far more effective than eradicants with only SL 567A and Tanos showing any activity against stem infections when applied post-infection, SL 567A giving 100% control when applied 7 days after infection (Figures 11 & 12). All protectant treatments except Tanos and Invader completely inhibited infection of the stem when applied either 4 or 7 days pre-infection.

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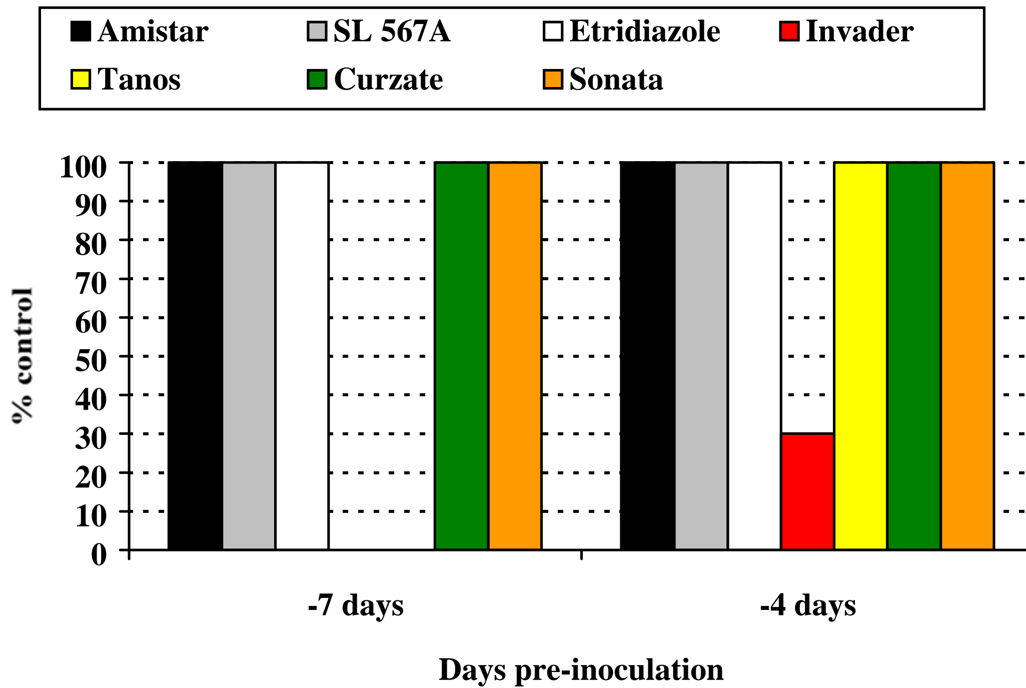

Figure 11. Effect of fungicides applied as protectant treatments on stem infections

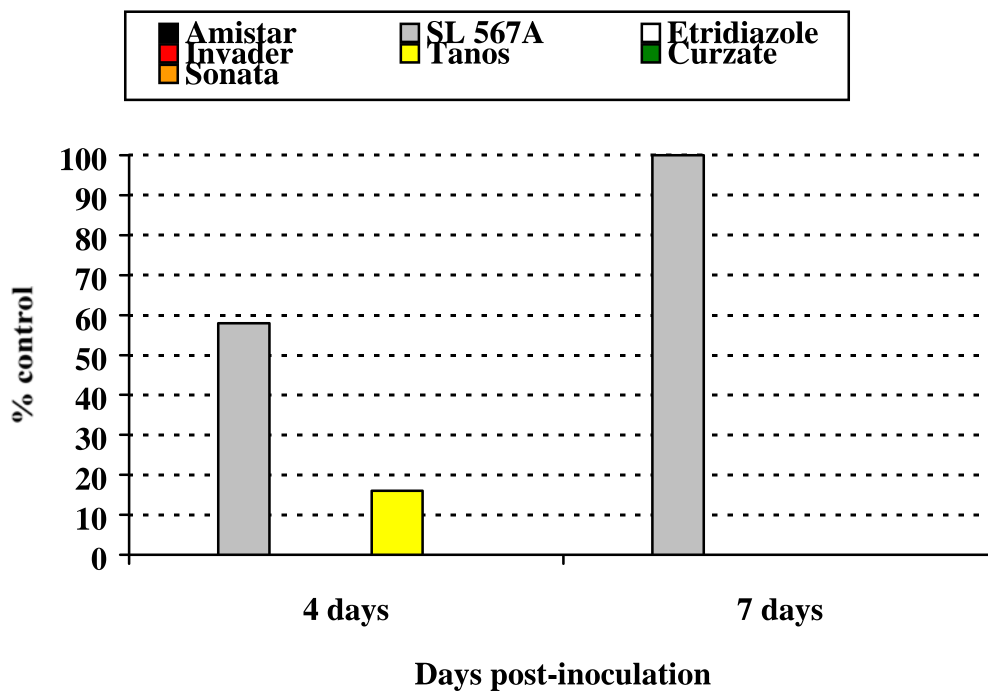

Figure 12. Effect of fungicides applied as eradicant treatments on stem infections

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Isolations from leaf material from the *in planta* tests were carried out to investigate the viability of the pathogen following treatment. In rhododendron, levels of viability were much lower after application with protectant treatments compared to those applied as eradicants (Figure 13). Tests showed that the pathogen could not be recovered from leaves after treatments of Amistar and SL 567A applied pre-infection or post-infection. In contrast to rhododendron, the levels of viability in viburnum were lower after application with eradicant treatments (Figure 14), with no recovery of the pathogen occurring on any of the treatments except Amistar.

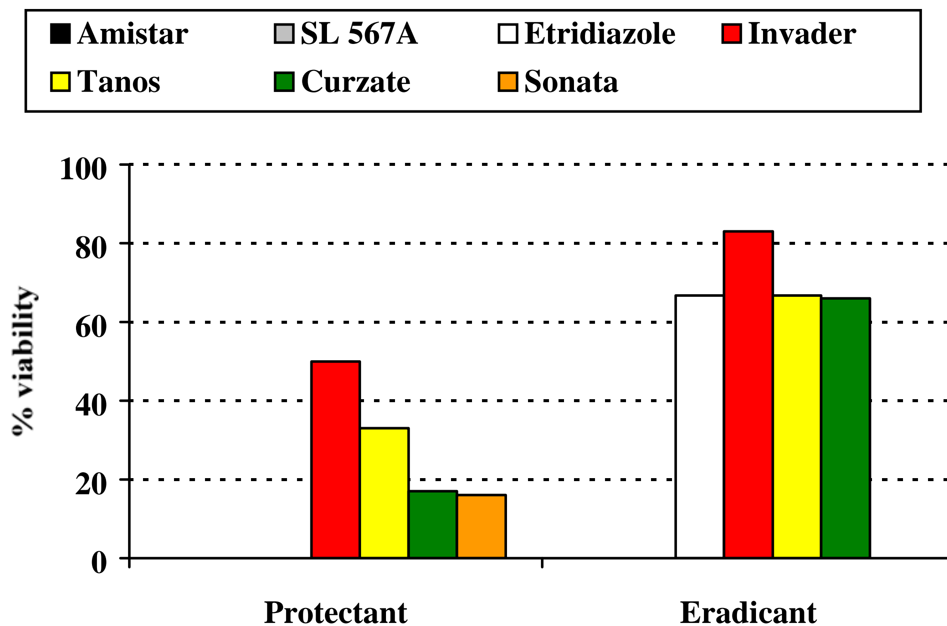

Figure 13. Viability of *P. ramorum* isolated from rhododendron leaves following fungicide treatment

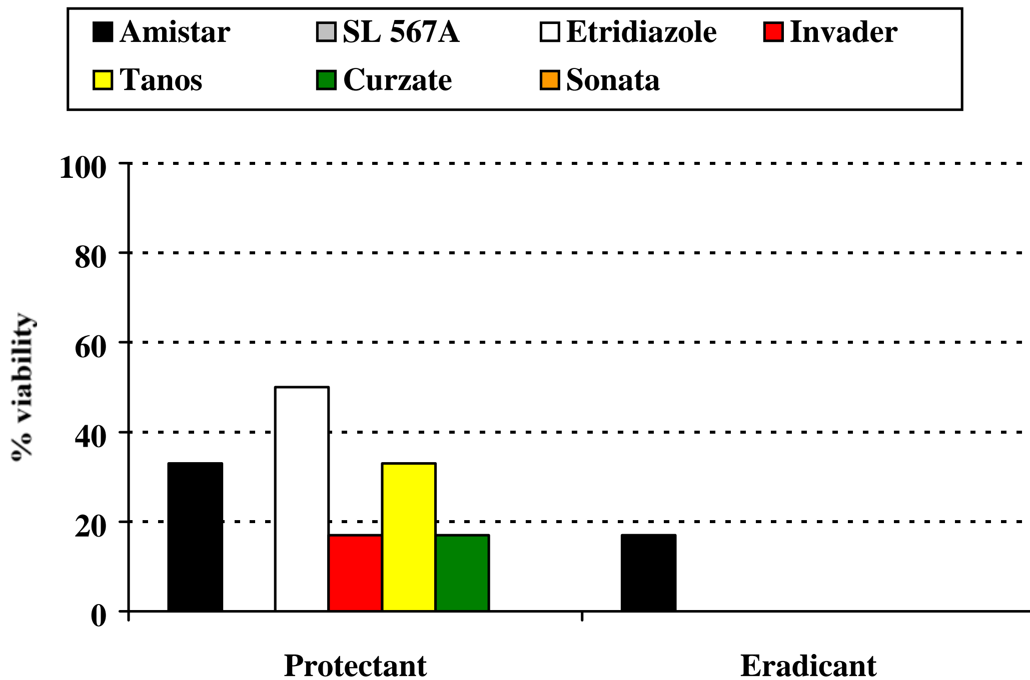

Figure 14. Viability of *P. ramorum* isolated from viburnum leaves following fungicide treatment

Summaries of the data from the *in planta* studies (Table 5) indicate that some treatments were fungitoxic, e.g. SL 567A applied as a protectant treatment, causing 100 % inhibition of growth and killing the fungus outright, whilst other fungicides were fungistatic, e.g. Sonata applied as an protectant on rhododendron, which caused 100 % inhibition of growth but the pathogen was demonstrated to be still viable. The effects of fungicides were markedly different on the two host plants tested with eradicant treatments far more effective on viburnum and many more of the treatments having a fungitoxic effect.

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Table 5. Summary of activity of fungicides *in planta*

	Rhododendron		Viburnum	
Fungicide	Protectant	Eradicant	Protectant	Eradicant
Amistar				
Level of control	100%	<100%	<100%	<100%
Viability	**NV**	**NV**	V	V
SL 567A				
Level of control	100%	100%	100%	<100%
Viability	**NV**	**NV**	**NV**	**NV**
Standon Etridiazole				
Level of control	<100%	<100%	<100%	<100%
Viability	**NV**	V	V	**NV**
Invader				
Level of control	<100%	<100%	<100%	<100%
Viability	V	V	V	**NV**
Tanos				
Level of control	<100%	<100%	<100%	<100%
Viability	V	V	V	**NV**
Curzate M68				
Level of control	<100%	<100%	<100%	<100%
Viability	V	V	V	**NV**
Sonata				
Level of control	100%	<100%	<100%	<100%
Viability	V	**NV**	**NV**	**NV**

NV – Non Viable

V – Viable

3. Efficacy of disinfectants against *P. ramorum*

Testing of treatments in a gravel/sand/soil mix showed that only Panacide-M at a rate of 1:60 was effective in eradicating *P. ramorum* after 48 hours exposure (Table 6). Other treatments tested were ineffective. The pathogen did not survive when inoculated onto concrete, possibly as a result of the caustic nature of the freshly prepared substrate.

Table 6. Effect of disinfectants after 48 h exposure on *P. ramorum* contamination in a gravel:sand:soil mix and on a concrete surface.

Treatment	Dilution Rate	Substrate	
		gravel:sand:soil mix	Concrete
Untreated - uninoculated		X	X
Untreated - inoculated		✓	X
Panacide-M	1:60	X	X
Hortisept	1:125	✓	X
Virkon S	1:100	✓	X
Jet 5	1:125	✓	X

X = no growth of *P. ramorum*; ✓ = *P. ramorum* growth

Further experiments were carried out using Panacide-M, Dettol and Jeyes Fluid to determine the minimum period of exposure required to eradicate *P. ramorum* from a gravel/sand/soil substrate. Jeyes Fluid was the most effective disinfectant tested being effective after 10 minutes at a dilution of 30 mL L^{-1} but ineffective at 7 mL L^{-1}. Panacide-M was effective at a rate of 17 mL L^{-1} after 30 minutes but ineffective at 500 mL L^{-1} even after 48 hours exposure (Table 7). Experiments by Lane (unpublished) have also demonstrated that Panacide-M or Antec FFS were very effective in disinfecting a range of substrates, including weed suppressent fabric, limestone chippings and wood, after an exposure period of 10 minutes.

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Table 7. Effect of period of exposure on activity of disinfectants

Treatment	Dilution Rate	Exposure time							
		0 min	10 min	30 min	1 hr	2 hr	4 hr	24 hr	48 hr
Untreated - uninoculated	-	X	X	X	X	X	X	X	X
Untreated – inoculated	-	✓	✓	✓	✓	✓	✓	✓	✓
Panacide	2 mL L^{-1}	✓	✓	✓	✓	✓	✓	✓	✓
	17 mL L^{-1}	✓	✓	X	X	X	X	X	X
Dettol	44 mL 120 L^{-1}	✓	✓	✓	✓	✓	✓	✓	✓
	25 mL L^{-1}	✓	✓	✓	✓	✓	X	X	X
Jeyes Fluid	7 ml L^{-1}	✓	✓	✓	✓	✓	✓	✓	✓
	30 mL L^{-1}	✓	X	X	X	X	X	X	X

X= no growth of *P. ramorum*; ✓ = *P. ramorum* growth

4. Control of transfer of P. ramorum through pruning

Results of isolations from the pruned leaves showed that none of the leaves were infected with *P. ramorum* even when no disinfectant had been used to sterilise the secateurs. It is possible that inoculum was present on the secateurs, probably as mycelium, and that the amount present was insufficient to establish an infection on the leaves. Work carried out by Lane (unpublished) has shown that secateurs were readily contaminated with *P. ramorum* when used to cut naturally infected plant material and that exposure to disinfectants such as Panacide-M or Antec FFS was effective in completely sterilising the equipment.

Conclusions

Results indicate that SL 567A was a very effective treatment against *P. ramorum*, showing efficacy against both mycelium and spores. Tests carried out on containerised rhododendron and viburnum plants confirmed the efficacy of SL 567A both as a protectant and an eradicant treatment. However, this fungicide contains a single active ingredient and there is considerable evidence in the literature to demonstrate that the risk of resistance to such phenylamide fungicides in oomycete pathogens, including *Phytophthora* spp., is high. Indeed FRAC & FRAG-UK guidelines for control of oomycete fungi e.g. blight in potato specifically recommend fungicide mixtures (protectant & eradicant and/or contact &

systemic) or alternating programmes of different mode of action products to mitigate such risk. There is considered to be a significant risk of the rapid development of resistance from the repeated use of SL567A as a stand-alone product and as such it has not been recommended. Co-formulations and mixtures need to be investigated in order to develop a protocol for durable fungicidal control of *P. ramorum*. Possible mixtures could include other products found to be effective in this study, including Amistar and Sonata. The influence of the host plant on the level of control also needs investigating, as fungicide effects on infected rhododendron were in some cases very different to those on infected viburnum. Experiments carried out on methods of disinfecting have demonstrated that Panacide-M and Jeyes Fluid were very effective after ten minutes exposure in decontaminating substrates.

Overall, the project has demonstrated that, subject to further investigation, chemical treatments could play a major role in future control and containment strategies for *P. ramorum.*

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Appendix I

V-8 agar

V8 juice	330 mL
$CaCO_3$	2 g
Agar Nº3	40 g
0.1M KOH	50 mL (0.280 g in 50 mL distilled water)
Distilled water	1620 mL

Autoclave at 121˚C for 15 min.

$PARP_5H$ agar (Jeffers and Martin, 1986)

Corn Meal 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}

Glucose peptone medium (GPM)

Dextrose	14 g
Bactopeptone	7.1 g
Yeast extract	1.4 g
Water	1000 ml

Autoclave at 121˚C for 15 min.

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RAMORUM DIEBACK (SUDDEN OAK DEATH)

CODE OF PRACTICE FOR NURSERIES

Background

Ramorum dieback is caused by *Phytophthora ramorum*, the same fungus which is causing sudden oak death in the USA. The disease was first observed in the mid- to late-1990s causing the death of thousands of tan oaks (*Lithocarpus densiflorus*), coast live oaks (*Quercus agrifolia*), Californian black oaks (*Q. kellogii*) and interior live oaks (*Q. parvula* var. *shrevei*) in central California. The disease was first recorded in the UK in 2002, originally in containerised stock of viburnums, and later rhododendrons, in nurseries. The disease has, more recently, been found in shrubs and trees in large established gardens on estates.

Susceptible hosts

In addition to viburnums and rhododendrons, other ornamental plants affected include species of *Arbutus*, *Camellia*, *Hamamelis* (witch-hazel), *Kalmia*, *Leucothoe*, *Pieris*, *Syringa* (lilac), Laurus nobilis (bay laurel) and *Taxus* (yew). The main threat from the disease is to environmentally important and plantation trees and key heathland species such as *Vaccinium* spp. (bilberry).

Symptoms

The pathogen can infect the trunk, shoots or leaves, or a combination of all three. Symptoms on the trunk are of large bleeding cankers. In trees, shoot and leaf infections have, so far, only been recorded on holm oak and sweet chestnut. Leaf infections are the most widespread symptom on HONS and appear, most commonly, as brown necrotic areas, frequently at the margins and tips of the leaves. Symptoms vary according to the host affected.

Spread

As a new disease, there is limited information on how the disease spreads in nurseries, and in the wider environment. However, it is known that two different “spores” are produced by the fungus – sporangia (thin walled and short lived) produced on the surface of infected leaves and shoots and chlamydospores (thick walled) and produced within infected tissue. The sporangia are spread by rain splash, wind-driven rain, irrigation or in ground water. The chlamydospores allow the long-term survival of the pathogen. The disease is found in temperate climates with an optimum of 20°C. The pathogen is thought to be spread locally by movement of contaminated plant material, growing media and in soil/dust particles carried on vehicles, machinery, footwear or animals, which can lead to long-distance spread.

Control

Phytophthora ramorum is a notifiable pathogen resulting in statutory action to prevent its introduction and spread. If plants are suspected of being affected by the disease the local Defra Plant and Health Seeds Inspectorate should be informed immediately.

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Phytophthora ramorum – Typical symptoms	
Camellia: Brown-black lesions on the tips and margins of the leaves.	*Camellia*: Shoot infection can occur leading to die back
Hamamelis (witch-hazel): Brown lesions on leaf tips and margins, generally delimited by the veins.	*Kalmia*: Brown-black lesions to the tips and margins of the leaves.
Leucothoe: Brown-black lesions to the tips and margins of the leaves.	*Pieris*: Brown stem lesions leading to aerial dieback.

<table>
<tr><th colspan="2">Phytophthora ramorum – Typical symptoms</th></tr>
<tr><td>
Rhododendron: Blackening of the petiole, leaf base or leaf tip extending along the mid-rib.</td><td>
Rhododendron: Affected shoots and twigs exhibit a brown-black discolouration which can spread into the leaves via the petioles.</td></tr>
<tr><td>
Syringa (lilac): Brown-black lesions to the tips and margins of the leaves.</td><td>
Taxus (yew): Necrosis of the young foliage leading to dieback of shoots.</td></tr>
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Viburnum: Commonly lesions are produced at the stem-base.	*Viburnum*: Leaf infections do occur also.

PRECAUTIONS TO BE TAKEN ON NURSERIES

There are probably two main causes of introduction of the disease onto nurseries - bringing in infected planting material and via contaminated soil or growing media. It is, therefore, important to keep informed of the susceptible host species, as they become known, and to maintain good hygiene practices.

Containerised stock and planting material

Whilst the PHSI is checking material entering the country, the following guidance will assist in minimising spread should the disease be found. It is also good precautionary practice to follow these guidelines to prevent the introduction of other diseases onto the nursery.

- Only buy plants which have been correctly passported.
- Consider requiring that plants have not been treated with a fungicide for at least six weeks prior to delivery, as infection could be masked.

Personnel

Personnel are the key to good disease management.

- Ensure all personnel working on the nursery are familiar with the disease and the reasons why precautionary measures are required.
- Ensure all staff are familiar with hygiene precautions, e.g. requirement for a separate set of tools for the quarantine area, disinfection of boots and leggings, maintaining disinfection baths.
- Ask all personnel to report unusual symptoms. Mark suspect plants for future reference and subsequent visit by PHSI.
- Maintain records of all imported stock so that the origin can be traced.

Quarantine

In risk situations:

- Establish a quarantine area for susceptible containerised stock.
- Likewise selected an area of land that can be isolated for bare rooted susceptible plants.
- Hold imported stock for at least two weeks, inspecting regularly for disease symptoms.
- Limit access to quarantine area via one entrance.
- Provide a loading/unloading area with direct access to the quarantine area that can be readily cleansed and disinfected.
- Restrict access to as few staff as possible until satisfied that plants do not show symptoms of the disease.
- Place footbaths of disinfectant at the entrance and make sure they are maintained. Provide brushes for removing any soil.
- Provide disposable leggings so that overalls do not become contaminated.
- Maintain a separate set of tools within the quarantined area.
- Large equipment should be cleansed and disinfected off-site.
- While it may not be possible to have a team dedicated to the quarantine area it would be best that staff visiting the area do not move directly to work on susceptible hosts in other parts of the nursery.
- Overhead sprinkler irrigation should be avoided. Drip irrigate if possible. If overhead irrigation must be used then apply the water at a time when the foliage will dry quickly.
- Minimise applied water.
- Ensure that plants do not stand in water by maintaining good drainage.
- Ensure that excess applied water, or rainfall, is contained within the quarantine area.
- Do not allow visitors to the quarantine area.
- Remove and destroy plant debris by bagging on-site and bury or burn waste safely (taking account of any environmental legislation in operation).

Disinfectants

The following disinfectants have been tested against the pathogen in a variety of situations and found to be effective.

Product	Manufacturer	Use
Antec Farm Fluid S®	Antec International Ltd	Disinfection of footwear and equipment
Panacide-M®	Coalite Chemicals	Disinfection of gravel and standing areas
Jeyes Fluid®	Jeyes Limited	Disinfection of gravel and standing areas

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Summary

- Ramorum dieback is a notifiable disease and plants suspected of having the disease should be reported to the PHSI.
- Maintain vigorous on-site hygiene.
- Establish a quarantined area of the nursery.
- Inspect plants regularly.
- Place all imported plants under quarantine for at least two weeks.
- Cleanse and disinfect when leaving the quarantined area.
- Restrict access to the quarantined area.

For further information on the disease see HDC Factsheet 19/03 – Sudden Oak Death/Ramorum die back – implications for the HNS industry and the Defra web-site at:

http://www.defra.gov.uk/planth/oak.htm.

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