Grower summary

HNS 123a

Chemical control of *Phytophthora ramorum* causing foliar disease in outdoor hardy nursery stock

Final report 2006

Project title:	Chemical control of *Phytophthora ramorum* causing foliar disease in outdoor hardy nursery stock
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Report:	Final Report (December 2006)
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Date commenced:	1 April 2005
Completion date:	30 September 2006
Key words:	*Phytophthora ramorum,* fungicides, control, hardy nursery stocks, Rhododendron, Camellia, Viburnum, Ranman (cyazofamid), Signum (boscalid/pyraclostrobin), Elvaron Multi (tolylfluanid), Electis 75WG (mancozeb/zoxamide), Consento (fenamidone/propamocarb hydrochloride), Epok (fluazinam/metalaxyl-M), Fubol Gold (mancozeb/metalaxyl-M), Folio Gold (chlorothalonil/metalaxyl-M), Cerf303 (mancozeb/ KIF230), SL 567A (metalaxyl-M), Citrox (fruit acids), DP98 (phosphonate), Aliette (fosetyl-aluminium), resistance, adjuvants, Activator 90 (alkylphenylhydroxyl-polyoxyethylene/natural fatty acids), Bond (synthetic latex/ alkylphenylhydroxyl-polyoxyethylene), Banka (alkyl pyrrolidones), soil drench, HNS 123a

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Headline

A number of fungicides have been identified with protectant and eradicant properties against *Phytophthora ramorum* and potential resistance management strategies have been developed.

Background and expected deliverables

Phytophthora ramorum, the cause of sudden oak death in the USA (Werres *et al*. 2001), 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. The pathogen attacks the foliage and twigs of some hosts but only the bark of others. The disease was first found on US nurseries in 2003 and national surveys carried out by USDA have detected 48 outbreaks between January and July 2006. In England and Wales, there have been a total of over 582 outbreaks of *P. ramorum* confirmed to date (July 2006), of which approximately 434 have been in nurseries and 148 in managed gardens, woods or other wild planting areas (Source: Defra website - www.defra.gov.uk). *P. ramorum* is present elsewhere in Europe, including The Netherlands, Germany, Belgium, Denmark, Sweden, Spain and Poland.

Phytophthora ramorum is a notifiable disease in the UK. It causes a range of symptoms including lesions and dieback on the leaves, shoots and stems of a range of hardy ornamental plants. The principal host species affected are *Viburnum, Rhododendron, Pieris, Camellia,* and *Kalmia.* There have also been findings on a pot-grown plant of *Taxus baccata* (yew) as well as on *Syringa*, *Magnolia*, *Parrotia persica* (ironwood), *Hamamelis virginiana* (witch hazel), *Hamamelis mollis* (Chinese witch hazel), *Laurus nobilis* (Bay laurel), *Drimys winteri* (Winters bark), *Umbellularia californica* (Californian bay laurel) and *Leucothoe*. 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 a finding was reported on a Northern Red Oak (*Quercus rubra*) in the Netherlands. In the south-west of England, further infected trees have been identified within previously infected managed gardens, including amongst others: *Fagus sylvatica* (beech), *Aesculus hippocastanum* (horse chestnut), *Castanea sativa* (sweet chestnut), *Quercis cerris* (turkey oak), *Quercus petraea* (sessile oak) and *Quercus ilex* (Holm oak).

Since 2002, emergency measures have been in place in the UK and EC with the specific aim of preventing spread of the diseases both within the nursery industry and the wider environment. Current UK legislation requires destruction of all plants within a 2 metre radius of a diseased plant. All susceptible plants within a 10 metre radius should be withheld for a period of 3 months for further assessment as should any remaining plants from the same consignment as the diseased plants. These measures are currently having a major impact on the UK hardy nursery stock (HNS) industry, resulting in large numbers of plants having to be destroyed. The use of fungicides for controlling the disease on these plants under official hold (or under a 'containment order') is prohibited due to the risk of masking an infection in plants moving in trade. The policy for eradication/containment of this disease is continually under review as more information on the epidemiology and control 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. This is important to limit both the spread of the pathogen and the damage done to the industry in terms of loss of plants and restrictions imposed on trade.

A pilot study project funded by HDC (HNS 123) to investigate the potential for use of fungicides to control *P. ramorum* in HNS found that metalaxyl-M (SL 567A) was the most effective fungicide. However, this fungicide contains a site-specific active ingredient with a single mode of action 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[1] guidelines for control of these types of fungi (e.g. blight in potato), specifically recommend fungicide mixtures (protectant and eradicant and/or contact and systemic) or alternating programmes of products with different modes of action, to mitigate such risk. There is considered to be a significant risk of the rapid development of *P. ramorum* resistance to SL 567A from its repeated use as a stand-alone product and, as such, it has not been recommended. The current project aims to further explore and develop the potential role of chemical control treatments, particularly formulated mixtures, in future strategies for control of foliar blights caused by *P. ramorum* in HONS.

Since December 2003, a new species of *Phytophthora, P. kernoviae* (formerly known as *Phytophthora* taxon *C* or PTC), has been found attacking beech, rhododendron and several other plant species within localised areas in Cornwall and South Wales. It has also now been found on rhododendrons at two nurseries, one in the north west of England and one in Cornwall. One isolate of *P. kernoviae* has been tested for sensitivity to fungicides under evaluation in this project. It is important to evaluate whether control strategies developed for *P. ramorum* can be implemented to control *P. kernoviae* in case this pathogen also becomes a problem on nurseries.

The expected deliverables from this project are:

- An evaluation of a number of additional active ingredients for activity against *P. ramorum.*
- An evaluation of various fungicide formulations and tank mixtures (including those containing metalaxyl-M) for efficacy against *P. ramorum,* using a range of *in vitro* and *in vivo* methods as appropriate.
- An evaluation of the role of adjuvants (wetters, stickers, spreaders etc.) in improving fungicide performance on a range of host species.
- An evaluation of application techniques, including doses and timing of fungicides, for improved control.

[1] Dr Judith Turner is Chairperson of the Fungicide Resistance Action Group in the UK (FRAG-UK) and Dr Martin McPherson is also a member of this national group. FRAC is the Fungicide Resistance Action Committee, an international consortium of scientists which track, report on and establish policy guidelines for effective resistance management.

- Provision of a simple robust and durable strategy, which provides effective disease control whilst minimising the risk of resistance developing.

Summary of the project and main conclusions

- A number of fungicides have been shown to be effective when used as a protectant treatment for the control of *Phytophthora ramorum* and *P. kernoviae.* These include SL 567A (metalaxyl-M), Epok (fluazinam/metalaxyl-M), Fubol Gold (mancozeb/metalaxyl-M), Folio Gold (chlorothalonil/metalaxyl_M) and Consento (fenamidone/propamocarb hydrochloride).
- Products containing metalaxyl-M also showed eradicant activity.
- None of the fungicides used caused phytotoxicity symptoms.
- As resistance to metalaxyl-M has already been detected in UK isolates of *P. ramorum,* fungicides need to be used within robust treatment programmes which minimise the risk of resistance development. Evidence in this project confirms that plant infections caused by these resistant isolates are less easily controlled by metalaxyl-M.
- Consento could be a key product within spray programmes for both management of the disease and minimisation of resistance development.
- Effective fungicides were lethal to the fungus and not fungistatic (i.e. they killed the fungus rather than merely halting it's development).
- There was no evidence from this work of fungicides causing latency in plant infections.
- The addition of adjuvants, especially 'stickers', to fungicide treatments can enhance levels of control of *P. ramorum* on certain leaf types, such as camellia.
- An effective soil drench treatment for *P. ramorum* still needs to be identified. SL 567A (metalaxyl-M) was shown to be very effective but cannot be recommended due to the risks of further development of resistance within the pathogen population.

Financial benefits

The financial benefits from this project cannot currently be realised as both *P. ramorum* and *P. kernoviae* remain subject to statutory action by Defra Plant Health

Division (to be reviewed after end December 2007). The use of fungicides for controlling the disease on plants under official hold is prohibited due to the risk of masking an infection in plants moving in trade. The policy for eradication/containment of this disease is continually under review as more information on the epidemiology and control of the pathogens becomes available. If UK and EU policy does change in the future to permit use of fungicides for control of *P. ramorum/P. kernoviae*, then the results from this project will have immediate benefits by assisting the industry to rapidly implement chemical control strategies, which are robust both in terms of efficacy and anti-resistance management.

Action points for growers

- Growers should continue to follow good hygiene measures on their nursery and report any suspected symptoms of *P. ramorum/P. kernoviae* to the local Defra Plant Health Seeds Inspector.

Science Section - HNS 123a

Chemical control of *Phytophthora ramorum* causing foliar disease in outdoor hardy nursery stock

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. The disease was first found on US nurseries in 2003 and national surveys carried out by USDA have detected 48 outbreaks between January and July 2006. In England and Wales, there has been a total over 582 confirmed outbreaks of *P. ramorum* to date (July 2006), of which approximately 434 have been in nurseries and 148 in managed gardens, woods or other wild planting areas (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, including The Netherlands, Germany, Belgium, Denmark, Sweden, Spain, Poland and the UK.

Phytophthora ramorum is a notifiable disease in the UK which causes a range of symptoms including lesions and dieback on the leaves, shoots and stems of a range of hardy ornamental plants. The principal host species affected are *Viburnum, Rhododendron, Pieris, Camellia,* and *Kalmia.* There have also been findings on a pot-grown plant of *Taxus baccata* (yew), *Syringa*, *Magnolia*, *Parrotia persica* (ironwood), *Hamamelis virginiana* (witch hazel), *Hamamelis mollis* (Chinese witch hazel), *Laurus nobilis* (Bay laurel), *Drimys winteri* (Winters bark), *Umbellularia californica* (Californian bay laurel) and *Leucothoe*. 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, further infected trees have

been identified, again within previously infected managed gardens, including amongst others: *Fagus sylvatica* (beech), *Aesculus hippocastanum* (horse chestnut), *Castanea sativa* (sweet chestnut), *Quercis cerris* (turkey oak), *Quercus petraea* (sessile oak) and *Quercus ilex* (Holm oak).

Emergency UK and EC measures have been in place since 2002 with the specific aim to prevent spread of the diseases both within the nursery industry and the wider environment. Current UK legislation requires destruction of all plants within a 2 metre radius of a diseased plant and holding all susceptible plants within a 10 metre 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 UK HONS industry, resulting in large numbers of plants having to be destroyed. The use of fungicides for controlling the disease on these plants under official hold is prohibited due to the risk of masking an infection in plants moving in trade. The policy for eradication/containment of this disease is continually under review as more information on the epidemiology and control 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. This is important to limit both the spread of the pathogen and the damage done to the industry in terms of loss of plants and restrictions imposed on trade.

A pilot study project funded by HDC (HNS 123) to investigate the potential for use of fungicides to control *P. ramorum* in nursery stock found that metalaxyl-M (SL 567A) was the most effective fungicide. 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 these types of fungi e.g. blight in potato specifically recommend fungicide mixtures (protectant and eradicant and/or contact and 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 SL 567A as a stand-alone product and as such it has not been recommended. Fungicides used in this project and details of mode of action and resistance risk are shown in Table 1.

The current project aims to further explore and develop the potential role of chemical control treatments, particularly formulated mixtures, in future strategies for control of foliar blights caused by *P. ramorum* in HONS. Since December 2003, a new *Phytophthora, P. kernoviae* (formerly known as *Phytophthora* taxon *C* (PTC)), has been found attacking beech, rhododendron and several other plant species within localised areas in Cornwall and South Wales. It has also now been found on rhododendrons at two nurseries, one in the NW of England and one in Cornwall. One isolate of *P. kernoviae* was tested for sensitivity to fungicides under evaluation in this project. It is important to evaluate whether control strategies developed for *P. ramorum* can be implemented to control *P. kernoviae* in case this pathogen also becomes a problem on nurseries.

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)	Mode of action and mobility	Resistance risk
Metalaxyl-M	SL 567A in Epok, Fubol Gold, Folio Gold	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.
Cyazofamid	Ranman	Inhibits fungal respiration and energy production at Qi site. Limited systemicity.	No resistance detected.
Boscalid	in Signum	Cytochrome respiration	No resistance detected to date.
Tolylfluanid	Elvaron Multi	Multi-site contact activity	No resistance detected to date.
Zoxamide	Electis 75 WG	ß-tubulin assembly in mitosis. Protectant, non-systemic.	No resistance detected to date.
Fenamidone	in Consento	Inhibits fungal respiration at Qo site. Locally systemic. Protectant and anti-germination activity.	Resistance known in various fungal species. No resistance detected in *P. infestans*
Fluazinam	in Epok	Multi-site inhibitor. Stops cellular energy production. Protectant, non-systemic.	Multi-site inhibitor. No resistance detected.
Mancozeb	in Fubol Gold in Electis 75 WG in Cerf303	Multisite. Protectant, non-systemic	Multi site inhibitor. No resistance detected.
Chlorothalonil	In Folio Gold	Cellular respiration	No resistance detected.
Propamocarb hydrochloride	in Consento	Cell membrane permeability. Systemic	Multi site inhibitor. No resistance detected.
Pyraclostrobin	in Signum	Cytochrome respiration. Locally systemic.	Resistance known in various fungal species. No resistance detected in *P. infestans*.
KIF230	Cerf303	Information not available	Unknown
Fruit acids	Citrox	Enhancement of plant defences	No resistance detected.
Phosphonate	DP98	Enhancement of plant defences	No resistance detected.
Fosetyl-aluminium	Aliette	Highly systemic. Direct fungitoxic effect, enhancement of plant defences	Multi site inhibitor. No resistance detected.

Materials and Methods

Isolates used and inoculum production

A range of isolates of *P. ramorum* and a single isolate of *P. kernoviae* were used during the studies (Table 2).

Table 2. Phytophthora isolates used during the study

Isolate Code	Organism	Host species	Use	Country of origin
cc47	*P. ramorum*	Rhododendron	Fungicide testing, adjuvant testing, soil drench, application timing	UK
P1577	*P. ramorum*	Rhododendron	Fungicide testing	Germany
P1376	*P. ramorum*	Rhododendron	Fungicide testing	EU
cc70	*P. ramorum*	Rhododendron	Resistance testing	UK
1650	*P. ramorum*	Rhododendron	Resistance testing	Germany
1659	*P. ramorum*	Rhododendron	Resistance testing	Germany
1653	*P. ramorum*	Rhododendron	Resistance testing	UK
1658	*P. ramorum*	Rhododendron	Resistance testing	Germany
1664	*P. ramorum*	Viburnum	Resistance testing	Germany
cc92	*P. kernoviae*	Rhododendron	Fungicide testing	UK

Fungicides

A total of ten fungicides were assessed for efficacy against isolates of *P. ramorum* and *P. kernoviae* (Table 2) using a range of tests. These fungicides are detailed in Table 3.

1. Efficacy of fungicides in vitro

The effect of fungicides on mycelial extension and sporangial germination of three isolates of *P. ramorum* and one of *P. kernoviae* was examined *in vitro*. Agar plate assays were used to test the effect of fungicides on mycelial growth and an optical

densitometry (photometric) technique was used to determine effects on zoospore/sporangial germination.

1.1. Agar plate assays

Screening was carried out on all ten chemicals at concentrations of 0, 0.1, 1, 10, and 100 ppm. A 10% V-8 agar base medium (Appendix I) was amended with fungicide to give the 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 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 of the untreated controls.

Table 3. Fungicides: active ingredients and recommended application rates

Fungicide	Active ingredient	In field application rate (L ha^{-1} or kg ha^{-1})
Ranman	cyazofamid (400g l^{-1})	0.2 (A) +0.15 (B)
Signum	boscalid (267g kg^{-1}) + pyraclostrobin (67g kg^{-1})	0.75
Elvaron Multi	tolylfluanid (505g kg^{-1})	3.4
Electis 75WG	mancozeb (667g kg^{-1}) + zoxamide (83 g kg^{-1})	1.8
Consento	fenamidone (75g l^{-1}) + propamocarb hcl (375g l^{-1})	2
Epok	fluazinam (400g l^{-1}) + metalaxyl-M (200g l^{-1})	0.375
Fubol Gold	mancozeb (640g kg^{-1}) + metalaxyl-M (40 g kg^{-1})	1.9
Folio Gold	chlorothalonil (500g l^{-1}) + metalaxyl-M (37.5g l^{-1})	2
SL 567A	metalaxyl-M (480 g l^{-1})	1.3
Cerf303	mancozeb (700g kg^{-1}) + KIF230 (17.5g kg^{-1})	1.6

1.2. Photometric assays

The effect of fungicides on sporangial germination of three isolates of *P. ramorum* and one of *P. kernoviae* 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 3 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 were produced by inoculating a single plate of 10% V-8 agar (Appendix I) and incubating the plates 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. In order to produce large numbers of synchronous sporangia, fresh 10% V-8 agar plates were then inoculated with 100 µL of the resulting sporangial suspension and incubated under the same temperature and light regime, as previously described, for 3 days. Sporangia were harvested from each plate in 5 mL of GPM and sporangial suspension bulked for each isolate to be tested. Sporangia were counted and the spore suspension adjusted to 10^4 spores mL^{-1}. 150 µL of the spore suspension was added into each treatment well and 150 µL GPM was pippetted into control wells.

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 fungicides *in vivo* was investigated using either detached leaf or whole plant assays.

2.1. Detached leaf assay

The detached leaf assay was carried out using rhododendron and viburnum leaves. A

total of twelve fungicides were tested [Sonata (a.i. fenamidone), (Shirlan a.i. fluazinam), Amistar (a.i. azoxystrobin) and those shown in Table 3 (with the exception of Signum). All fungicides were applied according to the manufacturer's recommended rate (Table 3, plus Sonata at 1.5 kg ha^{-1}, Shirlan at 0.3 L ha^{-1} and Amistar at 1 L ha^{-1}).

Four application timings were tested on detached leaves of rhododendron and viburnum (fungicide applied 7 or 4 days before inoculation or 4 or 7 days after inoculation). For each fungicide treatment and timing, three rhododendron and three viburnum leaves of a similar age were sprayed. Sprays were applied to the abaxial leaf surface (underside) in a water volume equivalent to 200 L ha^{-1} using a battery powered ('lunch-box') sprayer (model LBP18; S/N3081) set at 2 bar, with a 1 m boom and three twin fan nozzles (Tee Jet XR 1100VK Yellow). Control leaves were sprayed with an equivalent volume of water.

A zoospore suspension comprising of spores from three *P. ramorum* isolates (Table 2) was used to inoculate the detached leaves. To produce the zoospore inoculum, plates of sporangia (see section 1.2) were flooded with 10 mL SDW, chilled at -20°C for 5 min and then returned to 20°C for 1 h. The zoospores suspension was filtered through a Whatman No. 113V filter (retention size >30 μm) to remove any sporangia/empty sporangial cases. Zoospore counts were carried out using a haemocytometer. Leaves were inoculated with 50 μl of a $1x10^6$ zoospore suspension applied to the abaxial leaf surface. The inoculum was applied equidistant between the leaf edge and the leaf midrib.

All control and treated leaves were incubated in a moist chamber at 20˚C. The length and breadth of leaf lesions were measured after 14 days' incubation and the % control calculated for each treatment and timing.

To test the viability of *P. ramorum* following fungicide treatment, leaf lesions were plated onto P_5ARPH agar (Appendix I). Where no lesions were present the area inoculated was tested. Plates were incubated at 20˚C and assessed for growth of *P. ramorum* after 7 days.

2.2. Whole plant assay

Sixteen rhododendron 'Cunninghams White' plants were placed in the quarantine CE room at least four weeks prior to the start of the experiment to ensure plants were fully acclimatised. Conditions in the CE room were 18˚C, 80 % humidity and a 12 h day/night light regime. By the start of the experiment, fresh shoots and leaves had developed. Three fungicide treatments were tested: DP98, Citrox and Aliette 80 WG (Table 4). These products are reported to work through stimulation of the plant defence system and as such their efficacy could not be tested using the detached leaf assay. All chemicals were applied according to the manufacturers recommended rate. Again, four application timings were tested (product applied 7 or 4 days before inoculation or 4 or 7 days after inoculation). A single rhododendron plant was sprayed for each product treatment and timing. Sprays were applied to the adaxial leaf surface (top side) using a battery powered sprayer (model LBP18; S/N3081) set at 2 bar, with a 1 m boom and three twin fan nozzles (Tee Jet XR 1100VK Yellow). Control plants were sprayed with an equivalent volume of water.

Table 4. Chemicals used for *in planta* testing: active ingredients and recommended application rates

Fungicide	Active ingredient	Application rate
DP98	Nitrogen (4%), Potassium (13%), Phosphate P_2O_5 (28%)	2 L ha^{-1} in 200 L water
Citrox	Natural biocide made from fruit acids	0.3 L ha^{-1} in 400L water
Aliette 80 WG	Fosetyl-aluminium (80%)	1 kg ha^{-1} in 200L water

A zoospore suspension comprising of spores from three *P. ramorum* isolates (Table 2) was used to inoculate plants. Zoospore suspensions were produced as described in section 2.1. Zoospore counts were carried out and the concentration adjusted to give a spore count of 10^6 spores mL^{-1}. Just prior to inoculation, 10 replicate leaves of equivalent age were selected on each plant and tagged. Each of the tagged leaves was wounded once on the adaxial surface using a dissection needle. Leaves were inoculated, over the wound, with 30 µl of the zoospore suspension within one hour of the spore count (to ensure zoospores were still motile). Following inoculation, plants were bagged overnight to raise humidity and aid leaf infection. The length and

breadth of leaf lesions were measured after 14 days' incubation and the % control calculated for each treatment and timing.

To test the viability of *P. ramorum* following chemical treatment, leaf lesions were plated onto P_5ARPH agar. Plates were incubated at 20˚C and assessed for growth of *P. ramorum* after 7 days.

3. *Fungicide efficacy against isolates resistant to metalaxyl-M*

A number of *P. ramorum* isolates, identified during testing carried out as part of the EU RAPRA[2] project, have shown increased resistance to metalaxyl-M (EC_{50} values ranging between 2.7 and >10 ppm compared to 0.04 ppm for an isolate tested in this project). The isolates sourced from EU partners were tested to identify whether the increased resistance shown in laboratory tests could lead to control problems in the field.

Four fungicides, SL567A, Fubol Gold, Consento and Ranman were tested in a detached leaf assay using three replicate rhododendron leaves per treatment (all leaves tested were of a similar age). The fungicides were applied according to the manufacturer's recommended rate (Table 3) at two preventative application timings (7 or 4 days before inoculation). Sprays were applied (as described in section 2.1) but this time to the adaxial leaf surface (upperside). Control leaves were sprayed with an equivalent volume of water.

Five *P. ramorum* isolates were tested (Table 2). Either four or seven days after fungicide treatment the leaf surface was damaged (cut <5mm) at a point equidistant between the leaf edge and the leaf midrib, a drop of water placed over the damaged area and an inoculum plug (cork borer Nº3), taken from the leading edge of a seven day old colony, placed on top of it. The methodology for inoculation of the leaf surface was different to that described in section 2.1 due to low levels of disease infection on control leaves experienced in a previous run of the experiment. All control and treated leaves were incubated in a moist chamber at 20˚C.

[2] Risk Analysis for *Phytophthora ramorum* (www.rapra.csl.gov.uk)

The length and breadth of lesions were measured after 14 days' incubation and the % control calculated for each treatment and timing.

4. *Effect of adjuvants on fungicide efficacy*

Results from project HNS123 indicated differences in efficacy of fungicides on different hosts. Many of the fungicides tested were originally formulated by the manufacturers for use on potatoes, and differences in leaf texture, plant architecture and leaf age of the different ornamental host species may affect run-off, adsorption and absorption. To try and improve the efficacy of fungicides on different hosts, representative examples of adjuvants were tested (Table 5) to determine whether improved efficacy could be achieved by prolonging the persistence of the application on and through the leaf surface.

Table 5. Adjuvants used during this study: active ingredients field use and recommended application rates

Adjuvant	Active ingredient	Field use	Application rate (% total volume)
Banka	Alkyl pyrrolidones (29.2%)	Spreader, sticker and wetter	0.1
Bond	Alkylphenyl-hydroxypolyoxyethylene (10%)/ synthetic latex (45%)	Extender, sticker and wetter	0.1
Activator 90	Alkylphenyl-hydroxypolyoxyethylene (75%)/ natural fatty acids (15%)	Wetter only	0.1

Tests were carried out on five fungicides: two with good activity against *P. ramorum* (SL567A and Consento) and three with poorer activity (Sonata, Electis and Elvaron Multi). Tests were carried out on detached leaves of rhododendron, camellia and viburnum (leaves tested from each plant type were of a similar age). All fungicides were applied at the manufacturer's recommended rate and adjuvants were mixed with the fungicides at a rate of 0.1%. Two preventative timings were tested, one where the fungicide/adjuvant was applied 7 days before inoculation and the other where the fungicide/adjuvant was applied 4 days before inoculation. Control leaves were sprayed with water only. Leaves were wounded and inoculated as described in section 3 (agar plugs) using isolate cc47 (Table 2). All control and treated leaves were incubated in a moist chamber at 20˚C.

The length and breadth of lesions were measured after 14 days' incubation and the % control calculated for each treatment and timing.

5. *Efficacy of soil drenches against* P. ramorum

Of the fungicides listed in Table 3, only SL567A has stated approval as a soil drench (off label approval, 30 April 2007 – Raspberry outdoor), and as a result this was the only drench tested.

Lidded clear plastic 'sandwich' boxes (17x11x8cm) were half filled with compost (John Innes No3) and then autoclaved on 3 consecutive days at 121˚C for 15 minutes. The sterile compost was then inoculated with 15 mL of a 1 x 10^5 sporangial suspension of *P. ramorum* (isolate cc47, produced as described in section 1.2) and incubated for 7 days at 20ºC to allow growth to occur.

SL 567A (at a rate of 0.52L/100L water) was applied to the soil in three replicate boxes until the soil was thoroughly wetted (approx 30 mL). Soil was sampled (0.5g) from each box just prior to, and immediately after treatment with SL567A and then every 30 minutes up to four hours after treatment. Control boxes were treated with an equivalent volume of water. Each sample was plated onto P_5ARPH, incubated at 20˚C and assessed for growth of *P. ramorum* after 7 days.

6. *Evaluation of appropriate spray intervals for the control of* P. ramorum

Eighteen plants of each host species (rhododendron 'Cunninghams White', *Viburnum tinus* and *Camellia japonica*) were placed in the quarantine CE room four weeks prior to the start of the experiment to ensure plants were fully acclimatised. Conditions in the CE room were 18˚C, 80 % humidity and a 12 h day/night light regime.

To test the most appropriate spray intervals for the control of *P. ramorum*, SL 567A was applied as a foliar spray at intervals of either 3, 7 or 14 days. Fungicide was applied at 1.3 L ha^{-1} in a water volume equivalent to 200 L ha^{-1}, with the first fungicide spray applied 24 h before the first inoculation. The study lasted two weeks so plants were sprayed on either 1, 2 or 5 occasions, depending on the time interval between treatments. Control plants were treated with an equivalent volume of water.

P. ramorum isolate cc47 was used to inoculate plants. Zoospores were produced as outlined in section 2.1. Just prior to inoculation, three leaves of equivalent age were selected per plant and each wounded twice with a dissection needle. Plants were inoculated with approximately 40 mL of a zoospore suspension containing $1x10^6$ zoospores mL^{-1} within one hour of the spore count (to ensure zoospores were still motile). Following inoculation, plants were bagged overnight to raise humidity and aid leaf infection. The inoculation process was repeated every other day throughout the course of the experiment.

A record was kept of symptom development on the control plants and a full assessment carried out on all plants 21 days after the first 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.

Results and Discussion

1. *Efficacy of fungicides* in vitro

1.1. Agar plate assays

The agar plate test was used to determine the effect of selected fungicides on the mycelial extension of three *P. ramorum* isolates and a single isolate of *P. kernoviae*. No significant difference in sensitivity was observed between any of the *P. ramorum* isolates tested or *P. kernoviae* (Table 5). SL 567A, and those products containing metalaxyl-M in the formulation (Epok, Fubol Gold and Folio Gold), were the most effective products. Ranman, Cerf303 and Electis 75 were also moderately effective in this test.

Table 5. EC_{50} values for selected fungicides against isolates of *P. ramorum* and *P. kernoviae* in agar plate test.

Fungicide	Active ingredient	*P. ramorum*			*P. kernoviae*
		p1376	p1577	cc47	cc92
Ranman	Cyazofamid	0.33	0.1	0.78	0.4
Signum	Boscalid	>100	>100	>100	38.5
Elvaron Multi	Tolylfluanid	5.5	5.4	5.6	5.4
Electis 75 WG	Mancozeb/zoxamide	2.4	2	2.9	4
Consento	Fenamidone/propamocarb-HCl	33	32.5	8	13.5
Epok	Fluazinam/metalaxyl-M	0.05	0.05	0.05	0.05
Fubol Gold	Mancozeb/metalaxyl-M	0.06	0.06	0.06	0.06
Folio Gold	Chlorothalonil/metalaxyl-M	0.09	0.1	0.09	0.13
Cerf303	Mancozeb/KIF230	0.56	0.59	0.6	0.96
SL 567A	Metalaxyl-M	0.04	0.04	0.04	0.04

Testing of a larger population of *P. ramorum* isolates (as part of the EU RAPRA project) has revealed resistance in a number of isolates to metalaxyl-M (EC_{50} values ranging between 2.7 and >10 ppm compared to 0.04 ppm for cc47 in Table 5). The majority of these isolates originated in Germany, however one was isolated from a nursery in the UK.

1.2. Photometric assays

The photometric test was used to determine the efficacy of fungicides against spore germination of *P. ramorum* and *P. kernoviae*. There were more differences between the isolates in sensitivity to fungicides in this test. The most effective chemicals were SL 567A, products containing metalaxyl-M in the formulation (Epok, Fubol Gold and Folio Gold) and Ranman (Table 6).

Table 6. EC_{50} values for selected fungicides against isolates of *P. ramorum* and *P. kernoviae* in the photometric test.

Fungicide Product	Active ingredient	*P. ramorum*			*P. kernoviae*
		P1376	p1577	cc47	cc92
Ranman	Cyazofamid	0.04	0.004	0.05	0.07
Signum	Boscalid	>100	>100	6	5.5
Elvaron Multi	Tolylfluanid	2.2	1.3	4.1	2.2
Electis 75 WG	Mancozeb/zoxamide	3.1	2.9	4.3	3.5
Consento	Fenamidone/propamocarb-HCl	5.3	8.8	0.73	0.48
Epok	Fluazinam/metalaxyl-M	0.01	0.04	0.05	0.04
Fubol Gold	Mancozeb/metalaxyl-M	0.08	0.08	0.26	0.18
Folio Gold	Chlorothalonil/metalaxyl-M	0.06	0.07	2.6	0.9
Cerf303	Mancozeb/KIF230	0.48	0.46	0.42	0.53
SL 567A	Metalaxyl-M	0.03	0.06	0.04	0.004

2. *Efficacy of fungicides* in planta

2.1. Detached leaf assay

A detached leaf assay was used to test the efficacy of fungicides for the control of *P. ramorum in planta*. Testing was carried out on rhododendron and viburnum plants with chemical application either 7 or 4 days before inoculation (protectant) or 4 or 7 days after inoculation (eradicant).

When fungicides were applied as protectants to either rhododendron or viburnum leaves, at either timing, SL567A, Epok, Fubol Gold, Folio Gold and Consento prevented lesion development (Figures 1 and 2). In addition, Amistar prevented lesion development on viburnum leaves but not on rhododendron.

Lesions developed on control leaves within four days of the inoculum being applied. Where lesions developed following fungicide application, the onset of lesion development was not delayed, indicating none of the fungicide applications caused latency in the infections.

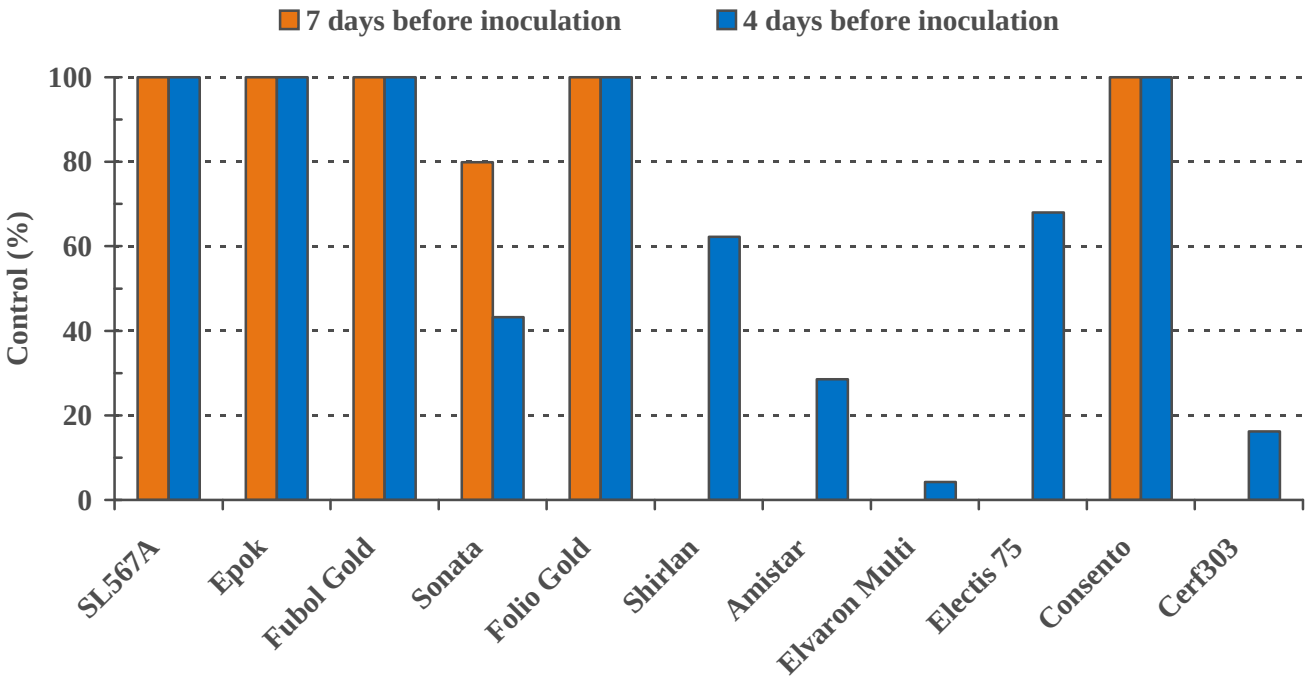

Figure 1. Effect of protectant fungicide applications on *P. ramorum* development (detached rhododendron leaves).

Isolations were carried out to determine whether the fungicides were fungistatic or fungitoxic in activity. *P. ramorum* was not recovered from any leaves where the fungicide had been 100% effective i.e. no lesion had developed, indicating that the chemicals had killed the inoculum and were not fungistatic.

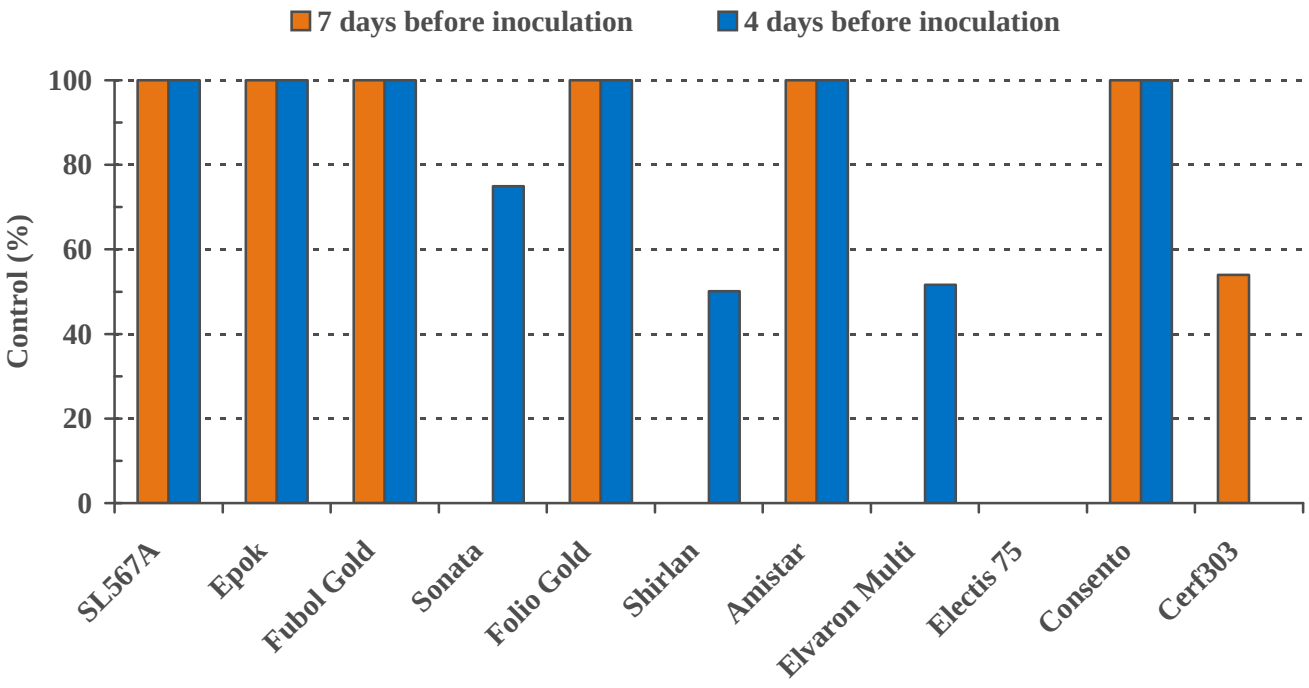

Figure 2. Effect of protectant fungicide applications on *P. ramorum* development (detached viburnum leaves).

When fungicides were applied as eradicants (after the lesion development) to either rhododendron or viburnum leaves, only products containing metalaxyl-M (SL 567A, Epok, Fubol Gold and Folio Gold) halted lesion development (Figures 3 and 4). No *P. ramorum* could be recovered from lesions following treatment with SL 567A, Epok, Fubol Gold or Folio Gold indicating treatments had eradicated the infection. *P. ramorum* was recovered from lesions following all other treatments.

2.2. Whole plant assay

A whole plant assay was used to test the efficacy of Citrox, DP98 and Aliette treatments, which are reported to work through stimulation of the plants own defence mechanisms. Testing was carried out on both rhododendron and viburnum plants with chemicals applied either 7 or 4 days before inoculation or 4 or 7 days after inoculation.

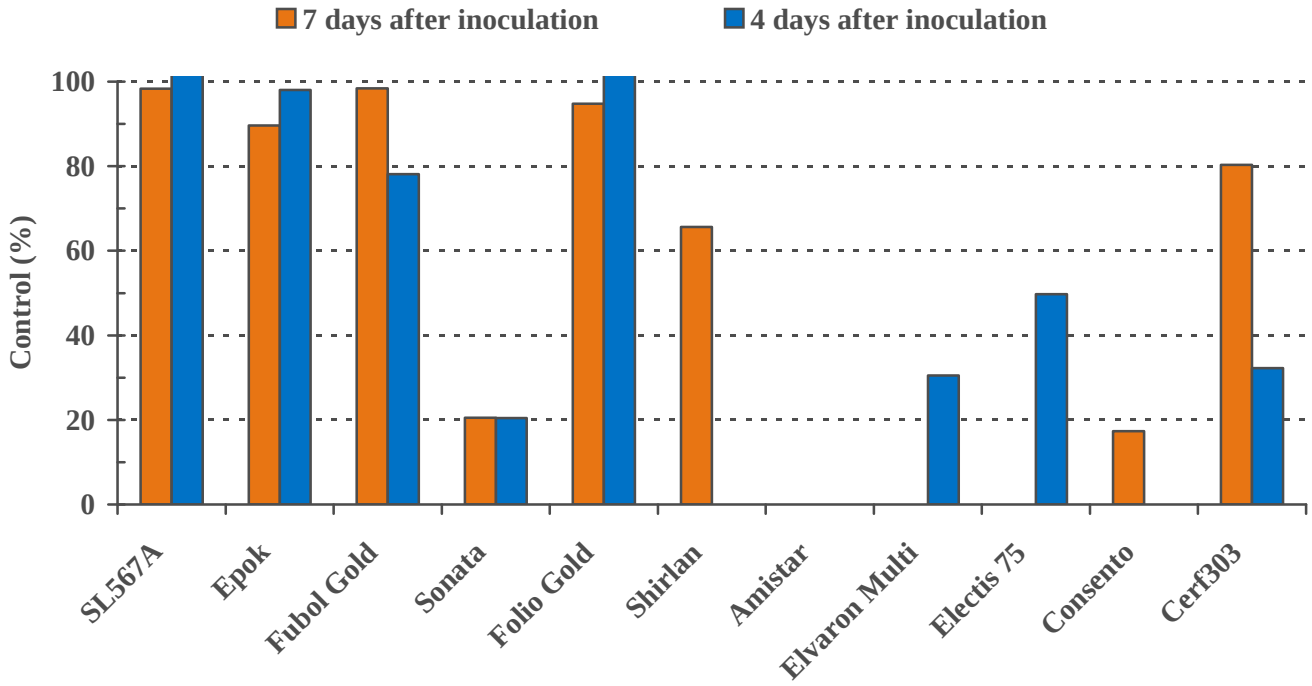

Figure 3. Effect of eradicant fungicide applications on *P. ramorum* development (detached rhododendron leaves).

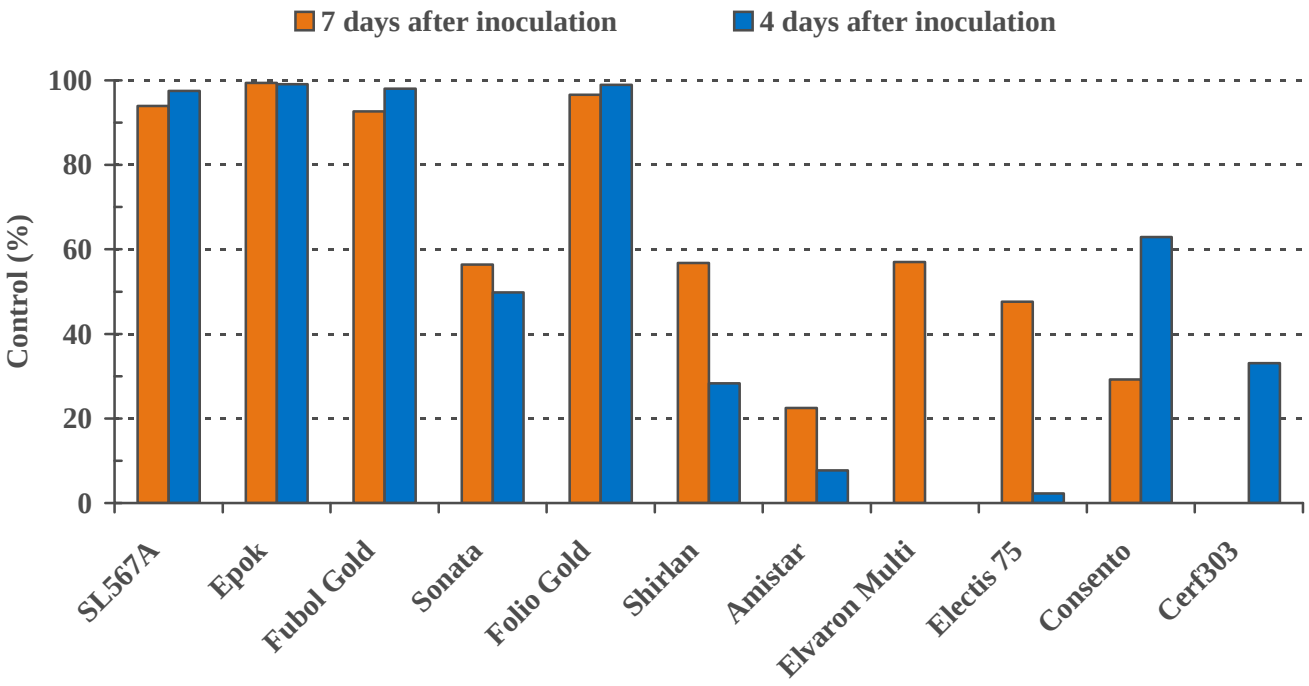

Figure 4. Effect of eradicant fungicide applications on *P. ramorum* development (detached viburnum leaves).

Testing of Citrox, DP98 and Aliette on rhododendron plants showed that none of the treatments, at any of the application timings tested, gave 100% control of *P. ramorum* (Figure 5). All treatments showed limited control of lesion development when applied as protectants seven days before the inoculum. Aliette was the only treatment to show any eradicant activity, and only when applied seven days after the inoculum.

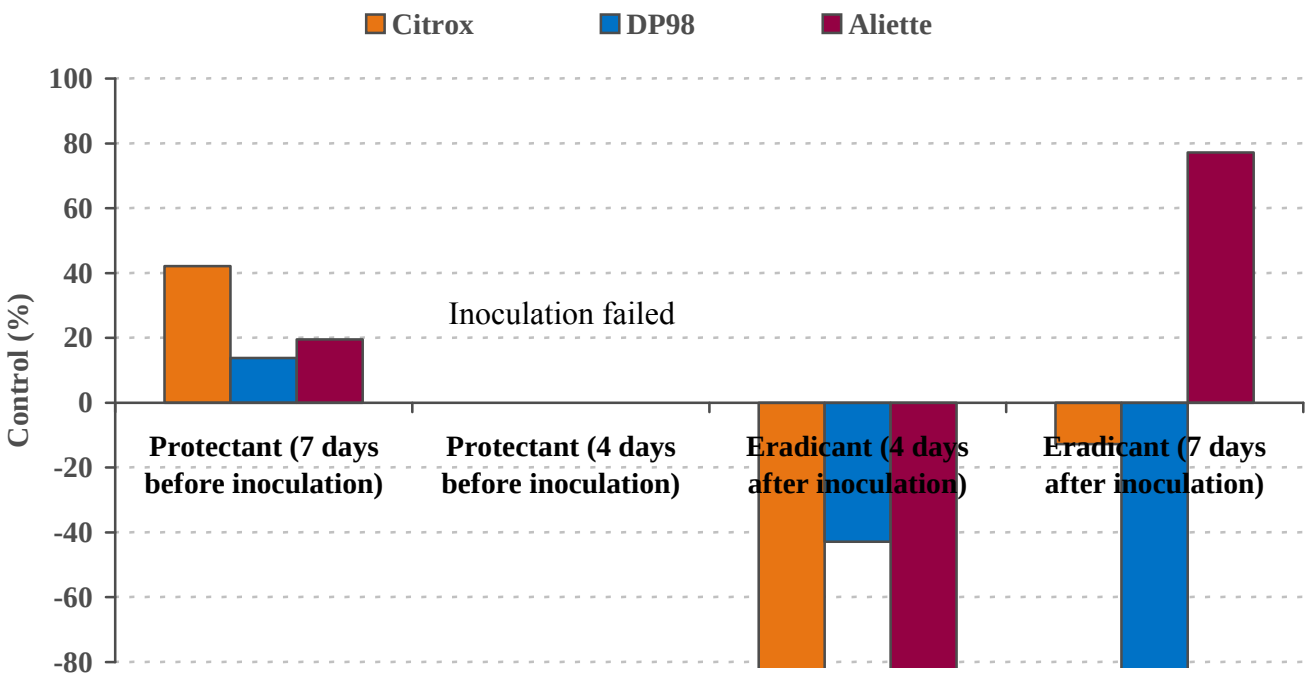

Figure 5. *In planta* (rhododendron) testing of the effectiveness of protectant (before inoculation) and eradicant (after inoculation) applications of Citrox, DP98 and Aliette against *P. ramorum*.

Results were difficult to interpret on viburnum plants as the inoculated leaves dropped off, especially on the control plant, before the end of the experiment.

3. *Fungicide efficacy against isolates resistant to metalaxyl-M*

A detached leaf assay was used to test the efficacy of fungicides for the control of five isolates of *P. ramorum.* These isolates had previously shown differing levels of resistance to metalaxyl-M during *in vitro* testing. Two of the fungicides, SL 567A and Fubol Gold, contained metalaxyl-M at 480 and 40g/L respectively. Testing was carried out on rhododendron leaves and fungicides were applied as a protectant treatment either 7 or 4 days before inoculation. Figure 6 shows the effects of the

treatments on the five isolates which are listed in order of increasing levels of resistance shown in *in vitro* tests (most sensitive first).

Treatment of leaves with SL 567A as a protectant four days prior to inoculation resulted in more than 95% control of all the isolates tested, with 100% control achieved for isolates 1650 and 1653 (Figure 6). Generally, treatment with SL 567A seven days prior to inoculation resulted in reduced control compared to the 4 day treatment, with only one isolate completely controlled by the treatment (1653). The poorest levels of control were for isolate 1664, where only 30% control was achieved. This isolate had the highest EC50 value and was the most resistant isolate identified during the *in vitro* testing.

Fubol Gold applied four days prior to inoculation was completely ineffective against the most resistant isolate (1664) and was less effective than SL 567A against isolates 1658 and 1653 (92.6 and 58.1% respectively). However, when Fubol Gold was applied seven days pre-inoculation the treatment increased disease control compared to the four-day treatment. This is in contrast to the results seen with SL 567A and may be due to the activity of the partner active (mancozeb) in Fubol Gold. Consento achieved greater than 95% control of all isolates but except 1659 at both application timings.

These results indicate that the resistance to metalaxyl-M detected in some isolates during *in vitro* testing could result in reduced efficacy of the product in controlling disease outbreaks on nurseries. However, there is no evidence that robust applications of formulated mixtures containing metalaxyl-M will still be effective. Results also show that Consento (fenamidone/propamocarb hydrochloride) is also highly effective as a protectant treatment against isolates showing resistance to metalaxyl-M. These, and results from previous experiments, suggest that products containing metalaxyl-M (SL 567A, Fubol Gold, Folio Gold and Epok) used in conjunction with Consento could form the basis of an spray strategy for the control of *P. ramorum* which would both control the disease and minimise the risk of further resistance development.

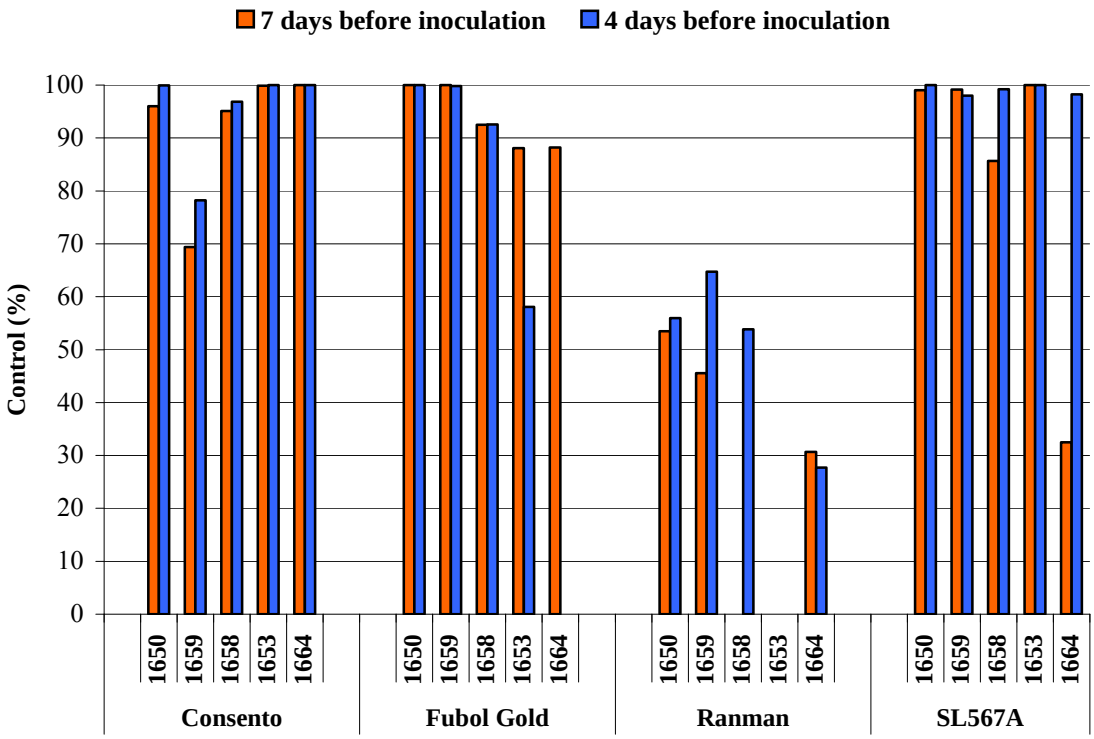

Figure 6. Effect of protectant fungicide applications against *P. ramorum* development in a detached rhododendron leaf assay (including isolates resistant to metalaxyl-M)

4. *Effect of adjuvants on fungicide efficacy*

A detached leaf assay was used to test whether the addition of an adjuvant improved the efficacy of fungicides applied to rhododendron, viburnum and camellia leaves for the control of *P. ramorum*. Three adjuvants were tested (Activator 90, Banka and Bond) in mixture with Consento, Electis, Elvaron Multi, SL 567A or Sonata. Fungicides were applied to the adaxial (upper) leaf surface either 7 or 4 days before inoculation. Inoculations were made to wounded leaves using agar plugs of inoculum and therefore results from use of the fungicide products alone are not directly comparable with earlier work in this report on detached leaves which used zoospore inoculum.

Overall, no beneficial effects on levels of disease control were achieved by the inclusion of adjuvants in any of the fungicides applied to rhododendron or viburnum leaves (Figures 7 and 8 respectively). However, the addition of Banka or Bond to

Elvaron Multi or Sonata gave increased control of *P. ramorum* on camellia leaves particularly when treatments were applied 7 days prior to inoculation (Figure 9). Both Banka and Bond have sticker activity (Table 5), which may aid the fungicide to adhere better to the waxy surface of the camellia leaf.

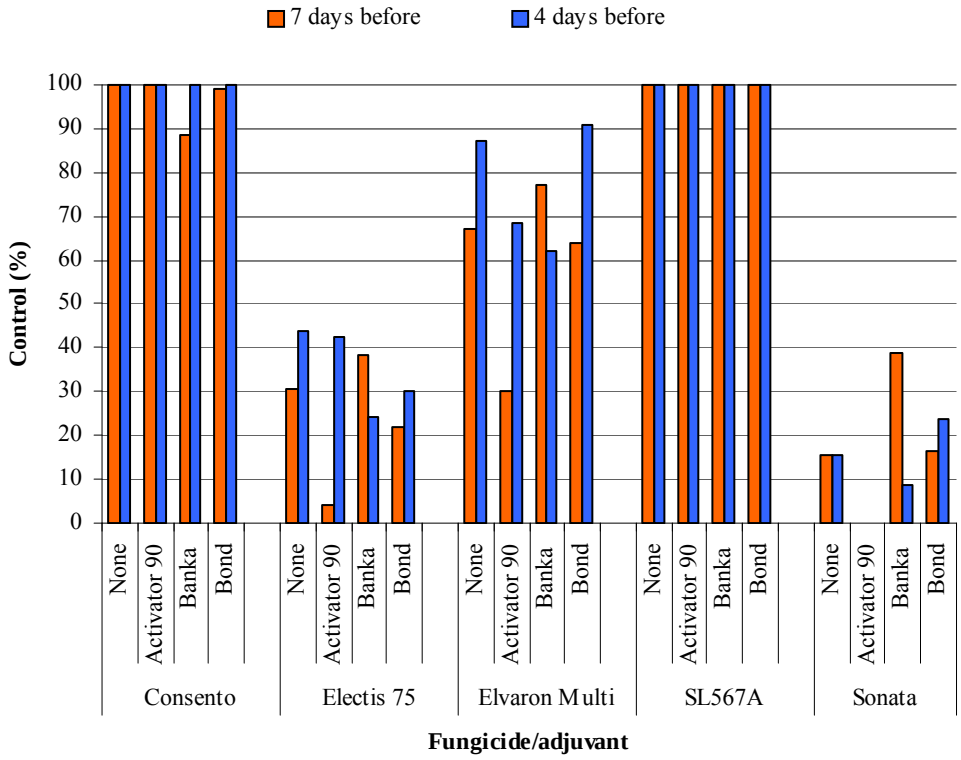

Figure 7. Effect of adjuvants on fungicide efficacy against *P. ramorum* (isolate cc47) in a detached rhododendron leaf assay.

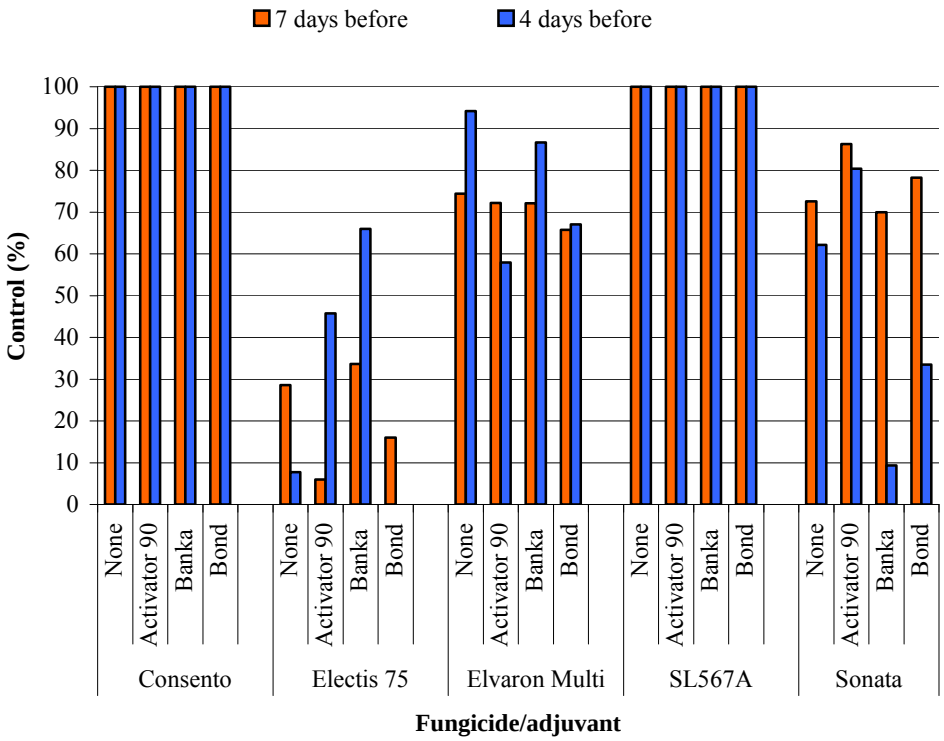

Figure 8. Effect of adjuvants on fungicide efficacy against *P. ramorum* (isolate cc47) in a detached viburnum leaf assay.

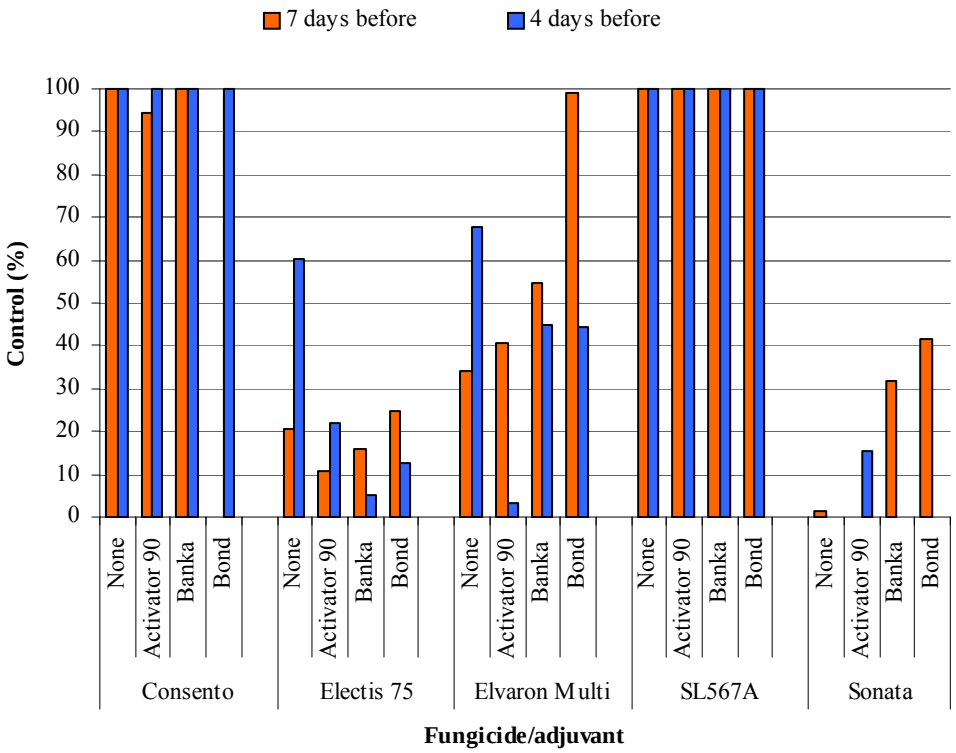

Figure 9. Effect of adjuvants on fungicide efficacy against *P. ramorum* (isolate cc47) in a detached camellia leaf assay.

5. *Efficacy of soil drenches against* P. ramorum

The efficacy of SL 567A as a soil drench for treatment of soil contaminated with *P. ramorum* was tested. *P. ramorum* was isolated from all control and pre-treatment soil samples taken, however it was not isolated from any of the post-treatment samples (including the one taken immediately after treatment). This either indicates that SL 567A is highly effective against *P. ramorum* in soil. However, this would not be recommended for use due to the risks of further resistance development in the pathogen.

6. *Evaluation of appropriate spray intervals for the control of* P. ramorum

Despite numerous repeats of the experiment it was not possible to produce consistent levels of disease on the control plants and as a result it was not possible to complete this element of the work. In the previous *in planta* work, carried out within this and the pilot project, plants brought into the CE rooms had shown active growth prior to the start of the experiment, however no such growth was seen in the latest series of experiments. It is well established that the susceptibility of a leaf to infection by *P. ramorum* declines as the leaf ages. There is also increasing evidence from monitoring of natural outbreaks that host plants show considerable seasonality in susceptibility to the disease. These experiments were conducted in late October and November and it may be that the plants were not susceptible to the disease as this time. It is recommended that in future this type of experiment be carried out in the spring when plants have younger leaves and are known to be more susceptible to infection.

Conclusions

- *In vitro* testing showed that metalaxyl-M (SL 567A) and co-formulations containing metalaxyl-M (Epok, Fubol Gold, Folio Gold) were the most effective against mycelium and spores of both *P. ramorum* and *P. kernoviae*.
- Application of SL 567A, Epok, Fubol Gold, Folio Gold and Consento (Fenamidone/propamocarb hydrochloride) as protectant treatments prevented lesion development on detached rhododendron leaves. In addition, Amistar prevented lesion development on viburnum leaves.

- Only products containing metalaxyl-M (SL 567A, Epok, Fubol Gold and Folio Gold) were effective as eradicant treatments.
- Where lesions developed following fungicide application the onset of lesion development was not delayed, indicating none of the fungicide applications caused latency of infection.
- *P. ramorum* could not be recovered from leaves where the fungicide had been 100% effective i.e. no lesion had developed (protectant applications) or there was no further lesion development (eradicant applications), indicating that the chemicals had killed the inoculum and were not, therefore, merely fungistatic.
- *P. ramorum* could be isolated where products had not been 100% effective.
- In general, the addition of the adjuvants Activator 90, Banka or Bond to the fungicides tested had no beneficial effect on control of *P. ramorum* on rhododendron or viburnum leaves. However, the addition of Banka or Bond to Elvaron Multi or Sonata increased the control achieved on camellia leaves particularly when applied 7 days prior to the inoculum.
- Some *P. ramorum* isolates showing *in vitro* resistance to metalaxyl-M could not be fully controlled using a field rate application of SL567A in a detached leaf assay, particularly when application timings were not optimal for control. Decreased efficacy was also evident in tests using formulated mixtures containing metalaxyl-M, where the metalaxyl-M component of the mixture is included at a much reduced rate.
- Consento was effective against isolates resistant to metalaxyl-M and could be useful as part of an anti-resistance strategy.
- Consento, and possibly Ranman, could be used in alternating programmes along with products containing metalaxyl-M (SL 567A, Epok, Fubol Gold and Folio Gold) for the control of *P. ramorum*.
- SL 567A was shown to be a very effective soil drench treatment against *P. ramorum*. However, further work on alternative products is recommended as this treatment cannot be recommended alone due to the risks of further resistance development in the pathogen as a result of its use by this means.

Future work

Further work is recommended to

- establish appropriate spray intervals between fungicide applications to prevent development of *P. ramorum* infection within periods of high risk of infection. This work will need to be carried out during the spring to prevent the problems encountered during this project.
- determine whether the resistance to metalaxyl-M carries a fitness penalty as occurs in *P. infestans* (potato blight). If so, then use of metalaxyl-M in integrated programmes may be feasible if managed carefully.
- examine the effect of adjuvants *in planta* using a wider range of products, e.g. Amistar which showed protectant activity on viburnum but not on rhododendron leaves.
- test further products as soil drenches. In order to fully control the disease on plants in trade, effective alternative drench treatments to eradicate the pathogen from soil will be an essential part of the management strategy. It is, of course, important to remember that metalaxyl-M is likely to be used by some growers for the control of root-infecting *Phytophthora* spp. e.g. *P. cinnamomi*, as per standard commercial practice. This could have an impact on the development of fungicide resistant strains of *P. ramorum/P. kernoviae* in the absence of alternative fungicides.
- Demonstrate effectiveness of control treatments in a nursery situation (subject to permission from Defra)

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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.

P_5ARPH 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.