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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: PHE/2122A
2. Project title: Investigation of Alternative Eradication Control Methods for P. ramorum and P. kernoviae on/in plants.
3. Contractor organisation(s): Central Science Laboratory, Sand Hutton, York, YO41 1LZ
4. Total Defra project costs (agreed fixed price): £ 10,000
5. Project: start date .... 15 May 2005

   end date .... 31 March 2006

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Wet and dry heat treatments were investigated for the eradication of sudden oak death pathogens *Phytophthora ramorum* and *Phytophthora kernoviae* on plant material from three common hosts. Initial experiments determined the lethal threshold temperature and exposure times for three isolates of each *Phytophthora* species using wet (hot water) and dry-heat treatments against mycelium and sporangia.

Mycelium: Mycelia of both *P. ramorum* and *P. kernoviae* were very resilient to dry heat treatment, with isolates from both fungal species surviving a 15, but not a 30 minute, treatment at 60°C. Extending the treatment time to 60 minutes for *P. ramorum* and 120 minutes for *P. kernoviae* reduced the lethal temperature to 50 and 42.5°C respectively. Wet heat treatments were effective in killing mycelia at 40°C (after 15 minutes) or 37.5°C (after 90 minutes).

Sporangia: A dry-heat temperature of 55°C was required to kill sporangia of both *Phytophthora* species after 15 minutes. Dry-heat temperatures could be reduced by extending the exposure time, 30 min at 50°C to kill sporangia of *P. kernoviae*, and 60 min at 52.5°C for *P. ramorum*. Wet-heat treatment was more effective, killing sporangia of both *Phytophthora* species at 42.5°C after only 10 minutes. Longer treatments reduced the effective wet-kill temperature to 40°C following treatment times of 60 and 30 minutes respectively for *P. ramorum* and *P. kernoviae*, and 37.5°C for 90 minutes for *P. kernoviae*.

Overall, wet heat treatments were more effective than dry heat treatments and *P. kernoviae* was consistently more temperature sensitive than *P. ramorum*. Mycelia of both *Phytophthora* species were more sensitive to wet heat treatment than sporangia, whereas sporangia were more sensitive to dry heat treatments than the mycelium

Whole *Camellia*, *Rhododendron* and *Viburnum* plants were subjected to wet heat treatments ranging from 45-60°C. Viburnum was found to be least heat tolerant, with a 45°C water treatment

causing severe damage. Camellia and rhododendrons were more heat tolerant showing little or no damage after a 20 minute treatment in hot water at 45°C. Plants were killed completely after a 20 minutes dry heat treatment at 55°C. Experimental work on the pathogens (above) had previously shown that a 30 minute dry heat treatment at 60°C was required to achieve complete kill for both *Phytophthora* species, therefore dry heat was eliminated as an effective eradication method.

The efficacy of a 20 minute wet heat treatment at 45°C was tested using detached leaves pre-inoculated with a sporangial suspension of *P. ramorum* or *P. kernoviae*. The treatment was 100% effective at eradicating both *P. kernoviae* and *P. ramorum*. However, it should be noted that this treatment is not suitable for viburnum plants, which appear too sensitive to high temperatures.

A hot water treatment at 45°C for a period of 20 minutes has therefore been shown to be highly effective in treating leaf material, which had been exposed to either *P. ramorum* or *P. kernoviae* within the previous 24 hours. Further work is needed to identify whether treatments would be effective after a longer period between pathogen exposure and treatment, and to confirm effectiveness on whole plants. The treatments could be used for management of pre-infection decontamination of plants which have been exposed to inoculum of the pathogens. The process of depotting was time consuming and therefore would be expensive in a nursery situation. However, the treatment could have applications for the protection of high value plants which have been in the vicinity of an outbreak.

Report Authors: Judith Turner, Phillip Jennings, Giles Budge, CSL

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
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   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Investigation of Alternative Eradication Control Methods for P. ramorum and P. kernoviae on/in plants.

The scientific objectives for the project (listed below) were all met in full.

1. Determination of cardinal and lethal threshold temperatures for growth of *P. ramorum* and *P. kernoviae* propagules

2. Determination of effect of pathogen lethal threshold temperatures on the growth of host plants e.g. *Rhododendron*, *Viburnum* and *Camellia*.

3. Examination of dry heat treatments for sanitation and prevention of infections

4. Examination of hot water treatments for sanitation and prevention of infections

5. Ensure that all results and observations are effectively and appropriately communicated within CSL, FR, PHD, PHSI and FC.

1. Determination of lethal threshold temperatures for different propagules of *P. ramorum* and *P. kernoviae*

(a) Effect of dry heat on mycelium

Isolates of *P. kernoviae* (CC95, CC102, CC113) and *P. ramorum* (CC47, 1376, 2046) were grown for 5-7 days on V8 juice agar (100 ml V8; 1.0 g Calcium carbonate; 20 g Tech. Agar No. 3; 25 ml 0.01 M Potassium hydroxide solution [14 g KOH in 25 ml H_2O]; 875 ml distilled water). Five plugs (No. 2 borer) of each culture, containing only mycelial growth, were transferred to empty 90 mm Petri dishes. The absence of both chlamydospores and sporangia was confirmed by visual inspection of each culture with a light microscope at x 100 magnification. To remove any edge effects, Petri dishes were then placed in stacks flanked by empty Petri dishes within a Hybaid oven set at various treatment temperatures; 30, 35, 40, 42.5, 50 and 60°C. A Latin square design ensured all isolates were present at each height within the stack. Stacks were removed after 10, 15, 30, 60, 90 and 120 minutes exposure to each heat treatment. Plugs were then transferred to V8 juice agar and incubated at 18°C. Survival was assessed 7 days after treatment.

Mycelia of both *P. ramorum* and *P. kernoviae* were very resilient to dry heat treatment, with isolates from both fungal species surviving a 15 minute, but not a 30 minute, treatment at 60°C (Table 1). Extending the treatment time to 60 minutes for *P. ramorum* and 120 minutes for *P. kernoviae* reduced the lethal temperature to 50 and 42.5°C respectively.

(b) Effect of dry heat on sporangia

Isolates of *P. kernoviae* and *P. ramorum* were grown on ¼ carrot potato agar (9.75g Potato Dextrose Agar; 5.25g Tech. Agar No. 3; 50g grated carrot, 1L distilled water) at 18°C, under daylight bulbs (12 h day/night light regime) for 7 days. Sporangial suspensions were produced from these plates and spread plated onto V8 juice agar, which were incubated as above for three days. This method produced profuse numbers of sporangia all of a similar age. Seven plugs (No. 6 borer) containing sporangia of each isolate were transferred into individual empty Petri dishes. The dishes were randomised in a Hybaid oven and placed at 40, 42.5, 50, 52.5 and 55°C. A plug of each isolate was removed after 10, 15, 30, 60, 90, and 120 minutes exposure to the heat treatment. Sporangia were then washed off each plug onto V8 juice agar using 0.5 ml SDW and the plate incubated at 18°C. Fungal survival was assessed 7 days after treatment.

A dry heat treatment of 10 minutes at 55°C successfully killed sporangia of *P. kernoviae* but failed to kill *P. ramorum* (Table 2). Complete kill of both pathogens occurred after 15 minutes at 55°C. Extending the treatment time reduced the temperature required to achieve 100% kill, 30 min at 50°C killed sporangia of *P. kernoviae*, whereas 60 min at 52.5°C was required for *P. ramorum*.

(c) Effect of wet heat on mycelium

Cellophane was cut into disks, boiled and autoclaved for 15 mins at 121°C. Isolates of *P. kernoviae* and *P. ramorum* were grown for 5-7 days on V8 juice agar covered by a single cellophane disk. Plates were sealed and incubated in the dark at 20°C to reduce sporangial production. Two rectangular strips of cellophane (approximately 10 x 50 mm) containing only mycelial growth were cut from the leading edge of each culture. Strips were checked to confirm the absence of chlamydospores and sporangia by visual inspection with a light microscope at x 100 magnification. Strips were transferred to 1.5 ml tubes containing 1 ml SDW and heated to 30, 35, 37.5, 40, 50 or 60°C on an aluminium hot block. Temperatures were monitored using a thermometer placed in an open tube containing 1 ml water. Strips were removed after 10, 15, 30, 60, 90 and 120 minutes exposure to each heat treatment. In addition, an untreated control was prepared by placing strips in SDW at room temperature. The cellophane strips

were then transferred to V8 juice agar and incubated at 18°C. Survival was assessed 7 days after treatment.

Temperatures above 40°C for duration times of longer than 10 minutes successfully killed mycelia of both *P. ramorum* and *P. kernoviae* (Table 3). All 3 isolates of *P. ramorum* survived a 10 minute wet heat treatment at 40°C, however this treatment was sufficient to kill *P. kernoviae*. Isolates of both *P. ramorum* and *P. kernoviae* survived 120 minute treatments at 30 and 35°C. Mycelia of *P. kernoviae* were killed after a 30 minute treatment at 37.5°C, however, *P. ramorum* required a 90 minute treatment for complete eradication.

(d) Effect of wet heat on sporangia

Sporangial were produced as described previously (section 1b). Sporangial suspensions were prepared by adding 5.0 ml SDW to a plate of each isolate and spreading the liquid to dislodge the sporangia. The resulting suspension was assessed for sporangial concentration using a haemocytometer, and the suspensions were adjusted to provide a concentration of approximately 1 x 10^4 sporangia/ml. A hot block was set to the treatment temperature and 1.5 ml eppendorf tubes prepared for each isolate and time combination containing 400ul of SDW. For each isolate, sporangial suspension (100ul) was added to the tubes containing pre-heated sterile distilled water. Test temperatures were 37.5, 40, 42.5, 45 and 50°C and tubes were removed after 10, 15, 30, 60, 90, and 120 minutes. After treatment the contents of each tube were spread onto an individual V8 juice agar plate and incubated at 18°C. Survival and growth were assessed 7 days after treatment.

Short hot-water treatments of 10 minutes at and above 42.5°C successfully killed sporangia of *P. ramorum* and *P. kernoviae* (Table 4). A longer treatment of 60 minutes for *P. ramorum* and 30 minutes for *P. kernoviae* was required to kill sporangia at 40°C. A treatment time of 90 minutes at 37.5°C was sufficient to kill sporangia of *P. kernoviae* but not *P. ramorum.*

2. Determination lethal temperature threshold of host plants and the effect of lethal temperature thresholds on plant growth

(a) Effect of hot water treatment

Batches of rooted cuttings of three different SOD host plants (rhododendron, viburnum, camellia) were depotted and the majority of soil removed from the root bowl. A temperature controlled water bath (Grant; model SB50) with circulated water was filled and heated to one of four test temperatures (45, 50, 55, 60°C). Triplicate plants of each host were randomly placed into a steel cage and weighted down before being totally immersed in the heated water for 20 minutes. As a control, one plant of each host was depotted and allowed to stand for an hour before being completely immersed in water at room temperature for 20 minutes. After immersion in water, the plants were re-potted in compost before being placed in a controlled environment cabinet set at 18 °C and 80% relative humidity. Plant growth was assessed 7 and 14 days after treatment and any adverse effects on growth recorded.

No plants of any species survived a 20 minute hot water treatment at either 55°C or 60°C (Figure 1). Viburnum plants were killed or severely damaged after immersion at 45°C and 50°C. Whilst camellia plants did not survive treatment at 50°C, they survived a 45°C treatment with only minor leaf discoloration, which was also present to a lesser degree on the control plant (Figure 2). Rhododendron was the most temperature tolerant host, with 2 of 3 plants surviving treatment at 50°C and all plants appearing healthy, but with a slight loss of leaf sheen, after a 45°C treatment. All control plants immersed in water at room temperature remained healthy.

(b) Effect of dry heat treatment

Data from Milestone 01 suggested 100% kill of mycelia of *P. ramorum* and *P. kernoviae* was achieved using a 30 minute treatment at 60°C, whereas 15 minutes at 55°C was required for sporangia. Therefore, as an initial test, three plants each of camellia, rhododendron and viburnum were depotted and subjected

to a 20 minute treatment at 55°C (a lethal treatment for sporangia, but not mycelia) in a Hybaid oven. Plants were repotted and plant health assessed after 7 and 14 days.

No rhododendron, camellia or viburnum plants survived the treatment at 55°C for 20 minutes. All plants were completely browned 1 week after treatment.

3. Examination of dry heat treatments for sanitation and prevention of infections
This was not pursued as data from experiments conducted under objective two clearly showed that the dry heat treatments were extremely damaging to the host plants and therefore not suitable as a treatment for infected plants.

4. Examination of hot water treatments for sanitation and prevention of infections
Cultures of *P. kernoviae* and *P. ramorum* isolates were prepared on ¼ carrot potato agar and grown for 7 days. Sporangial suspensions were prepared by adding 5.0 ml SDW to a plate of each isolate and spreading the liquid to dislodge the sporangia. The resulting solution was assessed for sporangial concentration using a haemocytometer, and the suspensions were adjusted to provide a concentration of approximately 1×10^4 ($\pm 0.5 \times 10^4$) sporangia/ml. 120 rhododendron and viburnum leaves and 60 camellia leaves were obtained and placed upside down in moistened trays. For each isolate a 50ul drop of sporangial suspension (approx. 50 sporangia) was pipetted onto 20 rhododendron and viburnum leaves; two 50ul drops were pipetted onto each of 10 camellia leaves. The leaves were then covered and allowed to incubate at 18°C for 24 hours.

For the wet heat treatment, a water bath (Tecam, model UB1) was prepared and heated to 45°C. For each isolate the exposed leaves were evenly split into test and control groups each consisting of 10 rhododendron, 10 viburnum and 5 camellia leaves per isolate. Leaves were placesd in a plastic container with holes punched on all sides. The test containers were immersed in the water bath for 20 minutes before being removed and drained. The control container was immersed in water at room temperature. After immersion, all leaves were patted dry and a 1cm² section of leaf removed from the location of the sporangial droplet. Leaf sections were placed onto PARP H media (17g Corn Meal Agar; 200ul Pimaricin; 10ml Ampicillin; 10ml Rifampicin; 2.5ml PCNB; 75ul Tachigazole; 1L distilled water). Half the leaves from the control containers were surface sterilised by immersing in a 10% bleach solution for 2 minutes before rinsing in water and plating as described for the test leaves. The plates were incubated at 18°C and assessed 7 days after treatment.

No *P. kernoviae* or *P. ramorum* was recovered from leaf material of viburnum, camellia, or rhododendron after 20 minutes hot water treatment at 45°C. *P. ramorum* and *P. kernoviae* were successfully recovered from the control leaves (Table 5). *P. ramorum* was frequently recovered from surface sterilised control leaves indicating the pathogen had entered the detached leaves within 24 hours. *P. kernoviae* was recovered from less leaf sections than *P. ramorum*. In addition, only one isolate of *P. kernoviae* was recovered from surface sterilised leaf material, indicating the pathogen was slower to colonise detached leaves than *P. ramorum*.

5. Communication of results
Progress on experimental work and updates on results has been reported via the SOD Co-ordination meetings in CSL and the Science sub group of the SOD programme board. Quarterly reports were submitted throughout the project life.

Conclusions
Dry-heat treatment has been shown to be extremely damaging to the host plant species tested, and therefore is not applicable as a treatment in this context. However, a hot-water treatment at 45°C for a period of 20 minutes was shown to be highly effective in treating leaf material of rhododendron and

camellia (but not viburnum due to host damage) which had been exposed to either *P. ramorum* or *P. kernoviae* within the previous 24 hours. Further work is needed to identify whether treatments would be effective after a longer period for infection to occur, and to confirm effectiveness on whole plants.

Experiments have shown that mycelium and sporangia of *P. ramorum* were more resistant to heat treatment compared to *P. kernoviae*. This indicates that heat treatments developed for pre-symptom infection by *P. ramorum* are also likely to be effective against *P. kernoviae*. These treatments could be used for management of pre-infection decontamination of plants, which have been exposed to inoculum of the pathogens. The process of depotting was time consuming and therefore would be expensive in a nursery situation. However, the treatment could have applications for the protection of high value plants which have been in the vicinity of an outbreak.

Table 1. Number of mycelial plugs (of 5) containing viable *P. ramorum* (PR) or *P. kernoviae* (PK) mycelia after exposure to various durations of dry heat treatments assessed 7 days post treatment. Control plugs were subjected to 120 minute exposure at room temperature.

	Exposure time (minutes)						
Isolate	**10**	**15**	**30**	**60**	**90**	**120**	**Control**
30 °C							
CC47 (PR)	5	5	5	5	5	5	5
1376 (PR)	5	5	5	5	5	5	5
2046 (PR)	5	5	5	5	5	5	5
CC95 (PK)	5	5	5	5	5	5	5
CC102 (PK)	5	5	5	5	5	5	5
CC113 (PK)	5	5	5	5	5	5	5
35 °C							
CC47 (PR)	5	5	5	5	5	5	5
1376 (PR)	5	5	5	5	5	5	5
2046 (PR)	5	5	5	5	5	5	5
CC95 (PK)	5	5	5	5	5	5	5
CC102 (PK)	5	5	5	5	5	5	5
CC113 (PK)	5	5	5	5	5	5	5
40 °C							
CC47 (PR)	5	5	5	5	5	5	5
1376 (PR)	5	5	5	5	5	5	5
2046 (PR)	5	5	5	5	5	5	5
CC95 (PK)	5	5	5	5	5	5	5
CC102 (PK)	5	5	5	5	5	5	5
CC113 (PK)	5	5	5	5	5	5	5
42.5 °C							
CC47 (PR)	5	5	5	5	5	5	5
1376 (PR)	5	5	5	5	5	5	5
2046 (PR)	5	5	5	5	5	5	5
CC95 (PK)	5	5	5	0	1	0	5
CC102 (PK)	5	5	5	5	0	0	5
CC113 (PK)	5	5	5	1	0	0	5
50 °C							
CC47 (PR)	5	5	5	0	0	0	5
1376 (PR)	5	5	4	0	0	0	5
2046 (PR)	5	5	2	0	0	0	5
CC95 (PK)	5	5	0	0	0	0	5
CC102 (PK)	5	5	0	0	0	0	5
CC113 (PK)	5	5	0	0	0	0	5
60 °C							
CC47 (PR)	5	5	0	0	0	0	5
1376 (PR)	5	5	0	0	0	0	5
2046 (PR)	5	5	0	0	0	0	5
CC95 (PK)	5	0	0	0	0	0	5
CC102 (PK)	5	0	0	0	0	0	5
CC113 (PK)	5	4	0	0	0	0	5

Table 2. Sporangial survival for *P. ramorum* (PR) or *P. kernoviae* (PK) after exposure to various durations of dry heat treatments. Survival assessed 7 days post treatment. Control sporangia were subjected to 120 minute exposure at room temperature prior to washing off plugs.

	Exposure time (minutes)						
Isolate	**10**	**15**	**30**	**60**	**90**	**120**	**Control**
40°C							
CC47 (PR)	1	1	1	1	1	1	1
1376 (PR)	1	1	1	1	1	1	1
2046 (PR)	1	1	1	1	1	1	1
CC95 (PK)	1	1	1	1	1	1	1
CC102 (PK)	1	1	1	1	1	1	1
CC113 (PK)	1	1	1	1	1	1	1
42.5°C							
CC47 (PR)	1	1	1	1	1	1	1
1376 (PR)	1	1	1	1	1	1	1
2046 (PR)	1	1	1	1	1	1	1
CC95 (PK)	1	1	1	1	1	1	1
CC102 (PK)	1	1	1	1	0	0	1
CC113 (PK)	1	1	1	0	0	1	1
50°C							
CC47 (PR)	1	1	1	1	0	1	1
1376 (PR)	1	1	1	1	1	1	1
2046 (PR)	1	1	0	0	1	0	1
CC95 (PK)	1	0	0	0	0	0	1
CC102 (PK)	0	0	0	0	0	0	1
CC113 (PK)	1	1	0	0	0	0	1
52.5°C							
CC47 (PR)	0	0	0	0	0	0	1
1376 (PR)	1	1	1	0	0	0	1
2046 (PR)	1	1	0	0	0	0	1
CC95 (PK)	0	0	0	0	0	0	1
CC102 (PK)	0	0	0	0	0	0	1
CC113 (PK)	0	0	0	0	0	0	1
55°C							
CC47 (PR)	1	0	0	0	0	0	1
1376 (PR)	1	0	0	0	0	0	1
2046 (PR)	1	0	0	0	0	0	1
CC95 (PK)	0	0	0	0	0	0	1
CC102 (PK)	0	0	0	0	0	0	1
CC113 (PK)	0	0	0	0	0	0	1

Table 3. Number of cellophane strips (of 2) containing viable *P. ramorum* (PR) or *P. kernoviae* (PK) mycelia after exposure to various durations of wet heat treatments assessed 7 days post treatment. Control strips were subjected to 120 minute immersion in water at room temperature.

	Exposure time (minutes)						
Isolate	**10**	**15**	**30**	**60**	**90**	**120**	**Control**
30°C							
CC47 (PR)	2	2	2	2	2	2	2
1376 (PR)	2	2	2	2	2	2	2
2046 (PR)	2	2	2	2	2	2	2
CC95 (PK)	2	2	2	2	2	2	2
CC102 (PK)	2	2	2	2	2	2	2
CC113 (PK)	2	2	2	2	2	2	2
35°C							
CC47 (PR)	2	2	2	2	2	2	2
1376 (PR)	2	2	2	2	2	2	2
2046 (PR)	2	2	2	2	2	2	2
CC95 (PK)	2	2	2	2	2	1	2
CC102 (PK)	2	2	2	2	2	0	2
CC113 (PK)	2	2	2	2	2	1	2
37.5°C							
CC47 (PR)	2	2	2	0	0	0	2
1376 (PR)	2	2	2	2	0	0	2
2046 (PR)	2	2	1	0	0	0	2
CC95 (PK)	2	2	0	0	0	0	2
CC102 (PK)	2	2	0	0	0	0	2
CC113 (PK)	2	2	0	0	0	0	2
40°C							
CC47 (PR)	1	0	0	0	0	0	2
1376 (PR)	1	0	0	0	0	0	2
2046 (PR)	1	0	0	0	0	0	2
CC95 (PK)	0	0	0	0	0	0	2
CC102 (PK)	0	0	0	0	0	0	2
CC113 (PK)	0	0	0	0	0	0	2
50°C							
CC47 (PR)	0	0	0	0	0	0	2
1376 (PR)	0	0	0	0	0	0	2
2046 (PR)	0	0	0	0	0	0	2
CC95 (PK)	0	0	0	0	0	0	2
CC102 (PK)	0	0	0	0	0	0	2
CC113 (PK)	0	0	0	0	0	0	2
60°C							
CC47 (PR)	0	0	0	0	0	0	2
1376 (PR)	0	0	0	0	0	0	2
2046 (PR)	0	0	0	0	0	0	2
CC95 (PK)	0	0	0	0	0	0	2
CC102 (PK)	0	0	0	0	0	0	2
CC113 (PK)	0	0	0	0	0	0	2

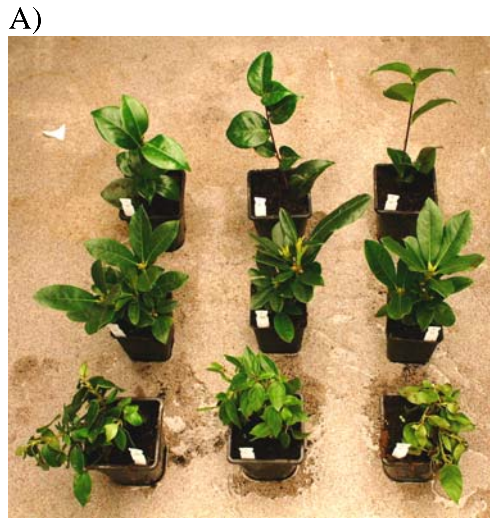
Table 4. Survival of sporangial suspensions of *P. ramorum* (PR) or *P. kernoviae* (PK) after exposure to various durations of hot water treatments. Survival assessed 7 days post treatment. Control suspensions were subjected to 120 minute exposure at room temperature.

	Exposure time (minutes)						
Isolate	**10**	**15**	**30**	**60**	**90**	**120**	**Control**
37.5°C							
CC47 (PR)	1	1	1	1	1	1	1
1376 (PR)	1	1	1	1	1	1	1
2046 (PR)	1	1	1	1	1	1	1
CC95 (PK)	1	1	1	1	0	0	1
CC102 (PK)	1	1	1	0	0	0	1
CC113 (PK)	1	1	0	0	0	0	1
40°C							
CC47 (PR)	0	0	0	0	0	0	1
1376 (PR)	1	1	1	0	0	0	1
2046 (PR)	1	1	0	0	0	0	1
CC95 (PK)	1	1	0	0	0	0	1
CC102 (PK)	0	0	0	0	0	0	1
CC113 (PK)	0	0	0	0	0	0	1
42.5°C							
CC47 (PR)	0	0	0	0	0	0	1
1376 (PR)	0	0	0	0	0	0	1
2046 (PR)	0	0	0	0	0	0	1
CC95 (PK)	0	0	0	0	0	0	1
CC102 (PK)	0	0	0	0	0	0	1
CC113 (PK)	0	0	0	0	0	0	1
45°C							
CC47 (PR)	0	0	0	0	0	0	1
1376 (PR)	0	0	0	0	0	0	1
2046 (PR)	0	0	0	0	0	0	1
CC95 (PK)	0	0	0	0	0	0	1
CC102 (PK)	0	0	0	0	0	0	1
CC113 (PK)	0	0	0	0	0	0	1
50°C							
CC47 (PR)	0	0	0	0	0	0	1
1376 (PR)	0	0	0	0	0	0	1
2046 (PR)	0	0	0	0	0	0	1
CC95 (PK)	0	0	0	0	0	0	1
CC102 (PK)	0	0	0	0	0	0	1
CC113 (PK)	0	0	0	0	0	0	1

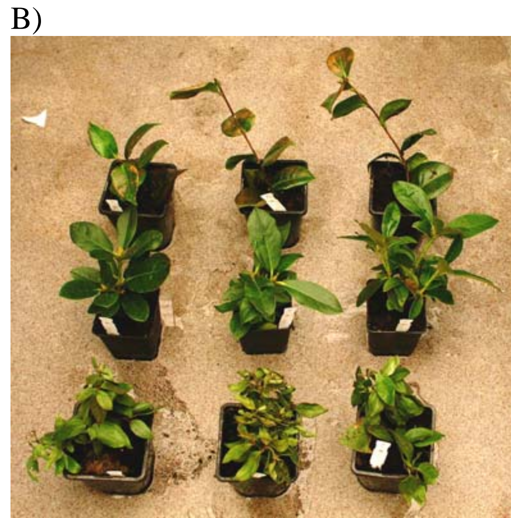
Table 5. Number of control leaf sections (of 5) showing growth of *P. ramorum* (PR) or *P. kernoviae* (PK) after a 20 minute immersion in water at room temperature. Leaf sections were either plated directly (direct) or surface sterilised for 2 minutes using 10% bleach solution (SS). Growth was assessed 7 days post treatment.

	Viburnum		**Rhododendron**		**Camellia**	
Isolate	**Direct**	**SS**	**Direct**	**SS**	**Direct**	**SS**
CC47 (PR)	5	5	4	5	5	5
1376 (PR)	5	5	5	5	5	5
2046 (PR)	5	5	5	5	5	5
CC95 (PK)	4	0	0	0	0	0
CC102 (PK)	5	0	4	0	4	0
CC113 (PK)	4	3	5	4	4	3

A)

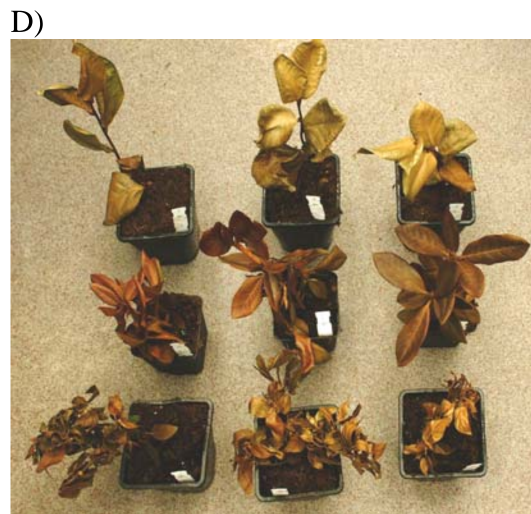


B)



C)

D)



E)

Figure 1: Images of viburnum, rhododendron and camellia plants after immersion in hot water for 20 minutes at (A) 45°C, (B) 50°C, (C) 55°C and (D) 60°C compared to (E) control plants.

A

B

C

D

Figure 2. Photographs showing leaf symptoms after hot water treatment at 45°C. (A) contortion of a viburnum plant; (B) slight browning on a camellia leaf; (C) loss of cuticle sheen on rhododendron leaves compared with the rhododendron control plant (D).

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

General enquiries on this form should be made to:
Defra, Science Directorate, Management Support and Finance Team,
Telephone No. 020 7238 1612
E-mail: research.competitions@defra.gsi.gov.uk

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Project identification

1. Defra Project code: PHE/2122B
2. Project title:
   Investigation of dry-heat treatment methods for sanitisation of P. ramorum and P. kernoviae on/in plants
3. Contractor organisation(s):
   Central Science Laboratory
   Sand Hutton
   York
   YO41 1LZ
4. Total Defra project costs (agreed fixed price): £ 10,000
5. Project: start date .... 01 September 2006
   end date .... 28 February 2007

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   Please confirm your agreement to do so...YES ☒ NO ☐

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   Defra recognises that in a small minority of cases there may be information, such as intellectual property or commercially confidential data, used in or generated by the research project, which should not be disclosed. In these cases, such information should be detailed in a separate annex (not to be published) so that the SID 5 can be placed in the public domain. Where it is impossible to complete the Final Report without including references to any sensitive or confidential data, the information should be included and section (b) completed. NB: only in exceptional circumstances will Defra expect contractors to give a "No" answer.
   In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

A pilot project to investigate the effectiveness of heat treatment for the control of *P. ramorum* and *P. kernoviae* showed that dry heat was less effective than wet heat in killing both mycelium and sporangia when used at similar temperatures and exposure times. Use of dry heat treatments at higher temperatures (55°C for 20 minutes) also caused significant damage to the plant and this treatment was discounted as having a practical role in strategies for control of *P. ramorum/kernoviae in planta*. However, further tests showed that dry heat treatments did kill *P. ramorum* and *P. kernoviae* mycelium and sporangia *in vitro* at lower temperatures (42.5 or 45°C) when exposure periods were more extended. The objectives of this project were to determine the potential of these longer exposure heat treatments (potentially achievable in glasshouses/polytunnels) to sanitise potentially contaminated or cryptically infected plants with a view to their potential future use on plants being held under statutory notice which have been within the 10m zone around infected plants on nurseries

Initial tests were carried out on detached leaves of rhododendron, camellia and viburnum to determine the required kill time for lower temperature treatments using dry heat. Leaves were inoculated with a sporangial suspension of either *P. ramorum* or *P. kernoviae* and subjected to dry heat treatments ranging between 37.5 and 45°C for between 20 and 240 minutes. Inoculated leaves were treated either: (a) two hours after inoculation (so inoculum was still present on the leaf surface); (b) approx. 12 hours after inoculation (so that the germination/infection process had started); (c) after 24 hours (so that infection and colonisation had occurred but symptoms not developed); and (d) after four days (so that early symptoms had developed but chlamydospores were not present). Following treatment, the effectiveness of each regime was determined by plating the inoculated leaf area onto agar and checking for the growth of *P. ramorum* or *P. kernoviae* after seven days. Results indicated that all temperature treatments were effective in controlling all stages of infection by *P. ramorum* and *P. kernoviae* on host plant leaves when the exposure time was at least 240 minutes. The highest temperature tested (45°C) was effective after 80 minutes and the lowest (37.5°C) required the full 240-minute exposure time to be effective. Exposure times required for efficacy of the intermediate heat treatment regimes decreased with increasing temperature. Overall, temperatures and exposure times required to kill infections by *P. kernoviae* were significantly lower than those required for infections by *P. ramorum*. There was some evidence that the more advanced infections on leaves (incubated for up to 4 days) required higher temperature and exposure treatments than less established ones.

Based on results from the first set of experiments, four dry heat treatments (240 min at 37.5°C, 120 min at

40 and 42.5°C and 100 min at 45°C) were tested on potted rhododendron, camellia and viburnum plants to determine whether the treatments would adversely affect plant growth and quality. Results from the tests showed that none of the treatments damaged the plants either immediately after, or by the end of the six-week monitoring period. In fact, by the end of the monitoring period all plants had produced fresh growth and the rhododendrons had flowered.

Finally, the heat treatments (240 min at 37.5°C, 120 min at 40°C and 42.5°C, and 100 min at 45°C) were tested on whole rhododendron, camellia and viburnum plants infected by *P. ramorum*. Post-treatment lesion measurement and isolations showed no consistent evidence that any of the treatments were 100% effective at the temperatures and timings tested. However, where the exposure time remained constant but the temperature was increased (exposure time of 120 min at 40 and 42.5°C), the level of control achieved was improved. The test was therefore repeated at 45°C with the treatment time increased to 130 min. The level of control achieved was much improved compared to the 100 min treatment. However, the treatment was still not 100% effective. Plants exposed to this longer treatment did not appear to be adversely affected when monitored over a two-week period following treatment. Results show that the treatment regimes, although effective in detached leaf assays, were not effective on infections on intact plants. This could be due to a number of factors including the possibility that conditions of the two experiments differed in levels of ambient humidity or the heat treatment may have been more penetrative in the detached leaf assay.

It is still possible that heat treatment could have a role in sanitising host plants with pre-symptomatic/early infections, however longer treatment times than the ones tested would be required. The consequent additional cost of using prolonged heat treatment and the problems of sanitising the compost within the pots of these plants would need to be considered.

Report author: Dr Philip Jennings, Central Science Laboratory, York

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Transfer).

Investigation of dry-heat treatment methods for sanitisation of *P. ramorum* and *P. kernoviae* on/in plants

Background

A pilot project to investigate the effectiveness of wet and dry heat treatments for the control of *P. ramorum* and *P. kernoviae* has shown that a hot water treatment at 45°C for a period of 20 minutes was highly effective in treating leaf material of rhododendron or camellia which had been exposed to either *P. ramorum* or *P. kernoviae* within the previous 24 hours. Dry heat treatments were shown to be less effective than wet heat treatments against both mycelium and sporangia when used at similar temperatures over a similar time period. Use of dry heat treatments at higher temperatures (55°C for 20 minutes) also caused significant damage to the host plants tested. However, when tested at lower temperatures (42.5 or 45°C) for more prolonged periods of time, dry heat treatments did control *P. ramorum* and *P. kernoviae* mycelium and sporangia *in vitro*. These treatments were not investigated *in planta* in the pilot project and further work was carried out within this project to examine these effects.

The aim of this work was to determine the potential for lower temperature dry heat treatments to sanitise potentially contaminated or cryptically infected plants. It was anticipated that these treatments might have a potential future role on nurseries for disease control on plants held under quarantine notice.

The scientific objectives for the project (listed below) were all met in full.

1/ Determine the kill time for *Phytophthora ramorum* and *P. kernoviae* infections of detached rhododendron, camellia and viburnum leaves using low temperature dry heat treatments.

2/ Determine how the most effective treatments for infected detached leaves affect whole rhododendron, camellia and viburnum plants.

3/ Determine the effectiveness of low temperature dry heat treatments for sanitising infected whole rhododendron, camellia and viburnum plants.

4/ Ensure that all results and observations are effectively and appropriately communicated within CSL, Forest Research, Defra Plant Health Division, Defra Plant Health and Seeds Inspectorate and Forestry Commission.

Materials and methods

<u>Detached leaf tests</u>
Initial tests were carried out on detached rhododendron, camellia and viburnum leaves to determine the kill time for low temperature dry heat treatments. Leaves were inoculated with either *P. ramorum* or *P. kernoviae* and then subjected to a range of dry heat treatments.

Sporangial production
Plugs of *P. ramorum* (CSL ref cc47) and *P. kernoviae* (CSL ref cc95) were taken from the CSL culture collection and grown on 10% V-8 agar (Appendix I) at 20°C, under day light bulbs (12h light/12h dark regime) until the colonies reached the edge of the plates. The agar plates were flooded with 5 mL of sterile distilled water (SDW) and sporangia removed from the agar surface using a sterile plastic rod. Fresh 10% V-8 agar plates were inoculated with 100 µL of the resulting sporangial suspension and incubated for 3 days under the same temperature and light regime as previously described. Sporangia were removed from the agar surface in 10 mL SDW and the concentration adjusted to $1x10^5$ spores/mL. This method resulted in synchronous production of sporangia ensuring that the sporangia used for the inoculations were all of a similar age.

Leaf inoculation
For each heat treatment and *Phytophthora* species, a single rhododendron, camellia and viburnum leaf was lightly damaged (a small scratch ~ 5mm) at four points on the upper leaf surface. One leaf of each plant species was then placed in a moisture chamber and 25µL of a sporangial spore suspension of either *P. ramorum* or *P. kernoviae* placed over each damaged area. Inoculated leaves were incubated for 2, 12, 24 or 96 h prior to heat treatment. The four incubation timings used provided leaf material at differing stages of infection: after 2 h incubation both *P. ramorum* and *P. kernoviae* were present on the leaf surface only, after 12 h the inoculum had germinated and the infection process just started, after 24 h infection and colonisation had occurred but symptoms not yet developed and after 96 h early symptoms had developed but chlamydospores had not been produced within the leaf tissue.

Heat treatment
Infected leaf material was tested under four heat regimes (37.5, 40, 42.5 and 45°C) using exposure times between 20 and 240 minutes. Four replicate lesions were exposed to each heat regime. Control lesions were left at 20°C for the duration of the experiment.

Following heat treatment, $1cm^2$ sections of the leaf, centred at the inoculation point, were removed from the leaves and plated onto $PARP_5H$ agar (Appendix I). Plates were incubated for 7 days at 20°C and then assessed for growth of either *P. ramorum* or *P. kernoviae* from the cut section of leaf.

<u>Whole plant tests (uninoculated)</u>
Based on results from the detached leaf tests, heat treatment regimes which appeared to sanitise the infected detached leaf material were identified (Table 1) and tested on whole rhododendron, camellia and viburnum plants to determine their effect on the plants.

Table 1. Dry heat treatment temperature regimes used in whole plant tests

Temperature (°C)	Time (min)
37.5	240
40	120
42.5	120
45	100

Whole rhododendron, camellia and viburnum plants were place under the temperature regimes outlined in Table 1. Following treatment, plants were monitored for six weeks to determine whether the treatments had caused any detrimental effects to the plants. Control plants were maintained at 20°C for the duration of the testing.

Whole plant tests (inoculated)
Dry heat treatments (Table 1) were tested to determine whether they were effective in sanitising infected rhododendron, camellia and viburnum plants. Initial experiments were carried out on whole plants using sporangial inoculum, however the infection rate was extremely low for all inoculation timings. As a result the inoculation method was altered for each host species as follows, three leaves per plant were wounded using a single stab on the adaxial (upper) surface. A 5 mm agar plug, taken from a seven day-old *P. ramorum* culture and containing sporangia, was placed over the wound (controls plants were inoculated with an agar plug only), the plug covered with a damp cotton wool swab and secured in place by wrapping with parafilm. Inoculated leaves were incubated for 2, 12, 24 or 96 h prior to heat treatment, inoculum plugs were removed just prior to treatment and the diameter of any lesion present measured. Treatments were carried out as outlined in Table 1 with an additional treatment of 45°C for 130 min. After treatment, plants were grown-on for seven days at 20°C, lesion diameters re-measured and the lesion or, where no lesion was present, the area around the inoculation was plated onto $PARP_5H$. Agar plates were incubated at 20°C for seven days and then assessed microscopically for the growth of *P. ramorum* from the leaf section.

Results and discussion

Detached leaf assay
All temperature treatments were effective in controlling all the stages of infection by *P. ramorum* tested on host plant leaves when the exposure time was at least 240 minutes (Table 2). The highest temperature tested (45°C) was effective after 80 minutes and the lowest (37.5°C) required the full 240-minute exposure time to be effective. Exposure time required for efficacy of the intermediate heat treatment regimes decreased with increasing temperature. There was some evidence that the more advanced infections on leaves (incubated for up to 4 days) required higher temperature and exposure treatments than less established ones.

All temperature treatments were also effective in controlling all the stages of infection by *P. kernoviae* tested on host plant leaves when the exposure time was at least 240 minutes (Table 3). The highest temperature tested (45°C) was effective after 40 minutes and the lowest (37.5°C) required the full 240-minute exposure time to be effective. Overall, temperatures and exposure times required to kill infections by *P. kernoviae* were significantly lower than those required for infections by *P. ramorum*. As for *P. ramorum*, there was some evidence that the more advanced infections on leaves required higher temperature and exposure treatments than less established ones.

Whole plant tests (uninoculated)
Based on results from the detached leaf assays, dry heat treatment regimes which sanitised the rhododendron, viburnum and camellia leaves of *P. ramorum* and *P. kernoviae* infections, were chosen to determine how they affected plant growth. The dry heat treatments used were 240 min at 37.5°C, 120 min at 40 or 42.5°C, and 100 min at 45°C. No plants were adversely affected by any of the sanitising treatments used either immediately after treatment or by the end of a six-week monitoring period (Figures 1-4). In fact, by the end of the monitoring period all plants had produced fresh growth and the rhododendrons had flowered (Figures 1c, 2c, 3c, 4c).

Whole plant tests (inoculated)
Rhododendron, camellia and viburnum plants infected with *P. ramorum* were treated for 240 min at 37.5°C (Figure 5), 120 min at 40°C (Figure 6) and 42.5°C (Figure 7), and 100 min at 45°C (data not presented). Post-

treatment lesion measurement and isolations showed no consistent evidence that that the heat treatments were 100% effective at the temperatures and timings tested. However, where the exposure time remained constant, but the temperature used was increased (exposure time of 120 min at 40 and 42.5°C (Figures 6 and 7)) an increase in the level of control was observed at the higher temperature. There was no clear evidence to indicate control differences between the host species tested. However, infections on camellia and viburnum were generally controlled more effectively than those on rhododendron, especially following exposure to the higher temperature (Figures 7).

The test was repeated at 45°C with the treatment time increased to 130 min (Figure 8). The control achieved was much improved compared to the 100 min treatment. However, the treatment still was not 100% effective: the rate of re-isolation was reduced from 92, 25 and 25% for rhododendron, viburnum and camellia respectively after 100 min exposure, to 42, 8 and 0% after 130 min exposure. Plants exposed to this longer treatment did not appear to be adversely effected following monitoring over a two-week period.

Results show that treatment regimes, which were effective in detached leaf assays, were not effective on infections on intact plants. This could be due to a number of factors including the possibility that conditions of the two experiments differed in levels of ambient humidity or the heat treatment may have been more penetrative in the detached leaf assay. Additionally different inoculation methods were used, sporangial suspension for detached leaf assay and mycelial/sporangial plugs for the whole plant assay. The use of agar plugs and wounded leaves for the whole plant assay may have allowed infection to develop earlier and hence have colonised the leaves to a greater extent.

Conclusions and implications for policy

The treatments as tested in this study were not proven to work. It is still possible that heat treatment could have a role in sanitising host plants with pre-symptomatic/early infections, however longer treatment times than the ones tested would be required. The consequent additional cost of using prolonged heat treatment and the problems of sanitising the compost within the pots of these plants would also need to be considered. As a result there are no policy implications.

Table 2. The effectiveness of dry heat treatments for the sanitisation of detached rhododendron, viburnum and camellia leaves infected by *P. ramorum* (applied as sporangial inoculum).

Heat treatment (°C)	Host plant	Infection period (days-hrs)	Exposure time (min)								Control
			20	40	60	80	100	120	180	240	
37.5	Rhododendron	0-2	n/t	++--	----	+---	++--	+---	----	----	++++
		0-12	n/t	+---	----	++++	++--	----	----	----	++++
		1-0	n/t	+++-	++++	++++	++--	+++-	+---	----	++++
		4-0	n/t	++++	++++	++++	+++-	----	+---	----	+++-
	Viburnum	0-2	n/t	----	----	----	----	----	----	----	+++-
		0-12	n/t	++--	+---	++--	----	+---	----	----	++++
		1-0	n/t	+----	+++-	+---	----	----	----	----	++++
		4-0	n/t	++++	+++-	+++-	++--	----	----	----	+++-
	Camellia	0-2	n/t	----	----	----	++--	----	----	----	++++
		0-12	n/t	+---	----	+---	----	----	----	----	++++
		1-0	n/t	+---	----	+---	++--	++--	++--	----	++++
		4-0	n/t	++++	++++	++++	++--	----	----	----	+++-
40	Rhododendron	0-2	n/t	----	+++-	----	----	----	----	----	++--
		0-12	n/t	+---	+---	++--	+---	----	----	----	++++
		1-0	n/t	++++	+++-	+---	----	----	----	----	+++-
		4-0	n/t	++--	++--	----	----	----	----	----	+++-
	Viburnum	0-2	n/t	----	++--	----	----	----	----	----	+++-
		0-12	n/t	++--	+++-	+---	+---	----	----	----	+++-
		1-0	n/t	++++	+++-	----	----	----	----	----	+++-
		4-0	n/t	+---	++++	----	----	----	----	----	++++
	Camellia	0-2	n/t	----	+---	----	----	----	----	----	++--
		0-12	n/t	+---	----	----	----	----	----	----	++--
		1-0	n/t	+++-	++--	----	----	----	----	----	++++
		4-0	n/t	++++	++++	----	----	----	----	----	++++
42.5	Rhododendron	0-2	n/t	----	----	----	----	----	----	----	+++-
		0-12	n/t	++--	++--	----	----	----	----	----	+++-
		1-0	n/t	+---	+---	----	----	----	----	----	++++
		4-0	n/t	+---	----	----	----	----	----	----	++++
	Viburnum	0-2	n/t	----	----	----	----	----	----	----	++++
		0-12	n/t	----	----	----	----	----	----	----	+++-
		1-0	n/t	++--	++--	----	----	----	----	----	++++
		4-0	n/t	++	----	----	----	----	----	----	++++
	Camellia	0-2	n/t	----	----	----	----	----	----	----	+++-
		0-12	n/t	++--	+++-	++--	+---	----	----	----	+++-
		1-0	n/t	----	----	----	----	----	----	----	++++
		4-0	n/t	+++-	----	----	----	+---	----	----	++++
45	Rhododendron	0-2	++++	++--	++--	----	----	n/t	n/t	n/t	++++
		0-12	++++	----	----	----	----	n/t	n/t	n/t	++++
		1-0	n/t	----	----	----	----	n/t	n/t	n/t	++++
		4-0	n/t	----	----	----	----	n/t	n/t	n/t	++++
	Viburnum	0-2	++++	----	----	----	----	n/t	n/t	n/t	++++
		0-12	++++	++--	----	----	----	n/t	n/t	n/t	++++
		1-0	n/t	----	----	----	----	n/t	n/t	n/t	++++
		4-0	n/t	----	----	----	----	n/t	n/t	n/t	++++
	Camellia	0-2	+---	----	----	----	----	n/t	n/t	n/t	+++-
		0-12	+---	+----	----	----	----	n/t	n/t	n/t	+++-
		1-0	n/t	----	----	----	----	n/t	n/t	n/t	++--
		4-0	n/t	----	----	----	----	n/t	n/t	n/t	++++

+ indicates growth and - indicates no growth of *P. ramorum* in the test. The number of '+'s or '–'s for an individual test indicates the number of replicates where growth of the *P. ramorum* either did or did not occur. n/t = not tested.

Table 3. The effectiveness of dry heat treatments for the sanitisation of detached rhododendron, viburnum and camellia leaves infected by *P. kernoviae* (applied as sporangial inoculum).

Heat treatment (°C)	Host plant	Infection period (days-hrs)	Exposure time (min)						Control
			20	40	60	80	100	240	
37.5	Rhododendron	0-2	n/t	----	+++-	++--	+---	----	++++
		0-12	n/t	++--	+---	++--	+++-	----	++++
		1-0	n/t	++++	++--	+++-	+++-	----	++++
		4-0	n/t	----	+---	----	----	----	++--
	Viburnum	0-2	n/t	+---	----	+---	+---	----	++++
		0-12	n/t	++--	----	----	----	----	++++
		1-0	n/t	++--	+---	----	+---	----	++++
		4-0	n/t	----	+---	----	----	----	+++-
	Camellia	0-2	n/t	++--	----	----	+++-	----	++++
		0-12	n/t	+++-	++--	++--	+---	----	++++
		1-0	n/t	++--	++--	++--	----	----	++++
		4-0	n/t	----	----	----	----	----	+++-
40	Rhododendron	0-2	n/t	----	----	----	----	----	+---
		0-12	n/t	++--	+---	----	----	----	++++
		1-0	n/t	+++-	+---	++--	----	----	++++
		4-0	n/t	+++-	+---	++--	----	----	++++
	Viburnum	0-2	n/t	----	----	----	----	----	----
		0-12	n/t	----	----	----	----	----	----
		1-0	n/t	----	----	+---	----	----	+++-
		4-0	n/t	----	----	----	----	----	++++
	Camellia	0-2	n/t	----	----	----	----	----	----
		0-12	n/t	+---	+++-	+---	----	----	+++-
		1-0	n/t	+---	+---	++++	----	----	++++
		4-0	n/t	++--	----	----	----	----	++++
42.5	Rhododendron	0-2	n/t	----	----	----	----	----	++--
		0-12	n/t	+---	----	+---	----	----	+---
		1-0	n/t	----	----	----	----	----	++++
		4-0	n/t	----	----	----	----	----	++++
	Viburnum	0-2	n/t	----	----	----	----	----	++--
		0-12	n/t	----	----	----	----	----	+---
		1-0	n/t	----	----	----	----	----	++++
		4-0	n/t	----	----	----	----	----	+++-
	Camellia	0-2	n/t	----	----	----	----	----	++--
		0-12	n/t	----	----	----	----	----	+---
		1-0	n/t	+---	----	----	----	----	+++-
		4-0	n/t	----	----	----	----	----	++++
45	Rhododendron	0-2	++++	----	----	----	----	n/t	++--
		0-12	+++-	----	----	----	----	n/t	++++
		1-0	++--	----	----	----	----	n/t	++++
		4-0	----	----	----	----	----	n/t	++++
	Viburnum	0-2	----	----	----	----	----	n/t	+---
		0-12	----	----	----	----	----	n/t	----
		1-0	----	----	----	----	----	n/t	----
		4-0	----	----	----	----	----	n/t	++++
	Camellia	0-2	++--	----	----	----	----	n/t	+---
		0-12	+---	----	----	----	----	n/t	++++
		1-0	----	----	----	----	----	n/t	++--
		4-0	----	----	----	----	----	n/t	++++

+ indicates growth and - indicates no growth of *P. kernoviae* in the test. The number of '+'s or '–'s for an individual test indicates the number of replicates where growth of the *P. kernoviae* either did or did not occur. n/t = not tested.

Figure 1. Effect of heat treatment on rhododendron, viburnum and camellia growth after a 240 min exposure to 37.5°C. a) pre-treatment, b) post-treatment, c) 6 weeks post-treatment.

Figure 2. Effect of heat treatment on rhododendron, viburnum and camellia growth after a 120 min exposure to 40°C. a) pre-treatment, b) post-treatment, c) 6 weeks post-treatment.

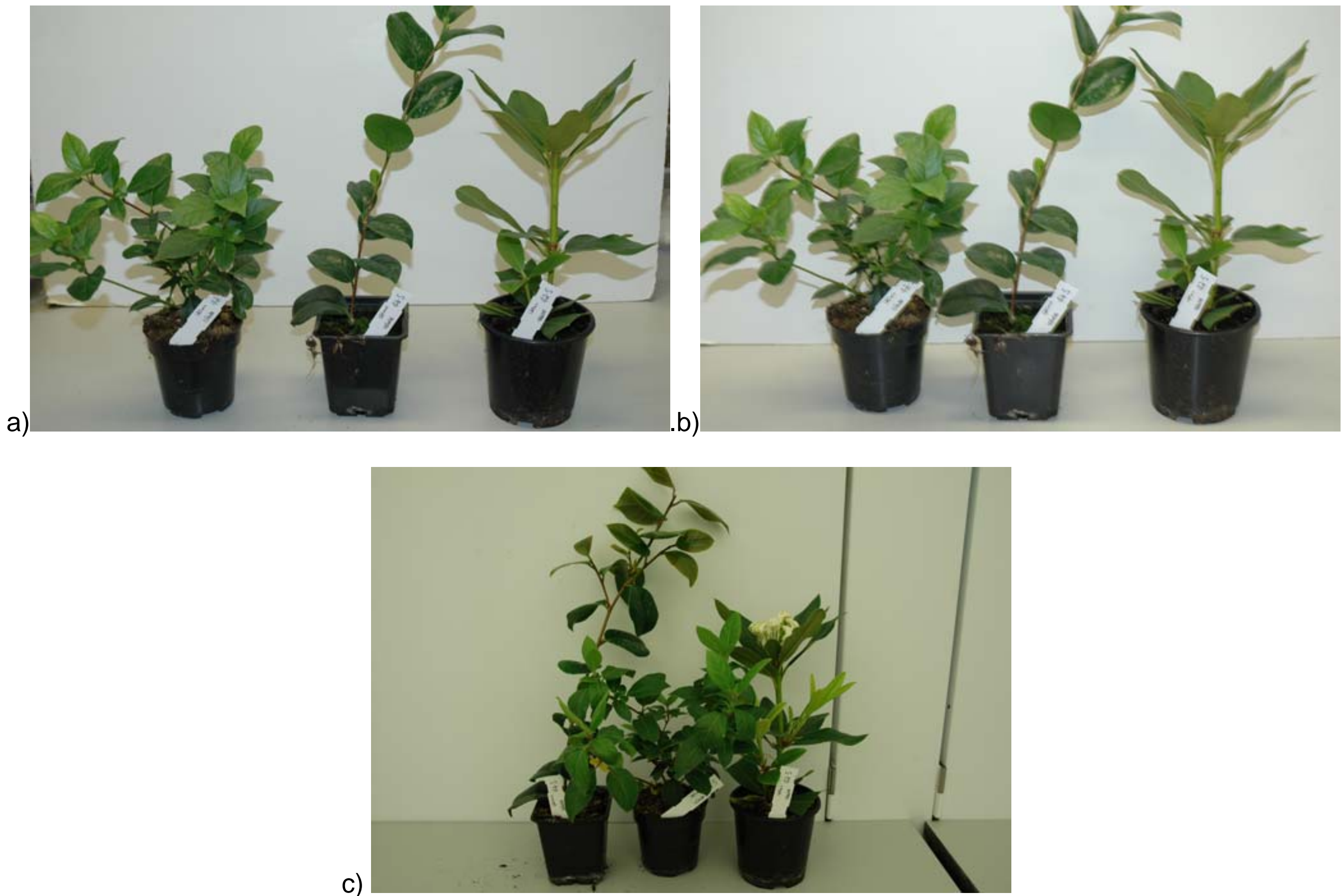

Figure 3. Effect of heat treatment on rhododendron, viburnum and camellia plants after a 120 min exposure to 42.5°C. a) pre-treatment, b) post-treatment, c) 6 weeks post-treatment.

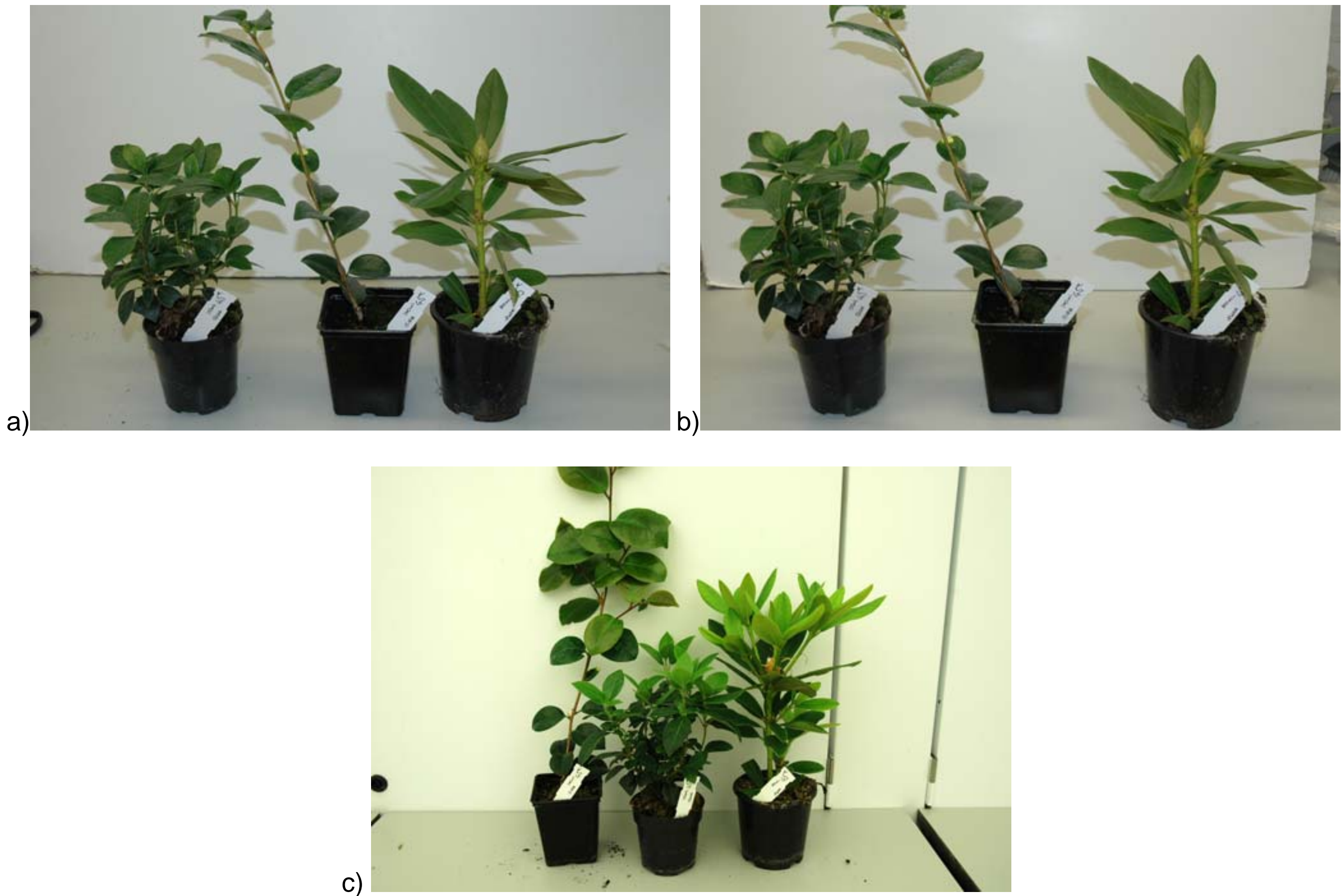

Figure 4. Effect of heat treatment on rhododendron, viburnum and camellia growth after a 100 min exposure to 45°C. a) pre-treatment, b) post-treatment, c) 6 weeks post-treatment.

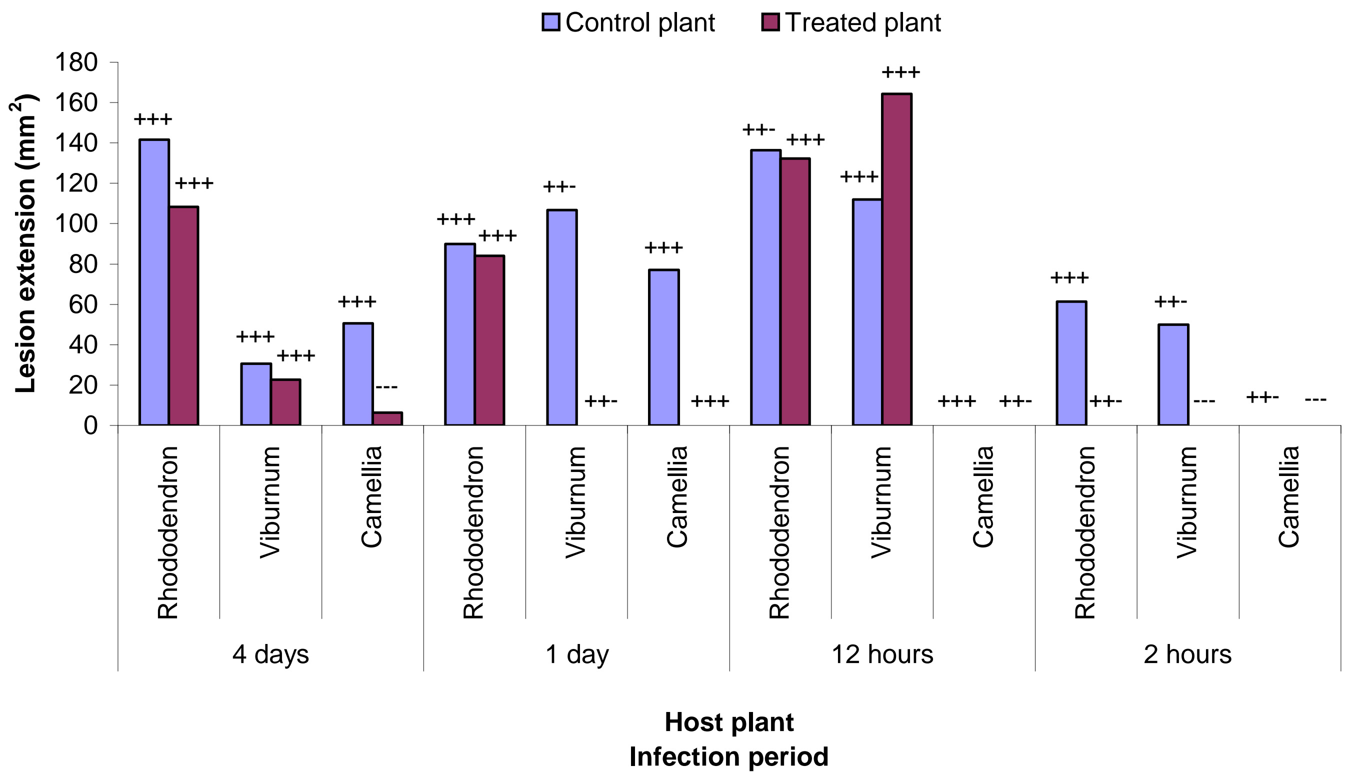

Figure 5. Effect of dry heat treatment on the extension of leaf infections, caused by *P. ramorum* (inoculum applied as a mycelial agar plugs) on whole plants of rhododendron, viburnum and camellia after a 240 min exposure to 37.5°C. The number of '+'s or '–'s above each bar of the chart indicates the number of replicates where growth of the *P. ramorum* either did (+) or did not (-) occur from a leaf lesion.

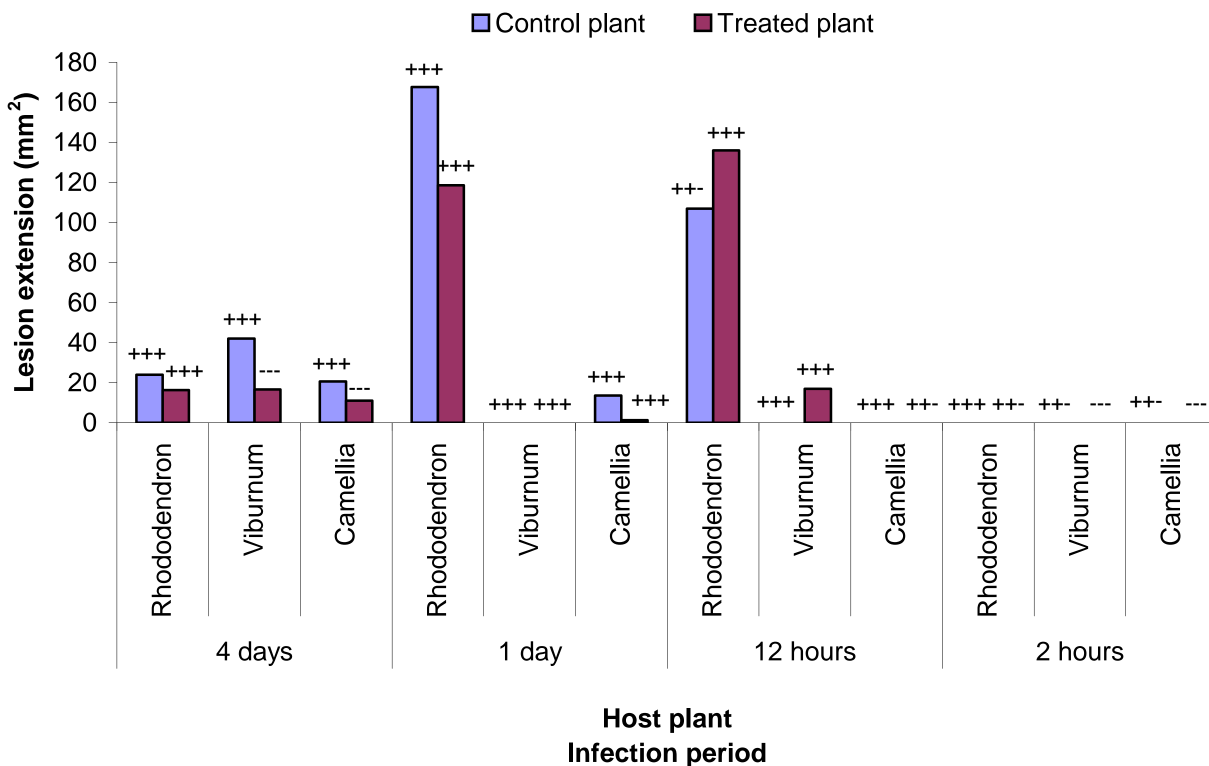

Figure 6. Effect of dry heat treatment on the extension of leaf infections, caused by *P. ramorum* (inoculum applied as a mycelial agar plugs) on whole plants of, on rhododendron, viburnum and camellia after a 120 min exposure to 40°C. The number of '+'s or '–'s above each bar of the chart indicates the number of replicates where growth of the *P. ramorum* either did (+) or did not (-) occur from a leaf lesion.

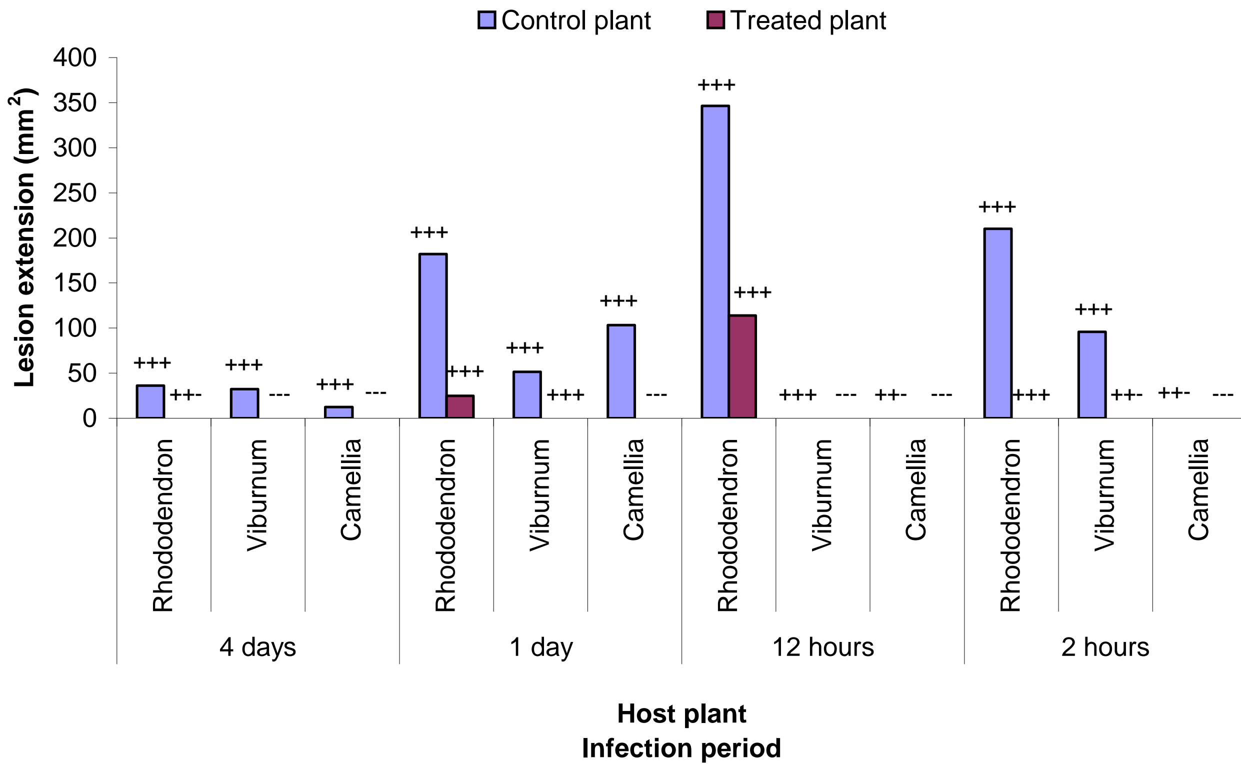

Figure 7. Effect of dry heat treatment on the extension of leaf infections, caused by *P. ramorum* (inoculum applied as a mycelial agar plugs) on whole plants of rhododendron, viburnum and camellia after a 120 min exposure to 42.5°C. The number of '+'s or '–'s above each bar of the chart indicates the number of replicates where growth of the *P. ramorum* either did (+) or did not (-) occur from a leaf lesion.

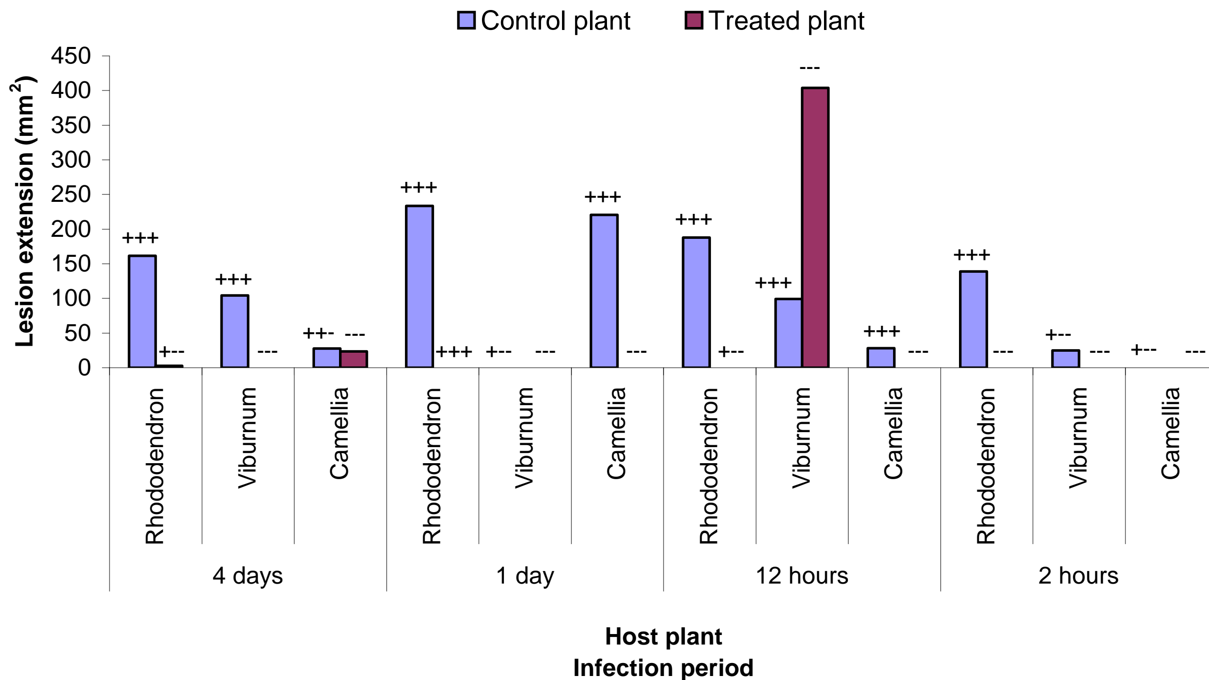

Figure 8. Effect of dry heat treatment on the extension of leaf infections, caused by *P. ramorum* (inoculum applied as a mycelial agar plugs) on whole plants of rhododendron, viburnum and camellia after a 130 min exposure to 45°C. The number of '+'s or '–'s above each bar of the chart indicates the number of replicates where growth of the *P. ramorum* either did (+) or did not (-) occur from a leaf lesion.

Appendix I

<u>10 % V-8 agar</u>

V8 juice	200 mL
$CaCO_3$	2 g
Agar N°3	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.

<u>PARP$_5$H agar (Jeffers and Martin, 1986)</u>

Cornmeal Agar (CMA) 17 g/L

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

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

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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