The Epidemiology of *Phytophthora ramorum* and *P. kernoviae* at Two Historic Gardens in Scotland[1]

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Abstract

This study looked at the factors that facilitated the spread of *Phytophthora ramorum* and *P. kernoviae* at two locations in the west of Scotland. Spore traps, river baiting, bait plants, and soil sampling were used to both confirm the presence of, and measure the amount of, inoculum in the environment in order to quantify the relationship between inoculum levels and disease development. *Phytophthora ramorum* was detected in spore traps at high levels under a sporulating host, but also at sites where hosts were not present, leading to the conclusion that inocula in low-level spore traps were the result of soil splash. *Rhododendron* and *Vaccinium* bait plants were also infected with *P. ramorum* via soil splash at sites where there was no sporulating host present. *Phytophthora kernoviae* was only detected in spore traps where there was a sporulating host overhead. Water baiting confirmed the presence of *P. ramorum* in two streams in one of the gardens, but *P. kernoviae* was not detected using this method at the other garden despite the large-scale *P. kernoviae* infection there. Inoculum continued to be detected in soil in areas where infected hosts had been removed 2 years ago, confirming that both of these pathogens can survive in soil for a considerable period. Evidence of the movement of infested mulch during horticultural activity was found. These findings have clear implications for the control of disease spread within the garden setting.

Keywords: *Phytophthora ramorum*, *Phytophthora kernoviae*, epidemiology, spread

Introduction

In Europe, *Phytophthora ramorum* was initially discovered within the nursery industry and was found infecting container grown *Rhododendron* and *Viburnum* plants (Werres et al. 2001). The extent of the host range and the damage that this pathogen could cause in parks and gardens was not immediately apparent; it was not until outbreaks occurred in forests in California that the true potential scale of damage was observed (Rizzo et al. 2002). During surveys undertaken to find *P. ramorum* in 2003, *P. kernoviae* was discovered infecting trees and shrubs in woods in Cornwall (Brasier et al. 2005). It soon became clear that *P. kernoviae* could cause as much damage as *P. ramorum* in gardens, particularly if the garden contained a large proportion of *Rhododendron* species (Webber 2008).

The trees and shrubs that have traditionally been grown in United Kingdom historic gardens have transpired to be particularly susceptible to both pathogens, particularly *P. ramorum*. Initially, ornamental *Rhododendron* species, *Viburnum*, and the invasive *R. ponticum* were found to be infected. As *P. ramorum* garden infections developed, many more commonly used horticultural species became infected such as *Magnolia*, *Pieris*, *Osmanthus*, *Fagus*, and *Camellia*. The garden hosts of *P. kernoviae* remained more restricted; *Rhododendron* is still an important host, but infections have also occurred on *Magnolia*, *Pieris*, *Fagus*, and *Vaccinium*. The proliferation of hosts

and the ideal environmental conditions in the west of England, Wales, and Scotland have provided these *Phytophthora* species with particularly conducive conditions for establishment and spread.

Methods

Field Sites

The first study location was a botanic garden in Argyll and Bute, where both *P. ramorum* and *P. kernoviae* were present. The second was a castle garden on the island of Arran, which was severely infested by *P. kernoviae*. A woodland *P. kernoviae* outbreak on *Vaccinium myrtillus* L. near the castle garden was also studied. Spore traps, river baiting, bait plants, and soil sampling were used to measure the amount of inoculum in the environment at the gardens. Four investigation sites within the botanic garden were chosen for spore trap locations and six at the castle garden. The sites at the botanic garden were: site 1, cleared infection site where both *P. ramorum* and *P. kernoviae* had infected a number of *Rhododendron* 'Elizabeth Hobbie' plants; site 2, under an infected *Magnolia kobus* DC.; site 3, cleared infection on *Kalmia latifolia* L.; and site 4, infection on two *Osmanthus* plants now cleared.

The investigation sites at the castle garden were: site 1, within an area of infected and partially cleared *Rhododendron ponticum* L. near the garden entrance; sites 2, 3, and 4, in the main infection area at the bottom of the garden, which was extensively infested with *P. kernoviae* and once contained a large number of *Rhododendron* species and cultivars which were partially cleared; site 5, under a grove of mature *Pieris japonica* (Thunb.) D. Don ex G. Don trees; and site 6, at the stump of a felled *Drimys* tree that was infected and cleared from the bottom of the garden.

Spore Traps

Two types of spore traps were designed to catch rain and water splashes, potentially containing sporangia and zoospores. The low-level traps were located at ground level and constructed by digging a 2-L bottle into the ground and placing a funnel on top. Wire mesh on the top of the funnel prevented blockage with fallen leaves and other debris. The high-level water traps were approximately 1 m above ground level. They consisted of a 2-L bottle on the ground, a funnel, and a length of hose connecting the two. The low-level traps are designed to record mainly rain splash dispersal from the surrounding soil, while the high-level traps record dispersal from rain and leaf splash from the nearby plants. The 2-L bottles were taken to the quarantine unit at Science and Advice for Scottish Agriculture (SASA) every 2 weeks. The water they contained was filtered through a 3μl membrane filter and the filters were processed to extract the DNA they contained using the Macherey-Nagel Nucleospin® Plant Kit. Any extracted DNA was then quantified using Real-Time PCR.

Bait Plants

To test the viability of the inoculum in the environment, and to test for a link between inoculum levels and infection, one potted *R. ponticum* bait plant and one *V. myrtillus* bait plant was placed monthly at each investigation site in both gardens for a 4-week period in order to see whether they became infected. The bait plants were taken to the quarantine unit at SASA after the 4-week period in the gardens, were kept for 3 months post-exposure, and were checked daily for symptoms. Infected leaves were plated onto V8 agar plus antibiotics medium and the resulting culture was identified under the microscope or by using PCR testing if required. Water baiting also took place in a number of streams around the gardens. The water baits were made by placing six cut *Rhododendron* leaf pieces in a muslin bag and attaching a length of string to be used to anchor the bait once it was placed in a stream. Recovered baits were surface sterilized and plated onto V8 agar plus antibiotics medium, and the resulting culture was identified using microscopy or PCR.

Soil Samples

To establish inoculum levels in the soil, soil samples were collected monthly from the investigation sites, but also extensively around selected sites within the gardens at 3 monthly intervals. Random soil samples were also taken around each garden at various times over the study period. Soil samples were dried, mixed, and then added to a mixing bowl from a large planetary ball mill with 12 steel ball bearings and 120 ml of cetyltrimethyl ammonium bromide (CTAB) buffer. They were milled at 300 rpm for 5 minutes. A robotic workstation for DNA extraction based on magnetic-particle purification (e.g., Qiagen Biosprint 15) and the Wizard Magnetic DNA purification system for food (Promega) was used for DNA extraction from the soil.

Water Baits

Four *Rhododendron* leaves were cut into pieces and placed in a square of muslin which was then tied with twine. The twine was about 1 m long, which allowed it to be anchored in the field. These baits were then placed in watercourses around each garden for 24 hours. As with the bait plants, the use of *V. myrtillus* and *Pieris* as water baits was tried in order to establish levels of *P. kernoviae* and *P. ramorum* in water courses at both gardens. Once the water baits had been recovered, the leaves in them were surface sterilized and plated onto V8 agar plus antibiotics. The plates were checked under a compound microscope after 5 days for the presence of mycelium and sporulation structures. If the presence of either of these *Phytophthora* species was suspected, the Nucleospin® Plant Kit protocol for DNA isolation from plants was used to extract any DNA and PCR was used for confirmation.

After initial investigation of the watercourses at the botanic garden, a stream which ran from where it entered the garden to a small pond and then down to site 1 was chosen to be baited for *P. ramorum* on a monthly basis. There were five baiting locations along this stream. Another regularly tested watercourse was near the car park and horticultural sheds; this stream originated from a different source.

Molecular Detection

Once the samples were processed using the extraction processes described above, Taqman Real-Time PCR, using a thermo-cycler, was used to quantify the levels of a target sequence, therefore quantifying the presence of these pathogens. This PCR method uses fluorophore-labelled DNA probes to measure the amount of amplified product in a sample in real time, giving results in cycle threshold (Ct) values. The Ct value is the number of cycles needed to get a fluorescent signal that is significantly higher than background levels. The lower the number of cycles, the more DNA was present in the sample. In order for the Ct value to be converted into a more useful measurement (e.g., the amount of DNA per sample in picograms), four standards of known concentrations were added to each PCR run along with the samples. Reaction mixtures of 25 µl were used for the Real-Time PCR containing:

Component	Amount (µl)
Double distilled H_20	8.0
Taqman Master Mix[7]	12.5
Forward Primer (5 molar concentration)	1.5
Reverse Primer (5 molar concentration)	1.5
Taqman Probe (5 molar concentration)	0.5
DNA (Sample)	1.0

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[7] Taqman Universal Master Mix, No AmpErase UNG (Applied Biosystems, part No. 4324018) containing DNA polymerase and dNTPs.

The primers and probes sequences are as follows:
P. ramorum:
Pram 114-FC	5' - TCA TGG CGA GCG CTT GA - 3'
Pram 1527-190-R	5' - AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT - 3'
Pram 1527-134-T	5'- [FAM] - TTC GGG TCT GAG CTA GTA G - [TAMRA] - 3'

P. kernoviae:
Pkern 615F	5' - CCG AAC AAT CTG CTT ATT GTG TCT - 3'
Pkern 722R	5' - GTT CAA AAG CCA AGC TAC ACA CTA - 3'
Pkern 606T	5'- [TET] - TGC TTT GGC GTT TGC GAA GTT GGT - [TAMRA] -3'

TET and FAM were the dyes used and TAMRA was the quencher.

The thermal cycler program used was an initial 10-minute denaturing stage of 94 $^{\circ}$C, then:

15 seconds at 94 $^{\circ}$C
60 seconds at 60 $^{\circ}$C   } X 40 cycles

Results

Spore Traps and Bait Plants

Phytophthora ramorum was detected in spore traps at the botanic garden 33 times over the 2 years (fig.1). Of these, 11 findings were at site 2 where the infected *M. kobus* had been left in *situ*. Four of these findings were in the high-level traps and seven in the low-level traps. When inoculum was recorded in the high-level traps, it was also recorded in the low-level traps in three of the four instances (April 10, December 10, November 11). Also, proportionally more inoculum was collected in the low-level trap (average 1.75 pg/µl) compared to the high-level trap (average 0.5 pg/µl). In fact, the high-level trap on average contained the least amount of inoculum found at any site throughout the study. At the other three botanic garden investigation sites, the infected hosts had been removed prior to the start of the study. Inoculum was recorded in these low-level traps on nine occasions at site 4, seven at site 3, and six at site 1. These data show that *P. ramorum* is readily splashed from soil into the low-level traps. *Phytophthora kernoviae* was only recorded on four occasions in spore traps at this garden; these four incidences were at very low levels (between 1.12 pg/µl and 2.13 pg/µl) and occurred at four different spore trap locations.
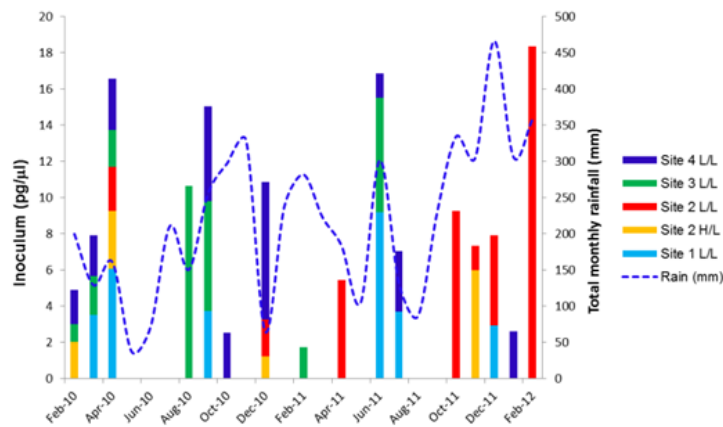

Figure 1—The incidence of *Phytophthora ramorum* inoculum (picograms/µl) in spore traps at the botanic garden.

Bait plants were found to be infected with *P. ramorum* on 16 occasions at site 2 under the *M. kobus* over the 2 years (fig. 2). This is not surprising because of the presence of an overhead sporulating host at this site. Site 4 bait plants, however, became infected almost as often (14 times) despite the removal of the infected host before the start of the study. This is also the case with the four infections at site 3. The bait plant infections at sites 3 and 4 have most likely resulted from inoculum splash from the surrounding infested soil, although the presence of infected plants without symptoms in these areas cannot be ruled out. The infection of bait plants indicates that the inoculum recorded under the sporulating *M. kobus* in April 11, October 11, December 11, and February 12 was viable because these were the months that the bait plants became infected (fig. 2). There were far more bait plant infections than inoculum detections in the trap, with a total of 10 instances of infection with no spore trap inoculum. The low level trap at site 3 only recorded inoculum with a bait plant infection once on April 10. There were also only three more bait plant infections at site 3 which all occurred when inoculum was not recorded in the spore trap.
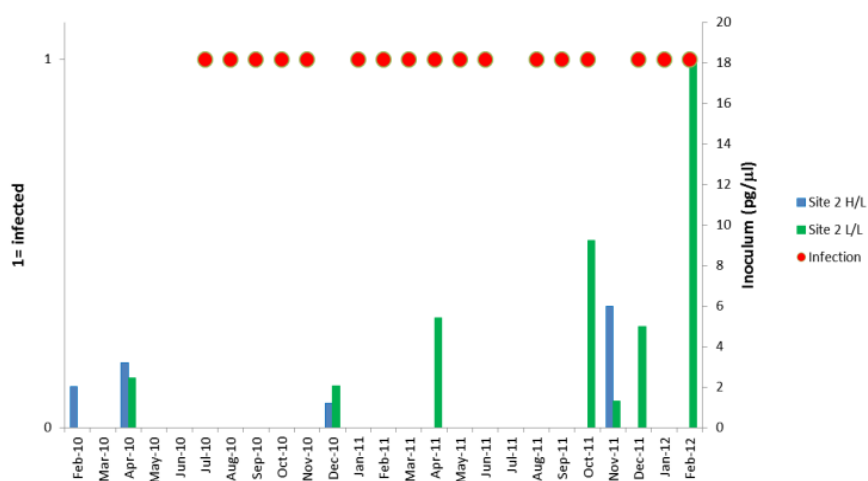

Figure 2—Spore trap inoculum (*Phytophthora ramorum*) at the botanic garden site 2 and bait plant infection.

At the castle garden, *P. kernoviae* was recorded on 12 occasions in the spore traps at site 5; nine of these were in the low-level trap and three in the high. The only other findings were in March 2010 at site 3 and site 4 (fig. 3). The high levels of inoculum recorded in the site 5 low-level traps in December 2010 (30.4 pg/µl) and January 2011 (19.7 pg/µl) were preceded by a month of high rainfall in November 2010 (total of 296 mm). The high-level traps did not record inoculum during this period, so these findings could have been due to water splashing off the soil and leaf litter around the low-level trap. Given the lack of *P. kernoviae* recorded at the botanic garden and most of findings at the castle garden occurring under the infected *Pieris*, it appears that high inoculum levels are required to be present in the environment before the spore traps pick up the presence of *P. kernoviae*. Another factor could be that there was less *P. kernoviae* soil contamination to splash up into the traps at the castle garden than there was *P. ramorum* soil contamination at the botanic garden.
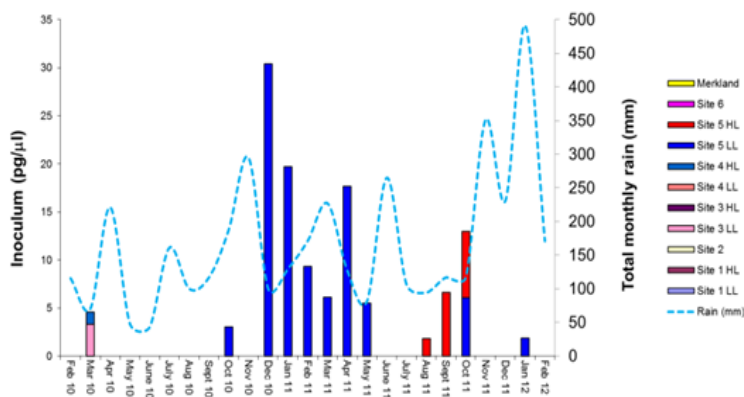
Figure 3—Incidence of *Phytophthora kernoviae* inoculum in spore traps at the castle garden with monthly rainfall.

The *Rhododendron* bait plants were largely ineffective at the castle garden. That was surprising because of the high prevalence of *P. kernoviae* infection of planted *Rhododendron* and *R. ponticum* at this garden. This could be because the bait plants were only exposed for one month whereas the *Rhododendron* plants in the garden may have been exposed for longer before succumbing to infection. It was not until a particularly susceptible batch of *Vaccinium myrtillus* bait plants were placed in the garden in January 2011 that infection started to occur. Once the *Vaccinium* bait plants were placed at site 5 in January 2011 infection was recorded in 10 of the subsequent 12 months (fig. 4). The bait plants were often very heavily infected upon their recovery from site 5b. Infection was also recorded on five occasions at site 4 which is in the heart of the original infection although the symptoms on the bait plants here were more subtle. Of the 11 instances of bait plant infection at site 5, inoculum was recorded in a spore trap (high and/or low) on six occasions. The lowest amount of *P. kernoviae* inoculum recorded whilst a bait plant was infected was 1.836 pg/µl in August 11 in the site 5 low level trap.
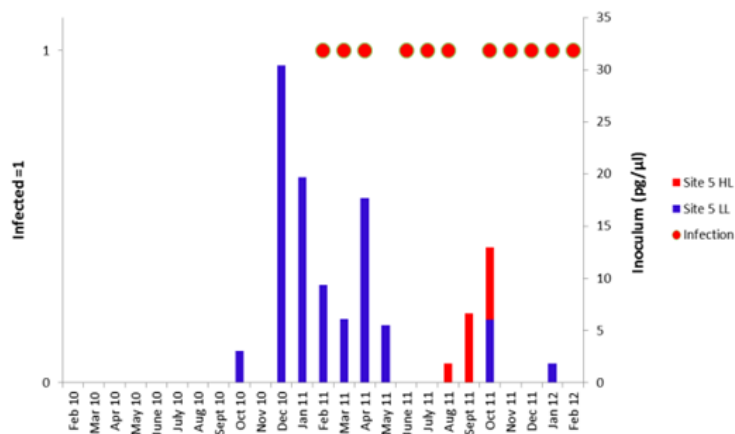

Figure 4— *Phytophthora kernoviae* inoculum in spore traps at site 5 at the castle garden and associated bait plant infection.

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Water Baits

Water baiting to establish the presence of these pathogens was only successful at detecting *P. ramorum* at the botanic garden; *P. kernoviae* was not detected in watercourses at either garden. *Phytophthora ramorum* was successfully isolated from the stream that enters the botanic garden then runs down to site 1 as follows: four times where the stream enters the garden, seven times 50 m downstream, once in the small pond, seven times above site 1, and 11 times at site 1. At the other baiting site near the horticultural sheds, *P. ramorum* was isolated 11 times. This stream originates from outside the garden at a former commercial forestry plantation above the garden. The only months where *P. ramorum* was not detected was when there was either not enough rain to fill the streams or the streams were frozen. If there was enough water in the streams for baiting, *P. ramorum* was usually detected.

Soil Infestation

The soil inoculum levels of both *P. ramorum* and *P. kernoviae* at these gardens did not deplete over the 2 year study, despite the removal of the sporulating hosts at most of the sites (fig. 5 and 6). There was some evidence of seasonal variation in *P. ramorum* soil inoculum levels at the botanic garden under the infected *M. kobus* at site 2, with findings of 11,205 pg/ml in August 2010 and 15,805 pg/ml in August 2011, but these high peaks coincide with low levels of rain, not high levels. This inoculum does not appear to 10 m away at site 3, as the average levels there remained relatively low (average 112 pg/ml) (fig. 5).
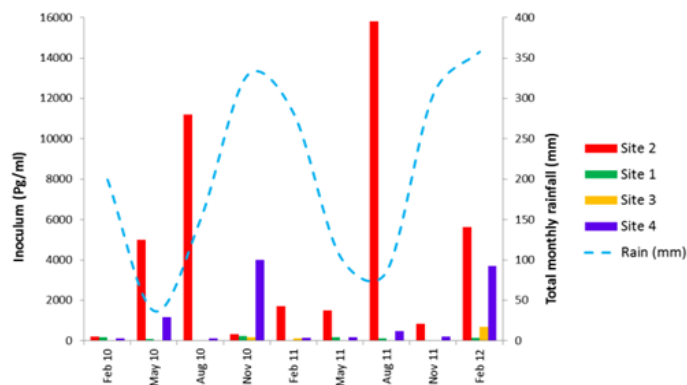

Figure 5—*Phytophthora ramorum* inoculum in soil at the botanic garden (picograms/ml) and total monthly rainfall.
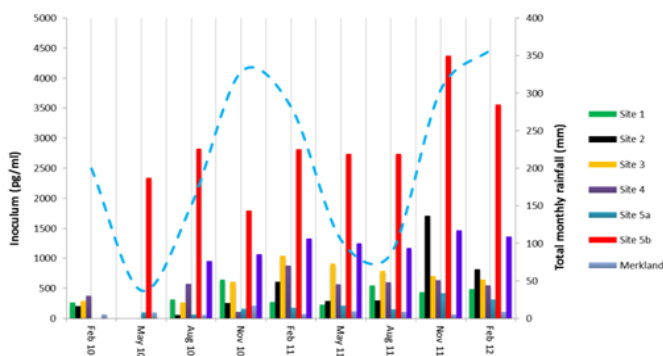

Figure 6—*Phytophthora kernoviae* inoculum in soil at the castle garden (picograms/ml) and total monthly rainfall.

There were peaks of 4,010.5 pg/ml at site 4 in November 2010 and 3,706 pg/ml in February 2012 which coincided with particularly wet months at the garden. This site is in a depression in the ground where the soil was often found to be saturated. It may be expected that, of all of sites where the host had been removed at the botanic garden, site 3 would contain the highest levels of soil inoculum (average 112 pg/ml) because it is only 10 m from sporulating *M. kobus* at site 2, but much higher levels were actually recorded at site 4 (average 1,129 pg/ml), the furthest site from this source of inoculum. The overall level of *P. kernoviae* inoculum in the soil at the botanic garden was low. It was only consistently found throughout the study period in the soil at site 1, with an average of 136.7 pg/ml. The inoculum level also depleted from 559 pg/ml in May 2010 to 74 pg/ml in February 2012. This site is where *P. kernoviae*-infected *Rhododendron* plants had been removed in 2009.

At the castle garden (fig. 6), inoculum levels at site 5b were particularly high with an initial finding of 2,347.5 pg/ml in May 2010, a maximum of 4,380 pg/ml in November 2011, and an average over the 2 years of 2,579.5 pg/ml. These findings combined with infected leaf litter on the ground indicated that one or more of the *Pieris* at this site were infected. Site 6 was added in August 2010 to measure inoculum around the stump of a large infected *Drimys* that had been removed before the study started. This was in the heart of the worst affected area and the soil inoculum levels there remained high, with an average of 958 pg/ml at site 6, 480.7 pg/ml at site 4, and 586.5 pg/ml at site 3.

The soil at the *V. myrtillus*-infected woodland site showed low levels of infestation over the 2 years with an average of 102.4 pg/ml. In addition, only small patches of soil were found to contain inoculum as opposed to parts of the castle garden where whole areas were infested, around site 4, for example.

Random soil sampling around the gardens detected very high levels of *P. ramorum* inoculum (8,540 pg/ml) near to a pond in the botanic garden, despite there being no confirmed cases of plant infections in this area. This sampling event was followed up with six more samples which were all positive; the highest level was found to be 4,670 pg/ml. The garden managers were able to confirm that the area had been redesigned the previous year and that mulch had been added to the soil. The mulch heaps in the garden were subsequently tested and *P. ramorum* was found to be present at an average level of 238 pg/ml. These findings were followed up by testing the wood chip piles which would have been used for mulching the beds. *P. ramorum* inoculum was detected in the wood chips.

On a number of occasions, the downhill sides of wooden slot drains were tested and found to contain high levels of *P. ramorum* inoculum, particularly the slot drain below the first confirmed case in the garden. This particular drain was first tested in November 2010 and a high-inoculum level of 3,975 pg/ml was found. Testing then continued over the next year, revealing that the area was heavily infested, with average inoculum levels ranging from 3,488 pg/ml to 6,305.5 pg/ml. Furthermore, the highest inoculum level found during this whole study (19,760 pg/ml) was found at this site in February 2012. This is higher than any of the samples taken from under the infected *M. kobus* (maximum 11,205 pg/ml). These drainage channels across the paths increase the amount of water on the downhill side of paths which in turn appears to concentrate the inoculum.

Random soil sampling at the castle garden detected *P. kernoviae* throughout the lower part of the garden, with 169 of the 211 samples collected containing inoculum. Most of the infections within this area were on planted rhododendron species and cultivars, or *R. ponticum*. Infested soil was also found in moderately high concentrations (510 pg/ml) in higher areas of the garden that contained no previously infected plants. It is assumed that *P. kernoviae* was introduced into these areas by horticultural activity. At the *Vaccinium*-infected woodland, random sampling uncovered no new infested areas of soil and the original patches of known infestation remained limited.

Discussion

Spore traps have been used widely for detecting the presence of plant pathogens in the past, and *P. ramorum* is no exception (Davidson et al. 2005, Hansen 2008, Turner et al. 2008).The overall findings of inoculum in spore traps at these gardens were relatively low, with only 13 percent of traps

recording the presence of inoculum. This could be due to the effects of the legislation that requires the removal of infected plants as soon as they show symptoms; the spore traps reliably detected inoculum under infected hosts. *Phytophthora kernoviae* was not detected in the spore traps at the castle garden despite the large extent of the initial infestation, which may suggest that *P. kernoviae* does not persist in the environment for as long as *P. ramorum*.

The low-level traps at the botanic garden where there were no sporulating hosts present, but where *P. ramorum* inoculum was still recovered, show that inoculum was most likely splashed from soil during rain events and into spore traps at these sites. This argument is strengthened by the frequent infections of bait plants at sites where the sporulating host had been removed. However, it must be noted that *P. ramorum* could be sporulating from an asymptomatic infected host nearby. Infection of conifer seedlings and *Rhododendron* plants via soil splash have also been found to occur under infected California bay laurel (*Umbellularia californica* (Hook. & Arn.) Nutt.) in the United States (Chastagner et al. 2008).

This study showed that both *P. ramorum* and *P. kernoviae* persist in soil in the west of Scotland for at least 2 years after their host is removed. This concurs with work carried out in other parts of the United Kingdom and further afield that have found that these pathogens persist in soil for at least 2 years (Turner et al. 2005, Shiskoff 2007, Widmer 2011; Alexandra Schlenzig, SASA, unpublished). An important consideration of inoculum survival in soil at the castle garden is that although the above ground parts of the main host, *R. ponticum*, had been largely removed, there was some evidence of regrowth and reinfection at some sites around the garden. This, combined with the possible asymptomatic infection of some of the other *Rhododendron* species present in the most infested parts of the garden, could effectively 'top up' the inoculum in the soil.

This study has also shown that infested soil is inadvertently moved around a garden by horticultural activity in infested mulch. This highlights the importance of correct composting techniques. In large scale compost heaps, Noble et al. (2011) found that *P. ramorum* had not survived after 5 days at a mean temperature of 41.9 $^{\circ}$C (32.8 $^{\circ}$C for *P. kernoviae*) or for 10 days at 31.8 $^{\circ}$C. If these composting procedures cannot be followed, horticultural practices should be modified in gardens that become infested by these pathogens so that infested material is burned and no compost or leaves are gathered to be used again.

These findings highlight the need for careful disease management to prevent the spread of these pathogens around a garden, the prevention of the establishment and spread of hitherto unknown pathogens that may be introduced in the future, and the potential risk of spread from gardens to surrounding plantations and forests.

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