Management of *Phytophthora ramorum* at a Botanical Garden in Washington State, USA

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Abstract

In March 2015 *Phytophthora ramorum* was detected at The Bloedel Reserve, a 150 acre botanical garden in Kitsap County, WA. Infected plants were destroyed and the soil in an area surrounding these plants was steam-sterilized to a depth of 15 cm during the summer of 2015. An IPM program was developed in an effort to control the spread of *P. ramorum* and other *Phytophthora* species in the garden, reduce the risk of *P. ramorum* spread to the surrounding landscape, and minimize additional destruction of valuable plants and visual impacts to the garden.

Several treatments were employed, including removing host vegetation, management of surface water, soil steaming, replanting affected areas with non-host or host plant species that have shown some resistance to *P. ramorum*, the use of *Phytophthora*-specific fungicides, and the use of *Trichoderma* biocontrol agents and mulch to reduce spread of disease from soil to plants. Surveys in the *P. ramorum* positive areas and perimeter were done during 2015-2018. Symptomatic foliage was collected and tested for *Phytophthora* using ELISA. Any ELISA positive samples were tested for *P. ramorum* with PCR. Isolates of *P. ramorum* were genotyped using microsatellite markers.

Surveys during 2015 detected *P. ramorum* on 17 plants at 12 sites. These sites were in two areas at the Reserve, the Rhododendron Glen and the Camellia Trail. Two samples from the Glen, one collected in the January survey and one in the February surveys in 2016, were positive for *P. ramorum* and all others were negative. No *P. ramorum* was found in the 2016 perimeter survey. No *P. ramorum* has been detected on plants since February 2016. Many of the earlier *P. ramorum* positives were detected on certain native hosts. In February 2016, the IPM strategy was therefore modified to include the removal of native host vegetation within the positive areas. Fungicides were applied in the positive areas during 2016-2018. The rate of ELISA+ plants was between 37% - 90% during this time period. The rate of ELISA+ samples has decreased in the positive areas since the peak of 90% in October 2016 and has stayed below the initial 72% measured in January 2016.

Fungicide applications and long term continual removal of native host plants in the positive areas, and the reapplication of Plant Helper (*Trichoderma atroviride*) in areas that were identified as higher risk due to slope and proximity of prior positive sites has been continued until the present. The Plant Helper is applied as a soil drench and then covered with a mulch that is made from chipped alder wood and other non-host material on the Reserve. Expanding the use of Trichodermas beyond previous positive areas at the Reserve is being considered to mitigate undetected infestations and limit the potential infestation of these areas.

The Birch Garden at The Bloedel Reserve. The shrub layer is made up of native plants, mainly salal (*Gaultheria shallon*).

1. ELISA+ host data to monitor *Phytophthora* and efficacy of fungicide treatments

As the monthly surveys continued during the winter of 2015-16, at least 50% of the *Phytophthora* ELISA positives detected in the Glen were on native hosts including *Gaultheria shallon*, *Mahonia* spp., and *Vaccinium* spp. In February 2016, the IPM strategy was therefore modified to include the removal of native host vegetation within the Glen area to reduce the amount of host material which could potentially become infested.

The rate of ELISA+ plants was between 37% - 90% during 2016-2018. Four hosts had high levels of ELISA+ samples in both perimeter and positive areas surveys. These were *Rhododendron*, *Gaultheria*, *Mahonia*, and *Vaccinium* (**Figures 1A and 1B**). The ELISA+ rate for all other hosts was 15% for both the perimeter and positive areas surveys. *Rhododendron* had the most ELISA+ samples in the positive areas surveys. In the perimeter surveys, *Gaultheria* and *Mahonia* had the highest rate of ELISA+ samples. These four species were sampled intensively and represent 83% of all samples taken in both positive areas and perimeter surveys.

Fungicides were applied in the positive areas during 2016-2018. Two fungicides were used in rotation, Segway (Cyazofamid 34.5%) and Stature (Dimethomorph 43.5%) (**Table 1**). Compass (Trifloxystrobin 50%) was applied at the same time as the Stature treatment starting in October 2017. Fungicides were applied to foliage using a backpack sprayer in rotation at 5 week average intervals. July or August treatments were skipped in some years. All hosts, including the native host plants, were sprayed, with the exception of sword fern (*Polystichum munitum*), because it was not considered to be a susceptible host. During the period of July – October 2016 the rate of ELISA+ increased in the positive areas and at a slower rate in the perimeter, and decreased during this same time period in 2017. Since fungicide applications in the positive areas were similar for both time periods, it is possible that the increase in 2016 was due to higher precipitation in October 2016 when compared to October 2017. This can be partially explained by the similar pattern of ELISA+ samples in the perimeter surveys, which were not treated with fungicides. The rate of ELISA+ samples has decreased in the positive areas since the peak of 90% in October 2016 and has stayed below the initial 72% measured in January 2016. Since the number of ELISA+ samples has also declined in the perimeter, which was not sprayed, it is likely that other factors, such as rainfall, are contributing to the low rate of *Phytophthora* positives.

High risk hosts include *Gaultheria shallon*, *Rhododendron*, and *Mahonia* spp.

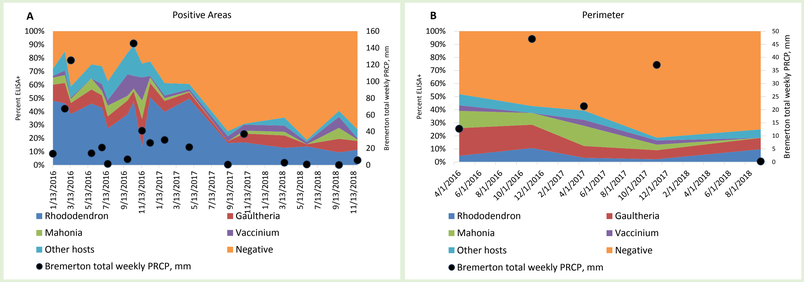


Figure 1. ELISA+ samples in the A) Positive Areas (Rhododendron Glen and Camellia Trail, Jan. 2016-June 2018) and B) Perimeter surveys (Jan 2016-Nov 2017) at The Bloedel Reserve. The four hosts with the highest rate of ELISA positives for *Phytophthora* were *Rhododendron, Gaultheria, Mahonia*, and *Vaccinium*. ELISA- samples shown in orange. Black dots = total weekly precipitation before the sampling date.

3. Evaluate *Trichoderma* spp. for effectiveness in controlling soilborne *Phytophthoras*.

Monitoring Phytophthora and Trichoderma populations

Two *Trichoderma* spp. were tested and compared to plots with no *Trichoderma* treatment (**Table 3**). Three 8' x 8' plots per treatment were established at The Bloedel Reserve. Each plot was divided into four subplots for sample collection purposes. Plots were laid out in three areas in the Reserve that have a history of *Phytophthora* infestation (**Figure 2, below**). Treatments were applied to the soil on February 1, 2016. Plant Helper (PH) was mixed according to the label directions and applied as a spray to the soil surface of three plots (**Figure 3A**). TA04-22 in a wheat bran formulation was spread over the surface of three plots, then raked into the top 2-3 cm of soil (**Figure 3B**). The six *Trichoderma*-treated plots were watered and then a 2" layer of mulch (60% wood chips, 30% aged dairy manure, 10% compost) was applied. Wood chips were made from non-host species, mostly red alder and compost was made from leaves collected onsite with the exception of the Glen and Camellia areas, which had been previously Pr+. Three plots without *Trichoderma* treatments only had the layer of mulch. *Phytophthora* populations in the soil were monitored using a buried bag technique. One week before each sampling time, rhododendron leaves were placed into mesh bags that were located at the soil surface/mulch layer interface (**Figure 3C**). Soil cores were sampled for *Trichoderma*.

Very little *Phytophthora* was detected in the plots during the one-year duration of this project. The highest level of *Phytophthora* was found at Site 3 in the Camellia Trail area of the Reserve. This was the wettest site. Soil moisture measurements during the year showed that the soil at this site was saturated for the entire time. Site 1 was the driest site, and Site 2 was between field capacity and saturated, tending to be closer to field capacity. These soil moisture levels may explain why no *Phytophthora* was detected at Site 1 and the positive detections at Sites 2 and 3. Since so little *Phytophthora* was found, it was not possible to determine efficacy of the *Trichoderma* treatments. Further studies are underway on soils that have been inoculated with *Phytophthora* under controlled conditions.

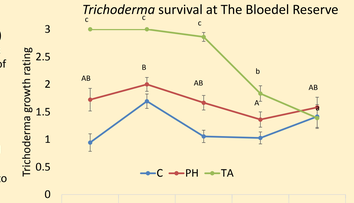
Trichoderma was recovered from soil cores from all of the treatment plots, including the non-inoculated checks during the one-year period of the study. There was no difference in survival of *Trichoderma* among the three sites. Of the three *Trichoderma* treatments, TA (*Trichoderma asperellum* in wheat bran formulation) had the highest survival rating. This difference was significant in all sampling times except for 1 year post-treatment (**Figure 4**). There was a dip in *Trichoderma* recovery for both the PH and TA treatments at the six-month measurement interval in July 2016, after which time populations increased. There was no significant difference among the sampling times in the amount of *Trichoderma* recovered from the untreated plots. *Trichoderma* recovery from the plots treated with TA dropped significantly between the 2 month and 6 month sampling periods.

Representative isolates of *Trichoderma* isolated from soil cores were sequenced to determine whether the *Trichoderma* spp. that were applied to the soils survived or were replaced by other species (**Figure 5**). *Trichoderma asperellum* (TA) had the best survival and persisted in large quantities throughout the study, although other species started to establish after one year. This organism was also detected on the C and PH plots, where the TA product was not applied.

Table 3. *Trichoderma* soil treatments used in the project.

Name	Product	Organism	Formulation	Inoculum concentration	Rate/unit area
PH	Plant Helper	*T. atroviride*	Wettable powder, aqueous suspension	100,000,000 cfu/cm^3	1 lb/100 gal water/acre
TA	Ta04-22	*T. asperellum*	Wheat bran, solid	10,000,000 cfu/g	Approximately 0.3 lb/10 ft^2
C	none	none	n/a	0	0

Figure 3. A) Aqueous suspensions of Plant Helper (*Trichoderma atroviride*) were applied to soil using a backpack sprayer. Blue flags mark the corners of each 4' x 4' subplot.
B) Formulation of *Trichoderma asperellum* in wheat bran was raked into the soil surface.
C) Mesh bags containing 3 rhododendron leaf baits were placed beneath 1 cm of soil in each subplot and exposed for 1 week, then taken to the lab for incubation, culturing, and isolation of *Phytophthora*.



It is not known whether the *T. asperellum* isolated from these plots is part of a resident population or had been moved from TA treated plots via movement of soil. *Trichoderma atroviride* (PH), was detected 60 days and one year after application. Plots treated with PH tended to have higher diversity of *Trichoderma* spp. than the untreated and TA plots.

Three species of *Trichoderma* and a closely related fungus were detected in the untreated plots. In addition to *T. asperellum*, the other species isolated were *T. hamatum, T. harzianum*, and *Clonostachys rosea* (= *Gliocladium roseum*). All of these fungi are known as biocontrol agents.

Due to the absence of data on *Phytophthora* populations at Bloedel, it is not possible to determine how effective the applied *Trichoderma* products were in reducing the populations of *Phytophthora* in soils. The larger amount of *T. asperellum* recovered from the soils, when compared with *T. atroviride*, indicates that it may be more likely to be ecologically fit and adapted to the site conditions. More research under field conditions is needed to determine what impact this might have on control of *Phytophthora*.

Figure 4. *Trichoderma* recovered from soil cores at all three sites at The Bloedel Reserve. Overall levels of all *Trichoderma* species are estimated by the *Trichoderma* growth rating scale (0 = no *Trichoderma* on plate, 1 = plate < 25% covered, 2 = plate 25-50% covered, 3 = plates 50 - 100% covered by *Trichoderma*). C = control, no *Trichoderma* added; PH = Plant Helper (*T. atroviride*); TA = *T. asperellum*. Points for each treatment at a given sampling time with different letters are significantly different at p=0.05 (ANOVA, Tukey HSD test). Details about the treatments are given in **Table 3**.

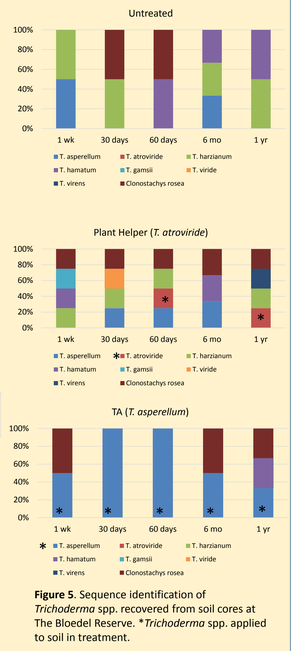


Figure 5. Sequence identification of *Trichoderma* spp. recovered from soil cores at The Bloedel Reserve. **Trichoderma* spp. applied to soil in treatment.

Plants in the Rhododendron Glen that are treated with fungicides on a regular basis.

Table 1. Fungicides and application rates used in the positive areas at The Bloedel Reserve between January 1 2016 - May 31 2018. Fungicides were applied to foliage until runoff using a backpack sprayer in rotation at 5 week average intervals.

Fungicide	Segway	Stature SC	Compass
Active ingredient	Cyazofamid 34.5%	Dimethomorph 43.5%	Trifloxystrobin 50%
FRAC group	21	40	11
Rate applied	6 fl oz/100 gal	12.25 fl oz/100 gal	2 oz/100 gal
Manufacturer	FMC Agricultural Products Group	BASF Corp.	Bayer Environmental Science

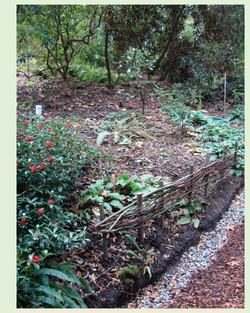
Summary

Continuing IPM practices include:

- Long term continual removal of native host plants in the Glen.
- *Phytophthora*-specific fungicide applications in the positive areas (Glen and Camellia Trail).
- Reapplication of Plant Helper (*Trichoderma atroviride*) in high risk areas.
- SOPs for reducing soil movement between areas such as signage and fencing for visitors, and sanitation for workers.
- Removal of prunings, fallen leaves, and other host material to prevent contamination of plantings.

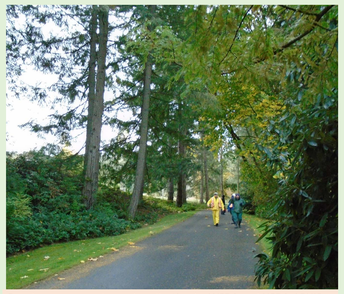
Possible future IPM practices:

- Ground applied systemic fungicide in Glen and Camellia Trail (but test for interactions with *Trichoderma* first).
- Expand areas where *Trichoderma* and mulching treatments are applied.



Improved drainage and low fencing in positive areas.

Signage to encourage visitors to stay out of planting areas, to prevent soil movement.



Regular surveys and sample collection by WSDA and USDA-APHIS personnel until it has been 3 years since the last plant positive.

Removal and safeguarding of plant material, and sanitation for workers tools and footwear in positive areas.

2. Genotypes of *P. ramorum* at The Bloedel Reserve

Multilocus genotypes were determined from *P. ramorum* collected at Bloedel and compared to other regulated sites, such as positive nurseries to examine the potential source of the Bloedel infestation, movement of the pathogen within the Reserve and the possibility of new introductions, as well as the effectiveness of mitigation practices. Seven NA1 microsatellite genotypes of *P. ramorum* were detected at Bloedel between March 2015 – February 2016 (**Table 2**). The two most commonly found genotypes were NA1-A13 (23 samples) and NA1-A17 (22 samples), which were identical to the genotypes of *P. ramorum* from a nursery in Clark County (2008) and a nursery in King County (2006), respectively. A *P. ramorum* isolate in the WSU collection taken from tanoak in Curry Co., OR was also typed as NA1-A17.

The remaining five genotypes have only been detected at Bloedel and are very similar to NA1-A13 and NA1-A17. These are probably derived from NA1-A13 and NA1-A17, rather than being new introductions. One sample from the first *P. ramorum* positive site (Site 1), which was the most intensively sampled, was a mixture of NA1-A13 and NA1-17, where the two original genotypes were also found. It is possible that *P. ramorum* moved from Site 1 to the Rhododendron Glen, and then to the Camellia Trail area based on the genotypes that were detected (**Figure 2**). Also, genotypes similar to NA1-17 were only found at Site 1, where genotypes similar to NA1-13 were found at all 3 locations.

Reflecting Pond

Table 2. Microsatellite markers used for genotyping *P. ramorum* isolates from The Bloedel Reserve. Eight NA1 microsatellite genotypes of *P. ramorum* were detected. Shaded cells in each row are different from the NA1-A13 genotype in the first column.

Marker	MLG / Repeat region	NA1-A13	NA1-A33	NA1-A34	NA1-A17	NA1-A30	NA1-A31	NA1-A32
		Allele sizes						
PrMS6	$(CGA)_8$	165/168	165/168	165/168	165/168	165/168	165/168	165/168
Pr9C3	$(CA)_{15}$	216/226	216/226	216/226	216/226	216/226	216/226	216/226
PrMS39	$(GA)_{11}$/ $(GA)4(GATA)_{33}$	130/250	130/250	130/250	130/250	130/250	130/250	130/250
PrMS45	$(TCCG)_{11}$	166/186	166/186	166/186	166/186	166/186	166/186	166/186
PrMS43	$(CAGA)_{71}$	376/485	376/489	376/493	356/485	356/489	356/485	356/485
Locus18	$(AC)_{39}$	219/275	219/275	219/275	219/275	219/275	219/275	219/275
Locus64	$(CT)_{16}$	342/379	342/379	342/379	342/379	342/379	342/379	342/379
ILVOPrMS131	$(AGAC)_{37}$	150/228	150/228	150/228	150/228	150/228	150/232	150/224

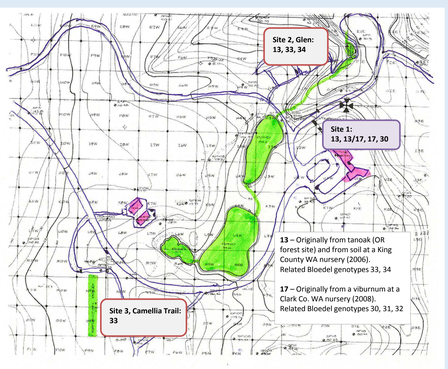


Figure 2. *Phytophthora ramorum* genotypes and their locations at The Bloedel Reserve. Other locations in WA and OR where genotypes 13 and 17 have been detected before 2015 are shown with the related genotypes 30-33 found only at Bloedel since 2015. All genotypes belong to the NA1 clonal lineage of *P. ramorum*.

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