

Effect of Chemicals on Hyphal Growth, Sporangia Production and Zoospore Germination of *Phytophthora ramorum*

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Introduction

Phytophthora ramorum, causal agent of Sudden Oak Death, has 23 regulated hosts. Some of these, such as coast live oak, tanoak and bay laurel, play a key role in the Californian forest ecology. There is one commercially available preventative treatment for two hosts-coast live oak and tanoak. New treatments that employ easier and cheaper application methods and protect both foliar and trunk/twig hosts are needed. Other products in the market registered to control other *Phytophthora* pathogens may be effective for controlling *P. ramorum*. This work will look at inhibition of different stages in the life cycle of *P. ramorum* (Figure 1).

Materials and Methods

In vitro studies

1. Hyphal growth inhibition

We used 12 isolates of *P. ramorum* (5 replications/strain) (Table 1). V8 agar was amended with different chemical agents (Table 2) at 4 dilutions EC10 (the concentration that will reduce colony size by 10 percent), EC50 and EC90 (real concentrations appear on Figure 3). The dilutions were calculated in a preliminary test that was done on plate Pr1. A 3 mm plug was placed at the center of each plate which was incubated at 22°C. Colony diameter was measured after 10 days (Figure 3).

2. Sporangia formation inhibition test

3 mm plugs of the isolates (Table 1) were placed in the center of each well of a 24 well plate. On each plug we poured 350µL of diluted chemical (Agrifos400 only) (figure 5). The plates were left to incubate for 72 hours in 20°C. Additional sporangia formation was stopped using cotton blue stain and sporangia were counted using a microscope.

3. Zoospore germination inhibition test

Same as above, except we used corn meal agar with concentrations as appear in Figure 7. 100µl of zoospore suspension was spread on each plate. Plates were read after 48 hours or put in 4 °C to suppress further growth.

In planta studies

1. Sapling injection experiments

6 feet tall saplings were treated a week before inoculation using a 10 ml Agrifos400 injection with 14 percent active ingredient. The trees were inoculated with the 12 isolates (Table 1). For each isolate we had inoculated 5 treated trees and 5 untreated trees. The lesion size was measured 6 weeks after inoculation.

2. Branch treatment

Bay laurel (*Umbellularia californica*) one meter branches were treated with Agrifos400, Champ, biological control (AgraQuest and TreeHelper) or water. The branches were placed under moist condition in a cool green house. 15 leaves were taken from each treatment and were inoculated with 2*10⁹ zoospores per milliliter concentration. The leaves were taken 1, 3, 7, 14, 21 and 28 days after treatment. The leaves were incubated on moist paper towels for 9 days. The leaves were scanned and analyzed using image analysis software Assess (APS, Winnipeg, Manitoba, Canada).

Data Analysis

The experiments were analyzed using analysis of variance (ANOVA). When comparing lesion or colony size or percent from control we used a log₁₀(X+1) transformation.



Figure 1- Life cycle of *P. ramorum* and possible inhibition steps.

Results

In vitro studies

1. Hyphal growth inhibition

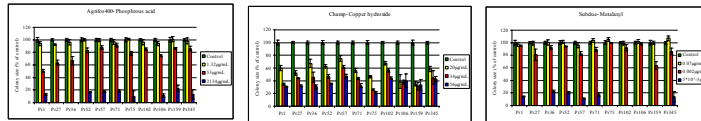


Figure 3- Mean colony diameter, represented as a percent of control, of *P. ramorum* ten days after inoculating petri dishes in different concentration and different isolates. The error bars stand for the standard deviation.

The tables below show the ANOVA analysis in respect to each graph above.

2-way ANOVA demonstrating effects of isolate, concentration and their interaction on the dependent variable log₁₀(control+1).
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ANOVA of chemical concentration across all isolates.
 Dependent variable is log₁₀(control+1).
 2Way, log, 10 0.00003 0.00000 4.192 0.0004
 17.9µg/ml, 10 0.00002 0.00000 12.253 0.0001
 33.8µg/ml, 10 0.00002 0.00000 12.253 0.0001
 215.9µg/ml, 10 0.00002 0.00000 12.253 0.0001

Hyphal growth control

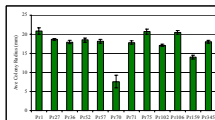


Figure 4- Mean colony diameter of *P. ramorum* ten days after inoculating petri dishes in the water control.

The tables below show the ANOVA analysis in respect to the graph above.

ANOVA of control plates across all isolates.
 Dependent variable is log₁₀(control+1).
 2-way, log, 10 0.00002 0.00000 83.102 0.0001

2. Sporangia formation inhibition test

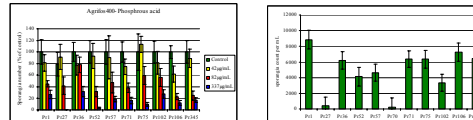


Figure 5- Mean colony sporangia count, represented as a percent of control, of *P. ramorum* ten days after inoculating petri dishes in the water control.

The tables below show the ANOVA analysis in respect to the graph above.

2-way ANOVA demonstrating effects of isolate, concentration and their interaction on the dependent variable log₁₀(control+1).
 2-way ANOVA demonstrating effects of isolate, concentration and their interaction on the dependent variable log₁₀(control+1).
 2-way ANOVA demonstrating effects of isolate, concentration and their interaction on the dependent variable log₁₀(control+1).

ANOVA of chemical concentration across all isolates.
 Dependent variable is log₁₀(control+1).
 2Way, log, 10 0.00002 0.00000 7.867 0.00000
 10.0µg/ml, 10 0.00002 0.00000 7.867 0.00000
 20.0µg/ml, 10 0.00002 0.00000 7.867 0.00000
 40.0µg/ml, 10 0.00002 0.00000 7.867 0.00000
 80.0µg/ml, 10 0.00002 0.00000 7.867 0.00000

3. Zoospore germination inhibition test

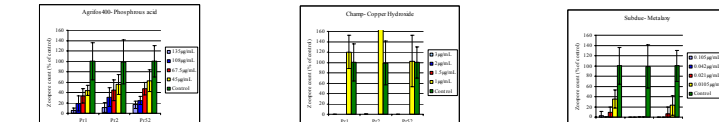


Figure 7- Mean zoospore count, represented as a percent of control, in different concentration and different isolates. The error bars stand for the standard deviation. The tables below show the ANOVA analysis in respect to each graph above.

2-way ANOVA demonstrating effects of isolate, concentration and their interaction on the dependent variable log₁₀(control+1).
 2-way ANOVA demonstrating effects of isolate, concentration and their interaction on the dependent variable log₁₀(control+1).
 2-way ANOVA demonstrating effects of isolate, concentration and their interaction on the dependent variable log₁₀(control+1).

ANOVA of chemical concentration across all isolates.
 Dependent variable is log₁₀(control+1).
 2Way, log, 10 0.00002 0.00000 2.379 0.1198
 0.4µg/ml, 2 0.00002 0.00000 2.379 0.1198
 0.7µg/ml, 2 0.00002 0.00000 2.379 0.1198
 1.4µg/ml, 2 0.00002 0.00000 2.379 0.1198
 2.8µg/ml, 2 0.00002 0.00000 2.379 0.1198

ANOVA of chemical concentration across all isolates.
 Dependent variable is log₁₀(control+1).
 2Way, log, 10 0.00002 0.00000 2.25 0.1248
 1µg/ml, 2 0.00002 0.00000 2.25 0.1248
 2µg/ml, 2 0.00002 0.00000 2.25 0.1248
 4µg/ml, 2 0.00002 0.00000 2.25 0.1248
 8µg/ml, 2 0.00002 0.00000 2.25 0.1248

ANOVA of chemical concentration across all isolates.
 Dependent variable is log₁₀(control+1).
 2Way, log, 10 0.00002 0.00000 1.638 0.2109
 0.010µg/ml, 2 0.00002 0.00000 1.638 0.2109
 0.020µg/ml, 2 0.00002 0.00000 1.638 0.2109
 0.040µg/ml, 2 0.00002 0.00000 1.638 0.2109
 0.080µg/ml, 2 0.00002 0.00000 1.638 0.2109

In planta studies

1. Sapling inoculations

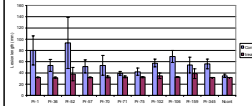


Figure 8- Mean lesion size (Square-Root-Forest-Width) represented as a percent of control, of *P. ramorum* 6 weeks after inoculating in different treatments and different isolates. The error bars stand for the standard deviation. The tables below show the ANOVA analysis in respect to the graph above.

ANOVA of control treatment across all isolates.
 Dependent variable is log₁₀(control+1).
 2-way, log, 10 0.00002 0.00000 4.737 0.0001

2. Branch treatment

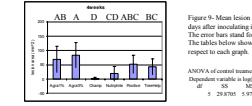


Figure 9- Mean lesion area of *P. ramorum* 9 days after inoculating in different treatments. The error bars stand for the standard deviation. The tables below show the ANOVA analysis in respect to each graph.

ANOVA of control treatment across all isolates.
 Dependent variable is log₁₀(control+1).
 2-way, log, 10 0.00002 0.00000 10.941 0.0001

Discussion

In the control treatments, there is a significant difference among the various isolates for hyphal growth (colony size) and sporangia production (Figure 4 and 6).

The response of *P. ramorum* hyphal growth, as a percent of the control, to the different chemical treatments shows a range of sensitivity within the different isolates. In the highest concentration used in this experiment, both in the Agrifos400 test and in the Subdue test, some of the isolates we used could grow and some could not. *P. ramorum* isolates might have more tolerance to higher concentrations of phosphoric acid than *P. cinnamomi* (Wilkinson 2001).

Sporangia production and zoospore germination is suppressed when using lower concentrations of chemicals compared to hyphal growth inhibition (Figure 5 and 7). The different isolates did not have a significantly different response to the chemicals at some concentrations. This might be due to the large standard deviation.

Lesion size was significantly reduced on coast live oak saplings that were treated with Agrifos400 (Figure 8) using a relatively small dose. By looking at the effect on the 12 different isolates that were screened we could not monitor any isolate to show much more tolerance compare to the others.

Copper hydroxide has shown to give a complete protection against *P. ramorum* zoospore infection on treated bay leaves up to four weeks from the initial treatment (Figure 9). phosphoric acid has shown to reduce the lesion size, but not completely. The biological control agents (AgraQuest and Tree Helper) were a very effective control *in vitro* but there was no reduction in lesion size when the products were applied in *planta*. The copper treatment led to bacterial contamination (Figure 10) and the phosphoric acid caused a severe phytotoxicity which is a common phenomena (Figure 11).



Figure 10- Bacterial contamination on Bay laurel leaves treated with Copper Hydroxide (Champ)

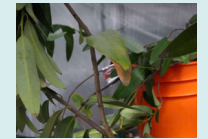


Figure 11- Phytotoxicity on Bay laurel leaves treated with Phosphoric acid (Agrifos400)

Conclusions

- We have found that different isolates can react differently to the different chemical concentrations, some being more tolerant than others.
- Zoospore germination and sporangia formation were more sensitive to chemicals than hyphal growth. This is very important because we suspect that most natural infection occurs by zoospores.
- Some of the less effective compounds were not expected to be effective *in vitro*, because they need to be transformed *in planta*. Phosphoric acid was found to be a very effective preventative treatment in the field, yet less effective *in vitro*. Copper Hydroxide was a very effective treatment *in vitro*, but so far was not effective in field studies as a coast live oak trunk treatment. However, it was effective as a foliar spray treatment on a foliar host.

Literature cited

- Garbelotto M, Rizzo DM and Marais L. 2002 Phytophthora ramorum and SOD: IV. Preliminary studies on Chemical control. In: Stanfor R, McCarry D, Purcell KB (eds.) Proc 55th Oak symposium: oak in California changing landscape. Oct 22-5 2001. San Diego CA USDA forest service. Gen Tech PSW-GTR 184-811-8
- Wilkinson CJ, Shearer BL, Jackson TJ and Hardy StJ, 2001 Variation in sensitivity of western Australian isolates of Phytophthora cinnamomi to Phosphite *in vitro*, Plant pathology 50: 83-9

Acknowledgments

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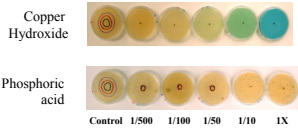


Figure 2- Petri dishes containing different compound concentrations. Marked lines indicate measurements of colony size.

Table 2-Chemical compounds and their concentrations. Active ingredient (ΔL)	1x concentration (%ΔL)
Phosphoric acid (Agrifos400®)	13.74
Metazalol (Subdue®)	10.65
Copper Hydroxide (Champ®)	3.00

Table 2- Isolates used in the experiments	Isolate #	Source	State, county	Year	Host	ATP	ATP/ [genotype]
Pr-1	Chovera	Agrifosa	California, Marin	2000	A2	1	
Pr-27	Q. agrifolia	California, Marin	2000	-	1		
Pr-36	Q. agrifolia	California, Sonoma	2000	A2	1		
Pr-52	Mitochondria on sp.	California, Santa Cruz	2000	A2	4		
Pr-57	Umbellularia californica	California, Santa Clara	2001	A2	1		
Pr-70	Frustrum	California, Monterey	2001	-	1		
Pr-71	Q. agrifolia	California, Sonoma	2001	A2	2		
Pr-73	Q. agrifolia	California, Monterey	2001	A2	1		
Pr-102	Q. agrifolia	California, Marin	2001	-	1		
Pr-106	Umbellularia californica	California, Sonoma	2001	A2	8		
Pr-109	Umbellularia californica	Uganda, Busoga	2001	-	4		

* Pr-106 and Pr-109 were from collection obtained at University of California, Davis.
 ** Q. L.P. genotype described in Harnik et al. 2004.
 † Genotype not described for both A1 and A2 isolate