Biocontrol of Phytophthora Root Rot of Avocado with Mulch and Biocontrol Agents

New Project: Year 1 of 3

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Benefit to the Industry

Biocontrol may provide an effective, long-term, non-chemical, environmentally acceptable method of controlling Phytophthora root rot of avocado through a combination of cultural methods and application of selected microorganisms. This approach will probably be most effective as part of an integrated system of resistant rootstocks, sanitation and cultural methods.

Objectives

1. Identify new biocontrol agents using conventional and new molecular biology methods.
2. Evaluate new biocontrol agents in the greenhouse and field.

Summary

Identification and Isolation of new biocontrol agents for suppression of Phytophthora root rot

1. <u>Survey for suppressive soils in California</u>

A survey of California avocado groves is continuing to identify local groves, which have soils which are suppressive to *Phytophthora cinnamomi*. Two criterions are used to identify a suppressive soil. It is a soil, which degrades *P. cinnamomi* hyphae or chlamydospores, or one which has high populations of *Phytophthora* but the trees continue to thrive. Thirty groves have been surveyed with four showing suppressiveness to *Phytophthora.* Individual trees in other groves also show suppressive characteristics. We have identified two microorganisms which are directly pathogenic to *P. cinnamomi. Rozella sp.* and *Lytobacter mycophilus* are known parasites of fungi and will attack and kill *P. cinnamomi* under laboratory conditions. These cultures have not survived in laboratory cultures. Efforts are now being made to recover them. We are now contacting experts who work with these organisms to obtain cultures from them.

We are utilizing several experimental approaches to extract biological control agents from local suppressive soils. One of them is to identify bacteria and fungi from soil mixtures with varying degrees of suppressiveness. We postulate that the microorganisms involved in the suppressiveness will be discovered by identifying species whose population levels correlate positively with the suppressiveness. One soil we are studying for this work is located in Somas CA and has been shown to consistently suppress *P. cinnamomi* for many years. We have created

soils with varying level of suppressiveness by heating the Somas CA soil to 21, 45, 60, 75, and 90 degrees. A greenhouse trial was set up with this microbially suppressive soil in which the soil was subjected to this same range of temperatures in order to determine at what point the suppressive effect disappeared. After takedown, the results showed a gradual decline in the percentage of healthy roots with increasing temperature, which indicated that the suppressiveness was declining with increased temperature. Samples of each soil treatment were taken to the lab, and *P. cinnamomi* hyphal mats were buried in the soil for one week. One ml of mat suspension was then plated onto various media to check for the predominant fungi and bacteria, which attacked *Phytophthora* at each temperature. *Galactomyces* was most common at the lower temperatures, *Verticillium* and *Trichoderma* at the higher temperatures and *Fusarium* was present throughout all temperature treatments. *Verticillium, Fusarium* and *Trichoderma* are all known antagonists of *Phytophthora* but their presence could not be correlated with suppressiveness. According to these results *Galactomyces* populations correlated well with suppressiveness. Bacterial identification of culture-based organisms and molecular identification of fungal and bacterial organisms were also done. Molecular examinations of the microbial communities confirmed *Galactomyces* as a major component of the suppressive soil. Molecular examinations led to the discovery of several other organisms whose populations correlate with suppressiveness. These organisms are related to *Chaetomium*, *Monographella*, *Galactomyces*, *Cryptococcus, Chytridium polysiphoniae* and beta-*Proteobacteria*. *Chytridium* is known to parasitize fungi and *Chaetomium* and *Mongraphella* are known to suppress *Phytophthora.* Subsequent studies will include verifying their association with the *P. cinnamomi* suppressiveness and then testing their ability to control *P. cinnamomi* in greenhouse and field trials. Future experiments will also endeavor to identify additional organisms from this soil.

This Somas CA soil demonstrating microbial suppression of *Phytophthora cinnamomi* was found from a survey of four avocado soils during 2001 and 2002. In 2001, lab trials demonstrated what appeared to be a seasonal effect of the suppressiveness, it was low in January, slowly increasing to a high in July, and then decreasing again in October. It was hypothesized that this pattern was due to the activity of the suppressive microbes in the soil which were less active during the winter months and more active in the summer. These lab trials were repeated in 2002 and this pattern of suppression was no longer evident. It appears that factors other than season are responsible for the fluctuations in suppressiveness. Soil enzymes such as cellulase and laminarinase were measured under different suppressive conditions. Soil enzymes were low under all soil conditions, and did not correlate with suppressiveness. It was concluded that soil enzymes are important *Phytophthora* inhibitors in mulches, but not in unmulched soil. Soil respiration, a measure of microbial activity, was measured under several suppressive conditions and did not correlate with soil suppressiveness to *Phytophthora.* It is concluded that microbial activity is not responsible for suppressiveness of soil to *Phytophthora.* Length of time after irrigation was also investigated and was not shown to affect the suppressiveness. The fact remains that the suppressiveness in this soil fluctuates seasonally and appears to have a patchy distribution in the field. These findings have not yet been adequately explained and are making the study difficult.

2. Searches where epidemics of *P.cinnamomi* have occurred

Epidemics of *Phytophthora* in individual groves have been studied. Populations of *P. cinnamomi* appear to decline precipitously immediately behind the leading edge of the epidemic in some

groves. Biocontrol fungi are being isolated from this region. We postulate that microorganisms, which fill a niche similar to that of the pathogen or that compete for chemical substrates important to the pathogen, such as root exudates, will be effective biological control agents. To identify such organisms, we have developed an in situ, culture-independent strategy to identify bacteria and fungi that rapidly grow in response to specified chemical substrates in environmental samples. To test this approach, we examined soils from a southern Californian avocado grove where a *Phytophthora cinnamomi* epidemic has produced three distinct zones. In Zone 1 where the pathogen epidemic has occurred, the trees are dying or dead but there is very little recoverable *Phytophthora*. Zone 2 is where the advancing margin of the *Phytophthora* epidemic is located and where the trees are relatively healthy but the roots are becoming infected and *Phytophthora* populations are high. In the region ahead of the advancing margin (Zone 3), the trees are healthy and the soil is conducive to avocado root rot. In Zone 1, microorganisms are reducing the populations of *P. cinnamomi* and the soil has become suppressive. Soil in Zone 1 is 467% more suppressive to *Phytophthora cinnamomi* than was Zone 3. Soil from Zone 1 is 10 fold more suppressive than fumigated soil that has removed all microorganisms. Using a substrate utilization approach, we molecularly identified six bacteria which responded to root exudates of avocado <u>and</u> were present only in Zone 1, where the *Phytophthora* epidemic had passed through. Four of these bacteria had varying similarities to *Bacillus mycoides*. The other two species were *Renibacterium salmoninarum* and *Streptococcus pneumoniae*. We have recovered four of these *Bacillus sp.* and are testing them in greenhouse trials. *Trichoderma aureovirde, Trichoderma harzianum, Gliocladium virens* and *Hyphodontia alutacea,* which were recovered behind the leading edges of *Phytophthora* epidemics, greatly damage *Phytophthora* chlamydospores. We are continuing testing of these biocontrol agents. New epidemic sites for testing have been identified. Using the new species specific DNA probes, many potential biocontrol agents are continuing to be identified from decomposing mats of *P. cinnamomi* hyphae. Attempts are continuously being made to isolate these microorganisms.

3. <u>Foreign exploration</u>

The rain forest of Papua New Guinea is thought to be the center of origin for *P. cinnamomi.* We have enlisted the aid of Bob Tombe, University of Goroka, Papua New Guinea, to cooperate with us and send samples every two months for the next two years. Unfortunately Dr. Tombe was robbed and lost his rented vehicle while collecting samples for us, so the cost and effort to get these samples has been great. We received only two new shipments of soil from New Guinea. Results from these shipments were rather disappointing with only a few soils showing suppressiveness to *Phytophthora cinnamomi.* These results were far less impressive than results we obtained last year. However, a group of these soils from the Mt. Kubor region of New Guinea gave exceptional suppressiveness. *Phytophthora* placed in these soils became vacuolated and died very quickly. Even chlamydospores were killed. We have now isolated the microorganisms responsible and with Dr Borneman's molecular technique we have identified two species of Pseudomonas - *Pseudomans tolaasii* and *Pseudomonas costantinii.* These bacteria are known pathogens of fungi. Experiments with *Pseudomonas costantinii*, show that this organism can severely damage *Phytophthora cinnamomi* hyphae *(*Table 1) and can damage up to 93% of the *Phytophthora* chlamydospores. However, *Pseudomonas costantinii* functions much better at high temperatures and damages nearly three times more chlamydospores at 30 C than at 15 C. Other experiments with *Pseudomonas costantinii* indicate that it significantly more inhibitory to *Phytophthora cinnamomi* in natural soil than it is in autoclaved soil. It also is more inhibitory to

Phytophthora cinnamomi in saturated and moist soils (field capacity) than in dry soils. We are now trying to arrange for quarantine facilities in which to test this biocontrol agent in greenhouse tests.

Contacts have been made in Malaysia and we received a shipment of 21 soils from that country. However, none of the soils were suppressive to *Phytophthora cinnamomi.*

4. <u>Commercial biocontrol agents</u>

No commercial biocontrol agents were tested this year.

Evaluation of biocontrol agents

The biocontrol agent *Pseudomonas putida*, which is known to parasitize *Phytophthora* has been rigorously studied in the greenhouse (Table2). Avocados were infested with *Phytophthora cinnamomi* and treated every two weeks with suspensions of *Pseudomonas putida.* This mimics treatment through the irrigation water with an EcoSoils bioject machine. Healthy roots of *Pseudomonas putida* treated *Persea indica* were 3.6 times healthier and weighed 1.7 times more than controls treated only with *Phytophthora cinnamomi.* Healthy roots of *Pseudomonas putida* treated Topa Topa roots were 2.4 times healthier and weighed 1.5 times more than controls treated only with *Phytophthora cinnamomi.* However, to put this experiment into perspective, Healthy roots of Ridomil treated *Persea indica* were 8.6 times more healthy and weighed 2.4 times more than controls treated only with *Phytophthora cinnamomi.* Healthy roots of Ridomil treated Topa Topa were 4.2 times more healthy and weighed 1.7 times more than controls treated only with *Phytophthora cinnamomi.* While it is apparent that *Pseudomonas putida* can significantly reduce the affects of avocado root rot, results with the biocontrol agent do not yet match those of commercial fungicides. However, *Pseudomonas putida* reduced populations of *Phytophthora cinnamomi* by 42% in the Topa Topa experiment and by 89 % in the *Persea indica* experiment. Perhaps over time field applications of the biocontrol agent *Pseudomonas putida* could be effective.

The EcoSoils Bioject machine has been thoroughly tested and has been found to produce high quality biocontrol agent inoculum in the field and deliver it reliably in the irrigation water. Large acreages can be treated by this machine since numerous treatments with low density inoculum of biocontrol agents eventually results in the same soil populations as numerous high density treatments. Continuous application of biocontrol agents in the irrigation water results in far better soil colonization than does single applications. A field project to test the efficacy of continuous applications of a biocontrol agent on avocado root rot has been established at South Coast Field Station. The biocontrol agent is the BpO2 *Bacillus sp.* (P. Douillet Co.), which showed promise as a biocontrol agent of *Phytophthora cinnamomi* in greenhouse tests in previous years. The trial is progressing well after one year, but root rot is not yet severe. While trees treated with phosphorous acid are significantly more healthy than controls, trees receiving the biocontrol agent are not. Control soil has 14% of the leaf disc baits colonized by *P. cinnamomi,* the soil treated with the biocontrol agent has 10% colonized, while the phosphorous acid soil has 8%. These values are not statistically different from one another.

Conclusions

We have not yet identified an effective biocontrol agent of *Phytophthora cinnamomi,* but we feel that we have made great progress. We have identified three sources of soil suppressiveness: 1) Suppressive soils from Southern California, 2) Soils that have experienced an avocado root rot epidemic, and 3) Soils from the region where *P. cinnamomi* originated, namely Papua New Guinea. Soils are now regularly being examined from all of these sources. Some of these sources are 10-fold more suppressive than the same soils which are fumigated to remove the biocontrol microbes. In some of the soils the microbes and the suppressiveness are transferable to other soils. We are regularly generating new, strong biocontrol candidates such as *Rosella sp., Pseudomonas constantinii* and *Lytobacter mycophilus* which are vicious predators of *P. cinnamomi* in the laboratory. Using new and novel microbial techniques we have identified, isolated and tested our first microbes from suppressive soil. We are now poised to initiate an assembly-line procedure to isolate and test new biocontrol organisms for use against *Phytophthora cinnamomi*. There probably are several types of soil suppressiveness toward *P. cinnamomi* and not all of them appear to be of microbial origin. *Pseudomonas putida* has been rigorously tested and has proven to be a fair biocontrol agent of *Pseudomonas putida*. We will initiate field tests with it in the future.

Table 1. Effect of temperature on the ability of *Pseudomonas constantinii* isolated from Papua New Guinea soil to damage *Phytophthora cinnamomi* [1]

Temperature	Mycelial mat rating (0-5; 5=degraded)	Damaged chlamydospores (Percent)	Mycelial colonies (per ml suspension)
15 C	1.42 e	34 d	361.2 a
20 C	2.02 d	58 c	532.8 a
24 C	2.71 c	70 b	483.3 a
28 C	3.65 b	86 a	316.7 a
30 C	4.21 a	93 a	289.0 a

[1] Mean values in each column followed by identical letters are not statistically different according to Waller's k-ratio t test.

Table 2. Effect of *Pseudomonas putida* and fungicide Ridomil Gold on *Persea indica* and Topa Topa seedlings inoculated with *Phytophthora cinnamomi* [1]

Treatments	*Persea indica*			Topa Topa		
	Root rating (0-5; 5=dead)	Root weight (gm dry wt)	Shoot weight (gm dry wt)	Root rating (0-5; 5=dead)	Root weight (gm dry wt)	Shoot weight (gm dry wt)
Control	86.14 a	2.61 a	6.30 a	94.93 a	10.40 ab	14.34 a
P. cinn alone	9.47 c	0.86 e	2.84 d	20.97 c	5.12 d	10.91 b
Ps. Putida alone	91.80 a	2.49 ab	4.81 bc	95.00 a	11.49 a	13.61 ab
Ridomil alone	90.67 a	2.15 abc	5.15 b	94.13 a	10.30 ab	12.51 ab
P.cinn + Ps putida	33.83 b	1.46 d	3.22 d	50.33 b	7.60 cd	15.07 a
P.cinn + Ridomil	81.53 a	2.09 bc	4.77 bc	87.77 a	8.60 bc	13.38 ab
Ps putida + Ridomil	83.43 a	1.74 cd	4.17 c	90.27 a	8.21 bc	15.15 a

[1] Mean values in each column followed by identical letters are not statistically different according to Waller's k-ratio t test.