Developing Improved Strategies for Management of Replant Problems

Project No.: **08-PATH1-Browne/Kluepfel**

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Objectives:

1. Determine the biological causes of and environmental contributions to replant disease (RD).
2. Support development of improved management strategies for RD and other replant problems.

Interpretive Summary:

In the 2009 project year we examined causes of replant disease (RD) on almond (Objective 1) by: 1) using culture-based diagnostics (i.e., based on microbe isolation in Petri dishes) and culture-independent diagnostics (i.e., based on DNA amplification and sequencing) methods to detect and identify "suspect" organisms that show up at elevated incidence in RD-affected root systems as compared to that in healthy root systems, and 2) testing the ability of the suspect microbes to cause RD by measuring their effects on growth and health of Nemaguard peach rootstock in greenhouse experiments. To date, both the population studies and pathogenicity trials have provided evidence that some isolates of *Cylindrocarpon destructans* (a true fungus) and *Pythium helicoides* (an oomycete, closely related to *Phytophthora*) contribute to development of RD, at least at some sites. We are using new sets of PCR primers (short DNA strands that "amplify" small amounts of DNA by the polymerase chain reaction [PCR]), some designed in our lab and others reported in the literature, to increase the breadth of our DNA-based examinations of RD-associated microbe populations. To support development of improved management approaches for RD and other replant problems (Objective 2), we monitored results of several orchard replant trials testing fumigant- and non-fumigant-based pre-plant treatments. To date, results of the trials, conducted in collaboration with B. Holtz, B. Lampinen, D. Doll, and B. Hanson, indicate the following: 1) after removal of an orchard on Nemaguard rootstock, taking 1 year out of production to maintain a bare fallow or to rotate once with sudan grass, wheat followed by sudan grass, or mustard provided a significant, measurable benefit to growth of almond or peach on Nemaguard rootstock, but the benefit was small and of doubtful economic significance; 2) spot fumigation, administered either through GPS-controlled shanks, or by spot drip application through an orchard's resident application system, can achieve acceptable if not optimal control of RD while reducing fumigant costs and emissions to the atmosphere. Finally, in an orchard replant trial led by Brent Holtz near Parlier, CA, we began monitoring microbial populations on roots of almond trees replanted following the use of a conventional push-and-burn approach to clearing an old orchard vs. following the use of an "iron wolf" to grind the old orchard trees into the resident soil. After each method of old tree removal was completed in replicate plots, the plots were subdivided. Half of the subplots were spot fumigated with Inline (a 61:35 mixture of 1,3-D:CP) and half were spot irrigated with water as a control. To date, no elevated incidence of RD or other pathogens has occurred in replanted almond trees on Nemaguard rootstock following orchard grinding (Holtz to report on tree growth responses).

Materials and Methods:

Objective 1

Using suppression subtractive hybridization (SSH) for culture-independent examination of RD.

In 2009 we attempted to use "suppression subtractive hybridization" (SSH, a PCR-based approach to subtractive hybridization) to identify microbes associated with relatively severe RD at an almond replant trial established in 2007 near Firebaugh, CA. SSH is a selective DNA amplification method that has many applications in molecular biology; it facilitates "subtracting out" some of the extraneous "haystack" of DNA from an investigation, thereby allowing an investigator to focus only on DNA fragments that differ in sequence in response to treatment or other effects. In our application the goal was to retain and sequence rDNA fragments from microbes specifically linked to roots affected by RD, or, alternatively to healthy roots, while eliminating rDNA fragments from microbes common to each class of roots. Another anticipated benefit of SSH in our application was elimination of host DNA (i.e., from the sampled Nemaguard roots). The host DNA, because it is so plentiful in samples and because it is amplified by many primers targeting microbe DNA, seriously complicates identification of potential microbe pathogens. We used SSH to amplify DNA fragments of rDNA bounded by PCR primers ITS 1 (forward) and ITS4 (reverse). The primers are known to amplify DNA from the rRNA gene, which is well represented in DNA sequence database of the National Center for Biotechnology Information (NCBI) and is therefore useful for microbial identification studies. The primers amplify Nemaguard peach DNA, but it was hypothesized that SSH would subtract out the unwanted host DNA.

Frozen samples of roots from healthy and RD-affected trees in the replant trial near Firebaugh were used for the tests with SSH in 2009. These samples were chosen for the tests because subsets of them had previously been used for culture-dependent microbe isolations, which revealed several fungi and *Pythium* spp. associated with RD (Browne et al., comprehensive Almond Board Report 2008). For SSH, total DNA (including host DNA and microbe DNA), was extracted and purified using conventional methods and kits. A published SSH protocol, including the mirror-oriented selection step, was followed. For the driver DNA (i.e., the DNA that in effect binds and subtracts out unwanted, extraneous DNA from samples), Nemaguard peach DNA, extracted from seedling leaves after surface sterilization, was used. Products of SSH were cloned. Forty-eight clones representing rDNA of microbes putatively unique to healthy samples and 48 clones representing rDNA putatively unique to RD-affected samples were sequenced to determine their source organism.

Using conventional PCR with new primers for culture-independent examination of RD.

In addition to using SSH to identify microbes associated with RD, conventional PCR amplification was used with new primer sets. One of the primers was formulated in our lab and others were obtained from literature reports. Further exploration of primers was needed because Nemaguard peach DNA was being amplified by primers used

previously to amplify fungal DNA from root samples, and oomycete primers had not been employed.

The new primer sets were tested with the same purified samples of DNA from healthy and RD-affected trees used to test SSH. In addition, control samples of DNA were used to evaluate primer specificity. When necessary primer sets were tested for intended specificity among the control samples by conducting PCR under a range of annealing temperatures. Primers with required specificity were further evaluated by cloning and sequencing products they amplified from the healthy and RD-affected root samples.

One of the primer pairs that performed effectively was used to examine the fungal community associated with incidence of RD at the orchard replant trial near Firebaugh. PCR was used to amplify rDNA from six replicate samples of healthy roots and six of RD-affected roots. The products were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI). DNA sequences were determined on 185 of the clones from healthy roots and 181 of the clones from RD-affected roots. Each DNA sequence was subjected to a BLAST search on the NCBI database to identify its source organism. Redundancy analysis (an ordination method) was used to examine associations of fungal incidence with RD.

Testing pathogenicity of organisms associated with RD.

In repeated greenhouse experiments designed to determine whether bacteria associated with RD (*Rhizobium* spp., *Pseudomonas* spp.) actually contribute to the disease, plants inoculated with the test bacteria grew as well as control plants, although in the same tests, plants grown in non-autoclaved RD soil grew less than plants in autoclaved RD soil (Browne et al., Almond Board Reports, 2007 and 2008). These results provided no evidence for contributions of bacteria to RD but suggested other microbes in the soil may be doing so.

In 2009 we completed two pathogenicity trials with fungal isolates and several species of *Pythium* that we found associated with RD. In each experiment, single-spored or hyphal-tipped isolates of the test organisms were grown for 6 weeks on a modified corn meal-sand-vermiculite substrate and then used to infest mixtures of field soil:course sand (2:1 ratio, v:v) ranging from 1 to 10% by volume. As alternative treatments, before inoculation, the field soil had either been autoclaved 3 times for 2 h or left non-autoclaved before mixing with the sand in infesting with the inoculants. The non-autoclaved and autoclaved treatments were used to examine pathogenicity of the test inoculants with and without interactions of the native soil microbial community. Immediately after soil infestation, 1-month-old Nemaguard peach seedlings were transplanted into 1.5 liter pots filled with the treated and non-treated test soil. Pathogenicity of all inoculants was judged according to effects on plant growth and root health. Three months after transplanting, top fresh weights were determined and, after washing free from soil, the root systems were weighed and, for roots $\leq$ 1 mm diameter, the percentage cortex length that was necrotic was estimated visually.

Objective 2

Effects of orchard residue grinding vs. burning on RD and other soilborne diseases.

We assisted B. Holtz and the USDA-ARS Soil Water Management Lab in establishing a trial to examine effects of grinding up orchards in place with an Iron Wolf machine (ironwolf.com) (see Liebelt and son, Reedley, CA) as compared to effects of conventional pushing out and burning of trees in piles. Each method of orchard removal was imposed on seven plots to be replanted to almond. Half of each plot was spot fumigated with Inline in October 2009. The fumigant was applied using the resident irrigation system, modified with a single 1 gph drip emitter placed at each tree site (i.e., to spot where a tree would be planted 3 months later). Each emitter was connected to a drip tube that delivered the fumigant 18 inches below the soil surface (0.2 lb of 1,3-D:CP [61:35] in approx. 4.5 gal water per tree site). The other half of each orchard removal plot was spot treated with water as a control. There were 12 tree sites per orchard removal plot (6 fumigated and 6 non-fumigated). All of the plots were planted to almond in January. In June 2009, fine roots were sampled from seven replicate trees for each combination of orchard-removal treatment and pre-plant fumigation treatment. One subset of the roots was frozen on dry ice immediately after collection in the field (for subsequent DNA analyses) and the other set was kept cool and subjected to culture-based isolations in the lab.

Evaluation of fumigant and non-fumigant pre-plant treatments for orchard replacement**.**

We continued to monitor tree performance in many orchard replant trials collectively testing the following types of pre-plant treatments: short-term fallowing and crop rotation, with and without strip fumigation with CP; spot and strip fumigation, with and without Sudan grass rotations; and spot, strip, and broadcast fumigation with different fumigants at different rates. We limit our report here to two trials started with Almond Board funding and continued with funding from the Pacific Area Wide Pest Management Program for Integrated MB Alternatives. In these trials treatment effects were assessed according to growth and yield of the replanted trees and incidence of soilborne pests and pathogens. In 2009, we began work with Bruce Lampinen and Shrini Upadhyaya to assess tree growth responses in the orchard replant trials using automated measurement of the proportion of photosynthetically active radiation (PAR) that is absorbed by tree canopies.

Results and Discussion:

Objective 1

Using suppression subtractive hybridization (SSH) for culture-independent examination of RD.

Among the 48 sequenced clones of rDNA fragments putatively unique to healthy roots, 40 of them (83%) were from the Nemaguard host (blast results *Prunus persica*, *P. ferganensis*) (Table 1). Similarly, among the 48 sequenced clones putatively unique to

RD-affected roots, 32 of them (67%) were from the host. Among rDNA fragments putatively unique to healthy roots, 12% were from fungi (unidentified basidiomycete and ascomycete), and the remaining fragments were mixed or incomplete (i.e., sequencing results had errors or indicated artifactual chimeric fragments) (**Table 1**). Among rDNA fragments putatively unique to RD-affected roots, 30% were from diverse fungi and 4% were of mixed or incomplete sequences.

The results indicate that SSH, at least as employed here, is not effective in selectively amplifying rDNA fragments unique to healthy and RD-affected roots. The host DNA, which was not unique to healthy or RD-affected roots, represented the majority (67 to 83%) of the rDNA fragments resulting from SSH. The dominance of host DNA among the rDNA products would result in costly and inefficient sequencing efforts.

Using conventional PCR with new primers for culture-independent examination of RD.

Among 14 primer pairs tested, three exhibited expected specificity among the fungal and oomycete control samples and did not amplify host root DNA (**Table 2**). As expected the ITS5 / ITS4 Oo pair amplified the oomycetes *P. cactorum* and *Pythium* sp. and did not amplify pure Nemaguard DNA. Similarly, the stramenopile primer pair amplified as expected among controls, and when a small sample of products from healthy and RD-affected roots were sequenced they were found to originate from diverse stramenopiles (*Labyrinthula* sp. and *Aplanochytriium* sp.) Also, the ITS304f primer developed in our lab, when paired with ITS4, amplified fungal DNA controls without amplifying oomycete or host DNA.

When PCR was conducted with the ITS304f / ITS 4 primer pair, followed by cloning, sequencing, and source determination for the amplified rDNA fragments, fragments from diverse fungi were detected without any interference from host DNA. The ordination of the incidence of fungal operational taxonomic units (OTUs; i.e., the identities of the fungi distinguishable by sequencing) vs. the health status of roots was statistically significant (**Figure 1**) (P=0.04 for each axis). *Cylindrocarpon destructans*, an unidentified ascomycete, and several other fungi were associated with RD-affected roots, while several other fungi were more abundant in association with healthy roots (**Figure 1**).

When results of the culture-independent identifications described above were compared with those from previous culture-dependent characterizations of the same samples, it was found that although both methods associated *C. destructans* and other fungi with the disease, each method detected some fungi that the other did not (**Figure 2**). For example, culture-independent sampling detected two OTUs of mychorrhizae and two other OTUs of soil fungi not detected by culturing (**Figure 2**). Mychorrhizae are obligate parasites and cannot be routinely cultured. Conversely, culturing detected several fungi not detected by PCR, including *Rhizopus*, *Aspergillus*, *Trichurus*, and *Trichoderma*. These results indicate that both culture-dependent and –independent approaches are needed for comprehensive examination of RD etiology. All of the culturable fungi strongly associated with RD by either culture-dependent or culture-independent methods are being testsed for pathogenicity.

Testing pathogenicity of organisms associated with RD.

In the completed pathogenicity tests, one isolate of *Cylindrocarpon destructans*-1 and one isolate of *Pythium helicoides* caused high levels of root cortex necrosis and significantly stunted the growth of Nemaguard peach seedlings (**Tables 3-5**). The other isolates of these genera and other fungi tested did not consistently affect plant growth.

In the first pathogenicity experiment 1, *Cylindrocarpon destructans*-1 reduced top and root plant weights whether or not the field soil was autoclaved before inoculation, and pathogenicity of the inoculants was not affected significantly by soil infestation rate (inoculants were added to the soil at 1, 5, and 10% by volume) (**Table 3**, means reflect averages of 1, 5, and 10% soil infestation rates). In the first experiment, root cortex necrosis was affected by significant interaction of the pre-inoculation autoclaving treatment and the inoculant; in the autoclaved soil, isolates *C. destructans*-1 and *Fusarium oxysporum*-1 both caused significant root cortex necrosis, compared to the control, whereas in non-autoclaved soil, all plants, including the controls, had relatively high levels of root cortex necrosis, regardless of inoculation treatment. Across inoculants, autoclaving the soil increased top and root fresh weights by 31and 41%, respectively ($P<0.0001$).

In pathogenicity experiment 2, inoculation with *Pythium helicoides* reduced top and root weights and resulted in high levels of root cortex necrosis and root rot regardless of whether the field soil was autoclaved before inoculation (**Table 4**). *Pythium helicoides* also was aggressive on peach in UC mix inoculated with the pathogen (**Table 5**). Pythium sp. 1 reduced top fresh weights in non-autoclaved soil of Hanford Sandy Loam (**Table 4**), but otherwise it caused no measureable disease (**Tables 4, 5**).

The results of our pathogenicity tests provide evidence that both *C. destructans* and *P. helicoides* can be important contributors to RD, at least in some replanted orchards. Isolates of these organisms were pathogenic whether or not the soil to which they were inoculated had been autoclaved, suggesting that interaction with other members of the soil microbial community is not needed for them to cause disease. The fact that root cortex necrosis and weight reduction resulted in all plants grown in non-autoclaved soil, even without a fungal inoculant, suggests that organism(s) present in the non-autoclaved soil were pathogenic.

Objective 2

Effects of orchard residue grinding vs. burning on RD and other soilborne diseases.

When root samples were collected from the orchard residue management replant trial in June, there was no evidence of high levels of root or other disease (replant disease or other disease) in any treatments, fumigated or non-fumigated. As trees developed foliage and root systems, the weather was relatively mild, and this may have suppressed development of RD. Although culture-based isolations were completed from subsamples of the roots and additional subsamples of the roots were frozen for

culture-independent assays, we have not completed the work and will report on it in the future.

Continuing evaluation of fumigant and non-fumigant pre-plant treatments for orchard replacement**.**

In the first of two orchard replant trials testing interactions of crop rotation with pre-plant fumigation treatments for management of RD, tree growth in the first year after planting was measurably improved ($P<0.02$) by the rotation, but by 2009, the rotation did not significantly affect the proportion of PAR absorbed by the canopy or the first year's fruit yield (**Table 6**). The temporary growth benefit in the peach trees following Sudan grass rotation was relatively small. In the same trial, the 8.3-ft.-wide strip treatment with Telone C35 and the GPS-controlled spot shank treatments with CP both produced good responses in the replanted trees, as measured by the increase in tree circumference in 2008, PAR absorbed in 2009, and fruit yield in 2009. Trees responded less favorably, although acceptably, to strip treatments with MB, GPS-controlled and drip-applied spot treatments with Telone C35 and Inline. A treatment with a root and soil drench with yeast extract at planting had no effect compared to the control (**Table 6**).

In the second trial, bare fallow for 1 year; a spring mustard rotation (*Sinapus alba*, Feb-Apr); and a wheat-sudan rotation (Penewawa wheat Feb-Apr, Piper sudan Jun-Aug) all improved almond tree circumference growth in 2008 and PAR absorbed in 2009, regardless of whether or not the treatments were followed by CP fumigation ($P=0.0009$ and 0.004, respectively) (**Table 7**). However, the benefit from fallowing or crop rotation was relatively small compared to that from pre-plant fumigation. The latter treatment increased PAR absorbed in 2009 by approximately two to three-fold, compared to the non-fumigated controls.

Based on the results of our tests, short term crop rotation are not expected to be as effective in orchards as they were in earlier microplot trials, at least on Hanford Sandy Loam soil. The fact that the spot treatments with Inline were not as effective as the strip treatment with Telone C35 or the spot treatment with CP, suggests that the limited area of treatment may be a problem, unless a highly effective fumigant for control of RD, such as CP, is used. Economic analysis of the treatments presented here as well as those of many other orchard replant trials are expected to be available by the end of 2009 on a website dedicated to the Pacific Area-Wide Pest Management Program for Integrated MB alternatives.

Recent Publications:

Browne, G., Lampinen, B., Holtz, B., Doll, Edstrom, J., Schmidt, L., Upadhyaya, S., Shafii, M., Hanson, B., Wang, D., Gao, S., Goodell, N., and Klonsky, K. 2008. Integrated pre-plant alternatives to methyl bromide for almonds and other stone fruits. Paper No. 12, pp. 12-1 to 12-4, Proceedings, 2008 Annual International Research Conference on MB Altern. and Emiss. Red., Orlando, FL. (online at mbao.org).

Wang, D. Browne, G., Gao, S., Hanson, B., Gerik, J. Qin, R., and Tharayil, N. 2009. Spot fumigation: Fumigant dispersion and emission characteristics. Environmental Science and Technology: Pub. Data (Web): July 8, 2009. DOI: 10.1021/es9015662

Table 1. Results of suppression subtractive hybridization (SSH) for identifying organisms associated with replant disease of almond

Organisms targeted by SSH procedure	Source organisms of DNA fragments sequenced	Number of detections	Frequency of detection (%)
Those from healthy roots	*Prunus persica*	28	58
	Prunus ferganensis	12	25
	Chrysosporium pseudomerdarium	4	8
	Unidentified basidiomycete	1	2
	Unidentified ascomycete	1	2
	Mixed or incomplete sequences	2	4
Those from diseased roots	*Prunus persica*	24	50
	Prunus ferganensis	8	17
	Zalerion varium	3	8
	Uncultured fungus	4	8
	Cercospora sp.	2	4
	Volvariella sp.	1	2
	Leptosphaeria sp.	1	2
	Lepiota lilacea	1	2
	Wilcoxina sp.	1	2
	Corticiaceae sp.	1	2
	Mixed or incomplete sequences	2	4

Table 2. PCR primers tested

Primer sets tested (and target organisms)	Expected rDNA PCR product (and approximate size)	Sample DNA and PCR amplification result ("-" = no bands, "+s = single bands, "+m = multip. bands, "NT"= not tested)								
		Healthy Nemaguard roots	RD-affected Nemaguard roots	*Phytophthora cactorum*	*Pythium sp.*	*Verticillium dahliae*	*Colletotrichum acutatum*	Nemaguard (pure)	Lovell (pure)	Marianna 2624 (pure)
ITS5/ITS4Oo (Oomycota)[a]	ITS 1 - 5.8S – ITS 2 (ca. 1200 bp)	-	-	+s	+s	-	NT	-	+m	+s
ITS5/ITS4Asco (Ascomycota)[b]	ITS 1 - 5.8S – ITS 2 (ca. 600 bp)	+s	+s	+s	+s	+s	+s	-	NT	NT
ITS1F/ ITS4 (All true fungi)[be]	ITS 1 - 5.8S – ITS 2 (ca. 550 bp)	+m	+m	+m	+m	+m	+m	+m	+m	+m
ITS1F / LR3 (All true fungi)[be]	ITS 1 - 5.8S – ITS 2 - 28S (ca. 1200 bp)	NT	-	+m	+m	+s	+s	+m	+m	+m
463/ITS4 (All true fungi)[e]	18S - ITS 1 - 5.8S – ITS 2 (ca. 1400 bp)	-	-	-	NT	+s	NT	+s	NT	NT
NS5/LR3 (All true fungi)[be]	18S - ITS 1 - 5.8S – ITS 2 – 28S; (ca. 2000 bp)	NT	+m	+m	NT	+s	NT	+m	NT	NT
NS5/LR16 (All true fungi)[bc]	18S - ITS 1 - 5.8S – ITS 2 – 28S; (ca 2000 bp)	NT	+s	+m	NT	+s	+s	-	NT	NT
NS5/LR5 (All true fungi)[be]	18S - ITS 1 - 5.8S – ITS 2 – 28S; (ca. 2200 bp)	NT	+s	+s	NT	+s	NT	+s	NT	NT
NS5/LR8 (All true fungi)[be]	18S - ITS 1 - 5.8S – ITS 2 – 28S; (ca. 3000 bp)	NT	+m	+m	NT	+m	NT	+m	NT	NT
NS5/LR6 (All true fungi)[be]	18S - ITS 1 - 5.8S – ITS 2 – 28S; (ca. 2700 bp)	NT	+m	+m	NT	+m	NT	+m	NT	NT
463/ITS2 (Nemaguard)[d]	18S - ITS 1 – 5.8 (ca. 1200 bp)	+s	+s	+s	+s	+s	+s	+s	NT	NT
ITS1/ITS4 (Eukaryotes)[f]	ITS1 – 5.8S – ITS2 (ca 750 bp)	+m	+m	+s	+s	+s	+s	+s	+s	+s
SSUF1/SSUR2 (Stramenopile selective)	18S (ca 680bp)	+s	+s	+s	+s	-	NT	-	+m	+m
ITS304f/ITS4 (True fungi only)	5.8S – ITS2 (ca 400bp)	+m	+m	-	-	+s	NT	-	-	-

[a] Useful for testing for the presence of Oomycetes
[b] Non-specific
[c] Amplification very weak overall, even at 35 cycles
[d] Fragment size not as expected
[e] Amplified host DNA
[f] Used for primer design

Table 3. Pathogenicity of fungi and oomycetes associated with RD (experiment 1)

Inoculant	Top fresh wt. (g)	Root fresh wt. (g)	Root cortex necrosis (%)	
			Soil autocl. before inoc.	Soil not autocl. before inoc.
Control (sterile substrate)	7.4	5.1	27	70
Cylindrocarpon destructans-1	*2.1*	*1.1*	*88*	90
Cylindrocarpon destructans-2	6.1	3.6	65	69
Cylindrocarpon sp.	7.0	4.8	*72*	54
Fusarium oxysporum-1	6.5	4.2	37	79
F. oxysporum-2	8.3	5.7	30	74
F. oxysporum-3	7.6	4.8	26	81
F. solani-1	6.3	4.4	*75*	66
F. solani-2	6.6	4.4	61	73
F. solani-3	6.7	4.5	31	75
F. solani-4	7.8	5.3	53	78
F. solani-5	7.9	5.5	26	81
F. solani-6	5.3	3.3	42	80
F. solani-7	5.7	3.6	53	79
F. solani-8	5.8	3.8	30	77
F. solani-9	6.8	4.7	31	71
F. solani-10	8.7	6.1	35	78
Nectria haematococcus	5.5	3.3	66	79
Pythium sp.-1	8.4	5.5	49	73
Pythium sp.-2	6.1	4.3	28	75
Pythium sp.-3	9.9	7.1	40	67
Trichoderma sp.-1	7.5	4.9	45	71
Trichoderma sp.-2	6.5	4.1	38	73
Unculutred Ascomycete-1	7.6	5.0	31	62
Uncultured Ascomycete-2	8.9	5.8	24	64
Uncultured Ascomycete-3	9.4	6.0	21	73
Uncultured fungus-1	7.7	4.6	36	67

Table 4. Pathogenicity of RD-associated *Pythium* isolates in Hanford Sandy Loam soil collected from a peach orchard affected by RD (experiment 2)

Soil treatment before inoculation	Inoculant	Top fresh wt. (g)	Root fresh wt. (g)	Root cortex necrosis (%)
Soil autoclaved	Control (sterile substrate)	31.8	23.1	10
	Pythium sp.-1	36.9	29.7	9
	Pythium sp.-2	44.4	35.5	14
	Pythium sp.-3	43.1	34.3	29
	Pythium sp.-4	32.5	31.9	8
	Pythium helicoides	*0.7*	*0.8*	*100*
Soil not autoclaved	Control (sterile substrate)	36.1	24.0	77
	Pythium sp.-1	*16.7*	13.5	64
	Pythium sp.-2	28.4	19.4	64
	Pythium sp.-3	35.2	26.1	68
	Pythium sp.-4	37.3	23.2	58
	Pythium helicoides	*10.3*	*6.7*	83

Table 5. Pathogenicity of RD-associated *Pythium* species in autoclaved UC Mix potting soil (experiment 2, continued)

Inoculant	Top fresh wt. (g)	Root fresh wt. (g)	Root cortex necrosis (%)
Control (sterile substrate)	72.2	41.9	7
Pythium sp.-1	64.2	43.8	11
Pythium sp.-2	67.6	43.4	7
Pythium sp.-3	73.5	47.9	12
Pythium sp.-4	65.3	49.9	13
Pythium helicoides	*4.6*	*4.2*	*74*

Table 6. Interactive effects of pre-plant fumigation treatments and crop rotation with sudan grass on performance of peach on Nemaguard rootstock

Fumigation treatment (Oct 2007)	Fum. per treated acre (lbs)	Fum. per orchard acre (lbs)	Sudan grass (Jul-Sep 2007)	Increase in trunk cir. 2008 (cm)	PAR absorption July 2009 (%)	Mkt. fruit yield July 2009 (kg)
Control (non fumigated)	0	--	no	3.9	1	2.0
			yes	7.1	6	5.3
MB, shank strip	400	168	no	10.4	17	12.1
			yes	9.5	15	10.0
Tel. C35, shank strip	540	227	no	12.5	20	21.1
			yes	13.9	21	20.3
Tel. C35, sh. spot 5x 6’	540	81	no	9.7	10	12.5
			yes	11.0	13	14.3
Inline, drip spot, 4’ dia	540	43	no	9.1	10	9.3
			yes	9.6	10	10.0
Chlorop. sh. spot 5x6’	400	60	no	10.5	14	14.7
			yes	11.6	16	16.1
None, yeast extract	0	--	no	5.5	5	3.6
			yes	6.6	5	5.5
MSD, 95% CI:				*3.5*	*6*	*9.2*
P value, fumigation trt:				*<0.0001*	*<0.0001*	*<0.0001*
P value, rotation trt:				*0.02*	*0.16*	*0.45*

Table 7. Interactive effects of pre-plant fumigation, crop rotation, and fallowing on performance of almond on Nemaguard rootstock

Pre-plant fumigation treatment	Pre-plant cropping	Increase in trunk circumference 2008 (cm)	PAR absorption July 2009 (%)
Control	Peach	5.6	10
	Fallow	6.9	15
	Mustard	7.2	14
	Wheat-Sudan	6.8	14
Chloropicrin 400 lb/A	Peach	10.5	21
	Fallow	11.6	30
	Mustard	12.2	30
	Wheat-Sudan	12.8	30
MSD, based on 95% CI:		*2.1*	*8*
P value, fumigation trt:		*<0.0001*	*<0.0001*
P value, pre-plant cropping trt.:		*0.0009*	*0.004*

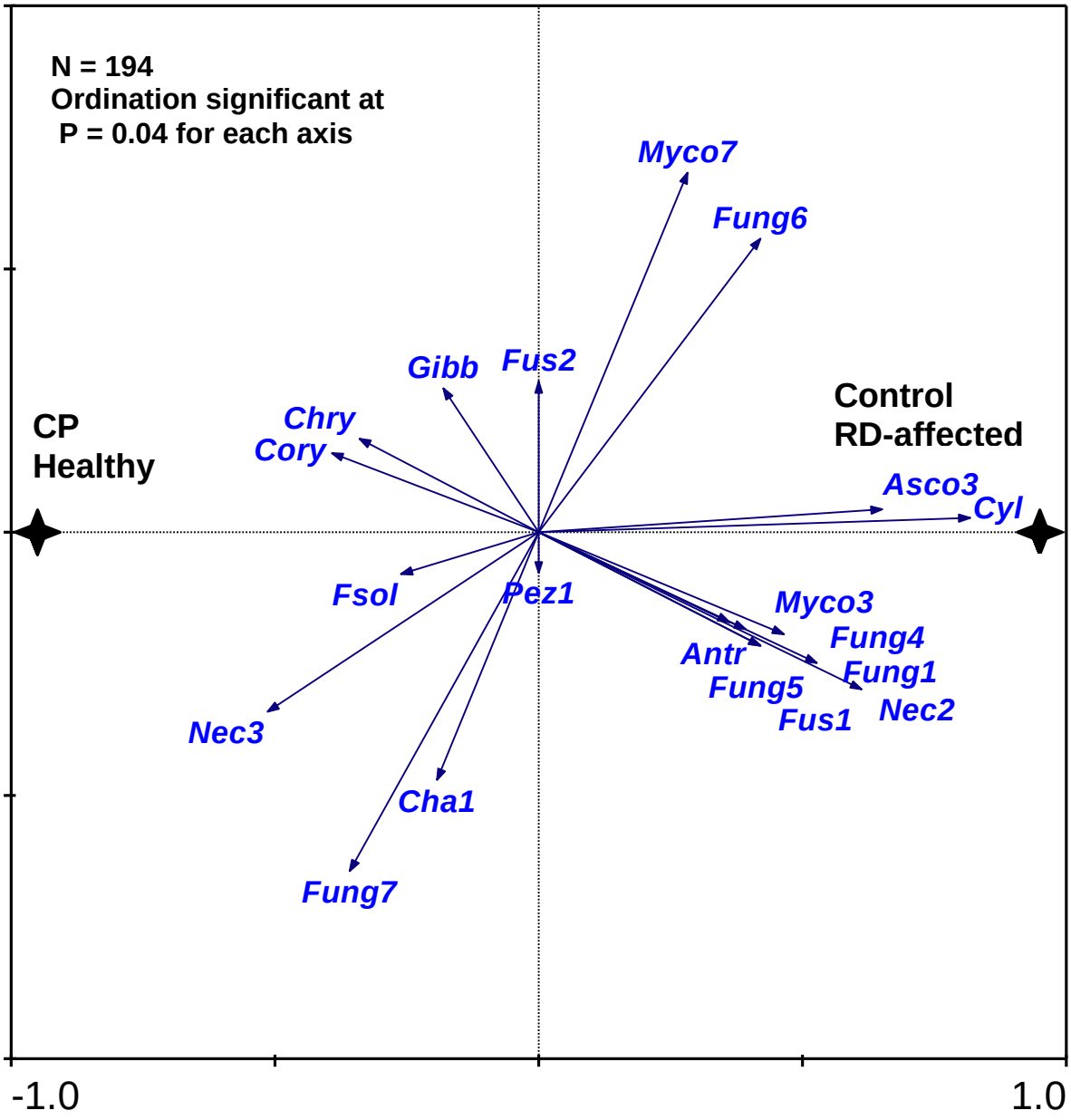
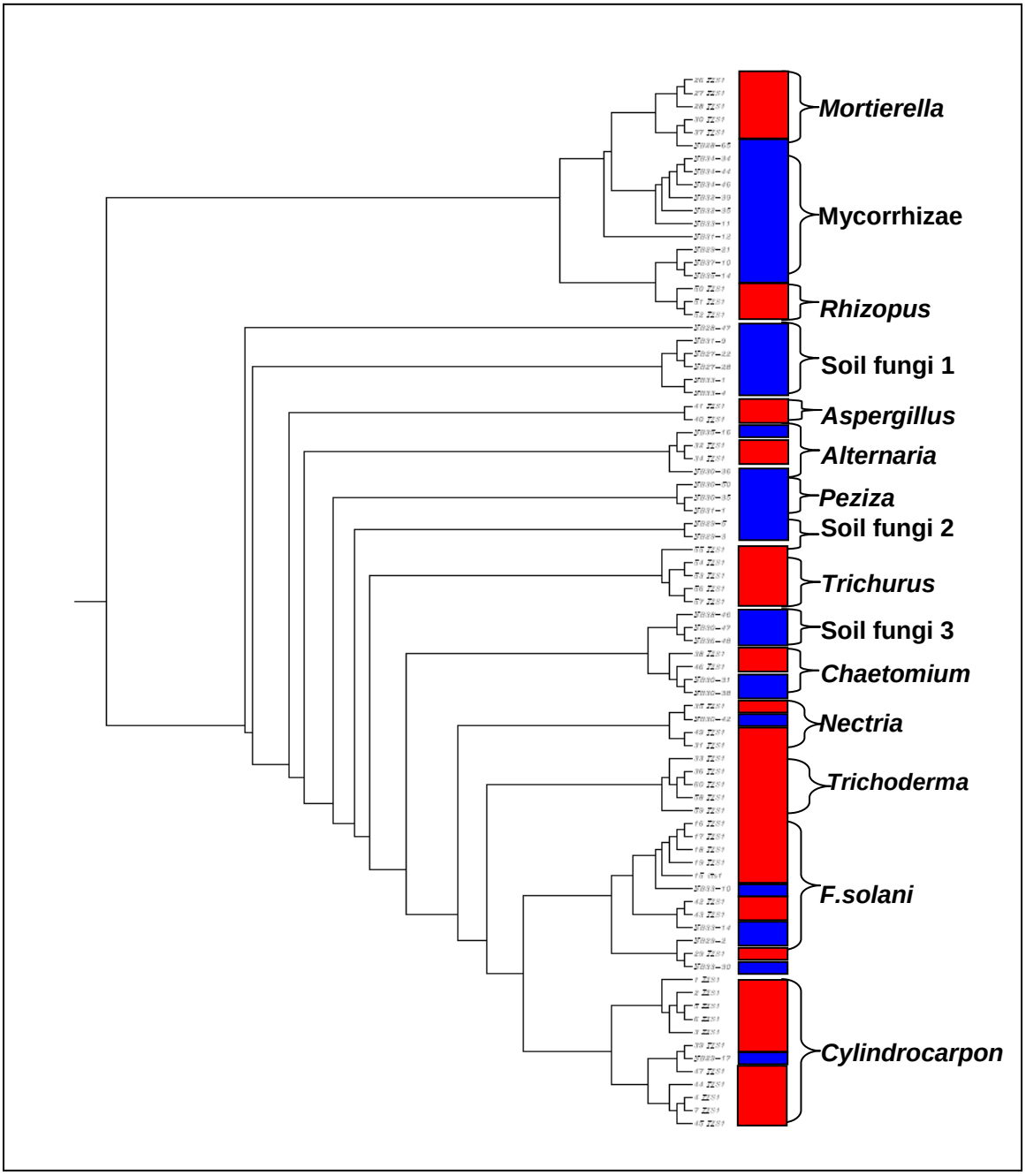


Figure 1. Ordination of culture-independent fungal incidence, Firebaugh replant trial, 2007. Abbreviations are as follows: Antr, *Antrodia camphorata*; Asco 3, Uncultured ascomycete clone; Cha 1, *Chaetomium globosum*; Chry, *Chrysosporium pseudomerdarium*; Cory, *Corynascus sepedonium*; Cyl 1, *Cylindrocarpon destructans*; Fsol, *Fusarium solani*; Fung 1, Uncultured endophytic fungus; Fung 4, uncultured soil fungus clone; Fung 5, Uncultured soil fungus clone; Fung 6, Fungal endophyte; Fung 7, Uncultured fungus isolate; Fus 1, *Fusarium* sp.; Fus 2, *Fusarium* sp; Gibb, *Gibberella avenacea*; Myco 3, Uncultured ectomycorrhiza (Laccaria)



Sampling method:

culture-based

culture-independent

Figure 2. Cluster analysis of OTU's detected by culture-based and culture-independent analysis. Clusters of known fungal species are marked as such. Soil fungi 1 include: uncult Peziza clone, uncult soil fungus clone and uncult ascomycete clone; Soil fungi 2: uncult soil fungus clone, *Chrysosporium*, *Zalerion*; Soil fungi 3 include: uncult soil fungus, fungal endophyte, *Zopfiella*.