Developing Improved Strategies for Management of Replant Problems

Project No.: **09-PATH1-Browne**

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Objectives:

1. Determine the biological causes of replant disease (RD).
2. Develop improved management strategies for RD and other replant problems.

Interpretive Summary:

Replant disease (RD) is manifested by moderate to severe suppression of tree growth and yield in successive plantings of almond and other stone fruit orchards (Browne et al., 2006). This report summarizes the background and current status of our research on: 1) causes of RD and 2) new approaches for management of RD and other replant problems.

Previously, we presented evidence that replant disease RD of almond is mediated by microorganisms in the soil. For example, heating RD soil to 140 °F for at least 30 min or fumigating it with chloropicrin prevented the disease. Culture-based isolations identified specific fungi (*Cylindrocarpon* sp., *Fusarium* spp.), stramenopiles (*Pythium* spp.), and bacteria (*Rhizobium* spp.) found more commonly in RD-affected roots than in healthy roots. It was determined that RD occurs in the absence of plant parasitic nematodes, and that fumigants most effective for controlling nematodes (i.e., 98% methyl bromide or Telone II) are less effective than chloropicrin or mixtures of it with other fumigants for preventing RD (Browne et al., 2006). Methods to apply spot fumigation treatments to tree sites using GPS-controlled

shanks and subsurface drip emitters were developed to help reduce fumigant costs and emissions and are being perfected (Upadhyaya and Browne, *unpublished*, Wang et al., 2009). However, soil fumigation is increasingly regulated and costly, and additional replant management strategies with minimal reliance on fumigants are needed.

This year under Objective 1 we completed new culture-independent (i.e., DNA-based analyses) of bacterial, stramenopile, fungal and populations in three almond orchards affected by replant disease in Sacramento and San Joaquin Valley orchards. Although we previously conducted culture-independent characterizations of bacterial communities at one Parlier replant trial and two Chico-area replant trials, we had not done so for several more-recent replant trials. In addition, before 2010, we had not completed comprehensive culture-independent examinations of fungi or stramenopiles (i.e., the latter grouping includes *Pythium*, *Phytophthora*, and many other diverse "water molds" and algae-like organisms). It is likely that this broad cross-section of the soil biota, i.e., bacteria, fungi, and stramenopiles, includes the key biological agents associated with RD, and both culture-independent and culture-dependent methods are needed to approach a realistic assessment of these populations. Many important soil microorganisms cannot be cultured, and conversely, in our experience, some organisms cultured from roots are not reliably detected by culture-independent PCR.

Our 2010 culture-independent analyses failed to reveal consistent associations between bacteria and RD, but they did confirm the positive association between *Cylindrocarpon* and RD both in the SV and SJV (this association was apparent based on culture-based isolations in previous years). The fact that both culture-dependent and culture-independent methods link incidence of RD with the presence of *Cylindrocarpon* affords confidence in this association. Interestingly, the culture-independent DNA-based analyses also revealed a positive association between *Phaeonectriella lignicola* and RD. *Phaeonectriella lignicola* is described as a saprophytic fungus found around freshwater environments (Raja et al., 2009), but we had failed to detect it (or at least did not recognize it) in our previous culture-based isolations. We will attempt to locate isolates of *P. lignicola* in our collection and from other investigators so that they can be tested for pathogenicity and used for testing of qPCR primers to detect *P. lignicola*.

In the same culture-independent assays, neither *Pythium* nor *Fusarium* was linked to incidence of RD. This was surprising because our culture dependent assays commonly found them at higher incidence in RD affected roots. A possible explanation for this is that the DNA extraction method we use may favor detection of DNA from organisms inside the root tissue rather than from organisms on the surface of the roots. *Pythium* and *Fusarium* may be more common on the surface of the roots. (We are checking this hypothesis with a modified extraction procedure). In a companion project (Development of qPCR diagnostics for RD pathogens of almond, Browne and Kluepfel, Almond Board of California [ABC] 2009,10) we developed and tested quantitative PCR (qPCR) primers for detection of *Cylindrocarpon*, *Pythium helicoides* (these organisms were isolated from almond trees affected by RD and exhibited some pathogenicity on peach rootstocks in the greenhouse) and *Hypocrea lixii* (the sexual state of *Trichoderma harzianum*, which we found associated with healthy trees in fumigated plots of the Firebaugh replant trial.

Also under objective 1 in this project year we assembled a collection of more than 200 *Cylindrocarpon* isolates saved from RD-affected almond trees over the years of our project. *Cylindrocarpon* has been the fungus most consistently associated with incidence of RD in our field replant trials, and our greenhouse pathogenicity trials have indicated that some isolates of

the fungus are pathogenic on Nemaguard rootstock. We are nearly finished extracting DNA from these isolates and have begun DNA sequence analyses (rDNA, beta tubulin, and mitochondrial gene regions of the isolates are being sequenced). The sequences will be used to cluster the isolates into similar genotype groupings. Representatives of each genotype grouping will then be tested for pathogenicity in the greenhouse on peach seedlings.

Under objective 2, we established tests of spot treatments with brassica seed meal (BSM, Mustard Products and Technologies, mptmustardproducts.com), UN 32, and steam in almond replant trials. The spot treatments with BSM were incorporated at 2000 and 8000 lb/treated acre in a Merced County almond replant trial conducted by Doll. In Nov 2009 the treatments were incorporated at tree sites planted in Feb 2010 using a 2-ft-diameter agar designed and built by collaborator Bob Weimer. “Auger-only” and non-treated control plots were included along with row-strip and tree-spot plots fumigated with MB and Telone C35. BSM treatments have helped to manage replant disease of apple (Mazzola, 2010), and the tests with almond will evaluate its effectiveness in managing RD on almond. In the same orchard, spot steaming treatments were applied in collaboration with Fennimore et al. (see ABC report Fennimore et al., 2009). Spot treatments with UN 32 were applied through microjet sprinklers (one per future tree site, 5 replicate plots of 10 tree sites) in a Madera County almond replant trial at a rate of 200 lbs N per treated acre. Efficacy of the BSM, steam, and UN32 treatments is being monitored by assessing growth of the trees and population counts of plant parasitic nematodes within and between the treated spots.

Materials and Methods:

Objective 1.

Culture-independent microbial examinations. In previous years, replicate non-fumigated and chloropicrin-fumigated plots were established at three almond orchards (SJV, SV-1, and SV-2) that had recently been replanted and expressed RD (2005-2009 Comprehensive Reports to the Almond Board of California [ABC], Browne et al.). The SJV orchard was in the San Joaquin Valley near Firebaugh, CA, and the SV-1 and SV-2 orchards were in the Sacramento Valley near Chico, CA. At each orchard, symptoms of severe RD developed in the almond trees in non-fumigated plots while trees in the fumigated plots remained healthy. Roots (< 1 mm dia) were sampled from four to six healthy and four to six RD-affected trees per orchard in June or July and stored at -80 C. DNA was purified from 150 mg of each root sample using a modified CTAB method (Bhat et al. 2009). PCR primers were used to amplify diagnostic rDNA fragments from bacteria, stramenopiles, and fungi (**Table 1**). The amplicons from each major taxonomic group were cloned (Promega P-Gem T Easy Vector System; Promega Corp, Madison WI) and sequenced (UC DNA Sequencing Facility). A total of 4608 fragment sequences were edited and aligned using Bioedit software (Hall, 1999) or MUSCLE (Edgar, 2004). Based on distance matrices (Feinstein, 1981), the sequences were grouped into operational taxonomic units (OTUs) by DOTUR (Schloss et al. 2005). OTUs representing 97% genetic similarity were established for the fungal and stramenopile amplicons while clusters representing 92% genetic similarity were established for bacterial amplicons. The largest OTU clusters representing 67% of the fungal and bacterial clones and 95% of the stramenopile clones were identified (BLAST ncbi.gov) and enumerated according to soil treatment (**Tables 2-4**). Redundancy analysis (Braak, 1990) was used to examine shifts in each microbial community associated with RD.

Characterization of *Cylindrocarpon* isolates. Isolates of *Cylindrocarpon* (250) from RD-affected almond orchards were removed from cold storage, transferred to fresh cultures of

potato dextrose agar, and purified by transferring hyphal tips from single conidia. The single-spored isolates were dried and preserved on strips of sterile filter paper. To identify the species of *Cylindrocarpon*, we initiated sequencing of rDNA ITS1 and ITS2 spacer regions (our culture independent sequences were for ITS2 only), the beta-tubulin gene, and the small subunit mitochondrial DNA (Petit, 2005). Fifteen to 20 isolates representing the genetic diversity among the 250 isolates will be tested for pathogenicity as we have done previously for a smaller subset of *Cylindrocarpon* isolates (comprehensive ABC report Browne et al., 2009).

Objective 2.
Spot treatments with brassica seed meal (BSM), UN 32, and steam were applied in almond replant trials. The spot treatments with BSM (Mustard Products and Technologies, mptmustardproducts.com) were incorporated at 4000 and 8000 lb/treated acre in an almond replant trial conducted by Doll in Merced County. Each treatment was mixed into the soil with a 2-ft-diameter auger (designed and built by Weimer) to a depth of approximately 2 ft;; controls included 21 tree sites that received the auger treatment without BSM, and 21 tree sites that received no treatment. For comparison, the trial included row-strip and tree-spot plots fumigated with MB and Telone C35. The spot steam treatments were applied in the Merced County replant trial as well as in another replant trial in Madera County (see ABC report Fennimore et al., 2009). The spot UN 32 treatments were drip applied in a Madera County almond replant trial. They involved applying 200 units of nitrogen per orchard acre through individual microjet emitters (one emitter per tree site). Efficacy of the BSM, steam, and UN32 treatments is being monitored by assessing growth of the trees and nematode population counts in and between the treated plots and controls.

Results and Discussion:

Objective 1.
Culture-independent microbial examinations. Frequency tables and ordinations of the SJV data revealed shifts in the bacterial, stramenopile, and fungal populations associated with RD (P = 0.01, 0.068, and 0.044, respectively) (**Tables 2-4**, **Figures. 1A, 2A, and 3A**; note that the tables contain keys for abbreviations on the figures). For example, incidences of *Streptomyces* sp., member(s) of alpha-proteobacteria, and unidentified uncultured bacteria (e.g., Bact-2 and Bact-13) were positively associated with incidence of RD, whereas incidences other bacteria were more prevalent among roots of healthy trees (**Table 2, Figure 1A**). Incidences of *Cylindrocarpon* sp. and *Phaeonectriella lignicola* OTUs were positively associated with incidence of RD, whereas several other fungal OTUs were detected more frequently from healthy samples (**Table 4, Figure 3A**).

Analysis of the SV-1 and SV-2 data also suggested that *Cylindrocarpon* sp. and *P. lignicola* were positively associated with incidence of RD (**Table 4, Figures 3 B,C**). However, overall ordinations of the bacterial, fungal, and stramenopile populations from these orchards were either not significant or were significant at relatively low levels (P=0.18 to 0.27) (**Figures 1 B,C; 2 B,C; and 3 B,C**).

The indications that *Cylindrocarpon* sp. and *P. lignicola* were positively associated with RD in two geographically distinct almond production regions suggest that the role of these fungi in the disease should be examined further. Our DNA sequences for ITS 2 were not sufficient to distinguish between *Cylindrocarpon macrodidyma* and *C. destructans* (both taxons were represented equivalently in our BLASTS for the *Cylindrocarpon* OTU. Currently, we are characterizing identity and pathogenicity of populations of *Cylindrocarpon* sp(p). and other

culturable fungi associated with RD in California. In our previous culture-based examinations of roots from the plots at SJV, SV-1, and SV-2,*Cylindrocarpon* sp., *Pythium* spp., and *Fusarium* spp. were positively associated with the disease, whereas *P. lignicola* was not detected. Using standard and qPCR, Bent et al. reported (2009) that *Sellophora* spp. and *Pythium* spp. were associated with replant suppression of peach plants under greenhouse conditions. Although we used the stramenopile primers employed by Bent et al., we did not detect *Sellophora* or *Pythium* spp. in our samples, suggesting that variations in edaphic factors (i.e. in field vs. greenhouse), PCR protocols, geographical location, and other factors may affect presence and detection of microbial community members when using culture-independent methods. Our results suggest that culture-dependent and culture-independent methods are important in examining PRD etiology.

Characterization of *Cylindrocarpon* isolates. DNA was successfully extracted from *Cylindrocarpon* isolates from replant trials at the following locations: Parlier microplot trial (108 isolates from Nemaguard peach rootstock); Durham replant trial 1 (26 isolates from almond on Marianna 2624 rootstock); Durham replant trial 2 (67 isolates from almond on Marianna 2624); Parlier 24C and 14N replant trials (20 isolates from almond on Nemaguard, 15 isolates from peach on Nemaguard); and Firebaugh replant trial (14 isolates from Nemaguard peach). This work will continue and provide insight on the role of this pathogen group in almond RD.

Objective 2.
Results are not yet available for our field trials testing spot treatments with BSM, steam, and UN32. We will report and discuss these results in the coming year.

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Table 1. Target groups of microorganisms and PCR primers used in culture-independent examination of roots from healthy and replant-disease-affected almond trees

Target microorganisms	Target site	Primer set	References
fungi	ITS 2 rDNA variable region	304F/ ITS4	Schmidt et al. (*unpublished*)/ White, 1993
bacteria	16S (SSU) rDNA	341f/ 1401r	Muyzer et al., 1996/ Nübel et al., 1996
stramenopiles	18S (SSU) rDNA	SSUF1/ SSUR2	Bent et al., 2009

Table 2. Identification and incidence of taxons detected with PCR primers targeting 16S rDNA of bacteria (Note OTU designations pertain to **Figures 1A-C**.)

Bacterial Taxon	OTU & designation on graph	Taxon incidence (no. of clones)					
		SJV orchard		SV-1 orchard		SV-2 orchard	
		Hea.	Dis.	Hea.	Dis.	Hea.	Dis.
Actinomadura sp.	Actino-3	-	-	-	-	1	3
Actinoplanes sp.	Actino-1	11	2	25	2	9	9
	Actino-2	-	-	-	-	-	-
α-proteo sp.	Alpha-p	2	22	-	-	-	-
Bradyrhizobium sp.	Brady	-	-	1	7	3	3
Caulobacter sp.	Caulo	-	-	4	1	-	-
Devosia sp.	Devo	8	4	-	-	4	0
γ-proteo sp.	Gamma-p	-	-	-	-	3	5
Myxobacterium sp.	Myxo	14	1	-	-	-	-
Paenibacillus sp.	Bacillus	-	-	3	1	-	-
Pseudonocardiceae	Pseudo	-	-	3	3	-	-
*Rhizobium*sp.	Rhizo-1	-	-	3	4	4	0
	Rhizo-2	-	-	2	2	-	-
Streptomyces sp.	Strep-1	8	15	8	7	36	51
	Strep-2	15	39	27	29	-	-
	Strep-3	12	17	-	-	-	-
Uncult β-proteo sp.	Beta-p1	15	1	4	3	-	-
	Beta-p2	4	10	5	2	-	-
Uncultured sp.	Bact-5	27	16	13	13	7	8
	Bact-6	-	-	0	7	-	-
	Bact-7	16	3	2	2	2	2
	Bact-2	2	17	7	4	11	11
	Bact-1	-	-	5	16	12	3
	Bact-3	-	-	3	2	-	-
	Bact-4	-	-	1	4	2	4
	Bact-9	-	-	-	-	11	6
	Bact-10	8	9	-	-	4	5
	Bact-11	-	-	-	-	4	2
	Bact-12	-	-	-	-	1	4
	Bact-13	0	11	-	-	-	-
	Bact-14	3	7	-	-	-	-
Variovorax sp.	Vario-1	31	14	2	8	7	14
	Vario-2	-	-	16	17	-	-
	Vario-3	-	-	-	-	4	4
	Vario-4	10	3	-	-	-	-

Table 3. Identification and incidence of taxons detected with PCR primers targeting 18S rDNA of stramenopiles (Note OTU designations pertain to **Figures 2A-C**.)

Taxon	OTUs and designation on figures	Taxon incidence (no. of clones)					
		SJV orchard		SV-1 orchard		SV-2 orchard	
		Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
Aplanochytrium	Aplano-1	91	184	35	29		
	Aplano-2	1	41	3	16	87	29
	Aplano-3	27	4				
	Aplano-4	1	19				
Halophytophthora	Halophy					2	32
Ochromonas	Ochro					1	13
Ochromonodaceae	Ochrom					2	2
Paraphrysomonas	Para-1					18	7
Paraphrysomonas	Para-2					2	3
Spumella	Spu-1	46	2	134	124	8	6
	Spu-2	18	2				
	Spu-3					15	2
	Spu-4					14	1
Thraustochytriidae	Thraus			4	1		
Uncultured eukaryote	Unc euk			0	6		
Uncultured marine eukar.	Marine euk					1	87

Table 4. Identification and incidence of taxons detected with PCR primers targeting ITS2 rDNA of fungi (Note OTU designations pertain to **Figures 3A-C**.)

Fungal Taxon	OTUs & designations on figures	Taxon incidence (no. of clones)					
		SJV orchard		SV-1 orchard		SV-2 orchard	
		Hea.	Dis.	Hea.	Dis.	Hea.	Dis.
Apiosordaria otanii	Apio	-	-	26	21	35	4
Bionectria sp.	Bionec	34	0	-	-	-	-
Cadophora sp.	Cadoph	5	5	-	-	-	-
Cercophora sp.	Cerco-1	-	-	36	1	-	-
Cercospora viticola	Cercosp	-	-	3	4	31	14
Chaetomium sp.	Chae	13	1	-	-	24	0
Chrysosporium sp.	Chrys	17	0	-	-	-	-
Ectomycorrhizae	Unk mycorr	1	36	-	-	-	-
Fusarium solani	F.sol	28	5	-	-	-	-
Fusarium sp.	Fus sp.	10	8	-	-	-	-
Gibberella avenacea	Gibb	14	3	-	-	-	-
Glomus intraradices	Glom-1	-	-	5	0	-	-
	Glom-6	-	-	-	-	0	5
Glomus sp.	Glom-2	-	-	22	1	3	2
	Glom-3	-	-	5	0	-	-
	Glom-5	-	-	-	-	3	2
	Glom-4	-	-	-	-	2	4
Hypocrea sp.	Hypo	10	0	-	-	-	-
Lepiota sp.	Lepio	-	-	6	7	-	-
Leucoagaricus sp.	Leuco	-	-	0	9	-	-
Melanophyllum haematospermum	Melano	-	-	8	0	-	-
Neonectria sp.	Cyl. sp.	2	97	3	10	0	11
Peziza sp.	Pez	28	0	-	-	-	-
Phaeonectriella lignicola	P. lig-1	0	35	0	27	0	16
	P. lig-2	0	26	-	-	-	-
	P. lig-3	-	-	0	16	4	20
Podospora communis	Podo	-	-	0	5	-	-
Podospora dimorpha	Podo-2	-	-	-	-	11	1
Sebacina sp.	Seba-1	12	0	-	-	-	-
	Seba-2	36	0	-	-	-	-
	Seba-3	17	0	-	-	0	14
	Seba-4	18	0	-	-	-	-
	Seba-5	33	1	-	-	7	32
Sebacina vermifera	Seba-6	-	-	-	-	16	0
Uncultured *Entoloma*	Unk Ento	-	-	6	0	-	-
Uncultured *Infundibulicybe*	Unk fung endo-2	1	38	-	-	-	-
Uncultured basidiomycete	Unk basidio	0	15	0	15	-	-
Uncultured endophyte	Unk fung endo-1	0	36	-	-	1	6
	Unk fung endo-3	6	8	-	-	-	-
Uncultured sp.	Unk fung	29	0	10	5	-	-
	Unk fung-2	-	-	-	-	11	0
	Unk fung-3	-	-	-	-	4	3
	Unk fung clone	20	0	-	-	-	-
Unknown soil fungus+P26	Unk soil fung	0	23	19	14	-	-
Zalerion varium	Zaler	0	15	-	-	-	-
Zopfiella karachiensis	Zopf	41	1	8	0	-	-

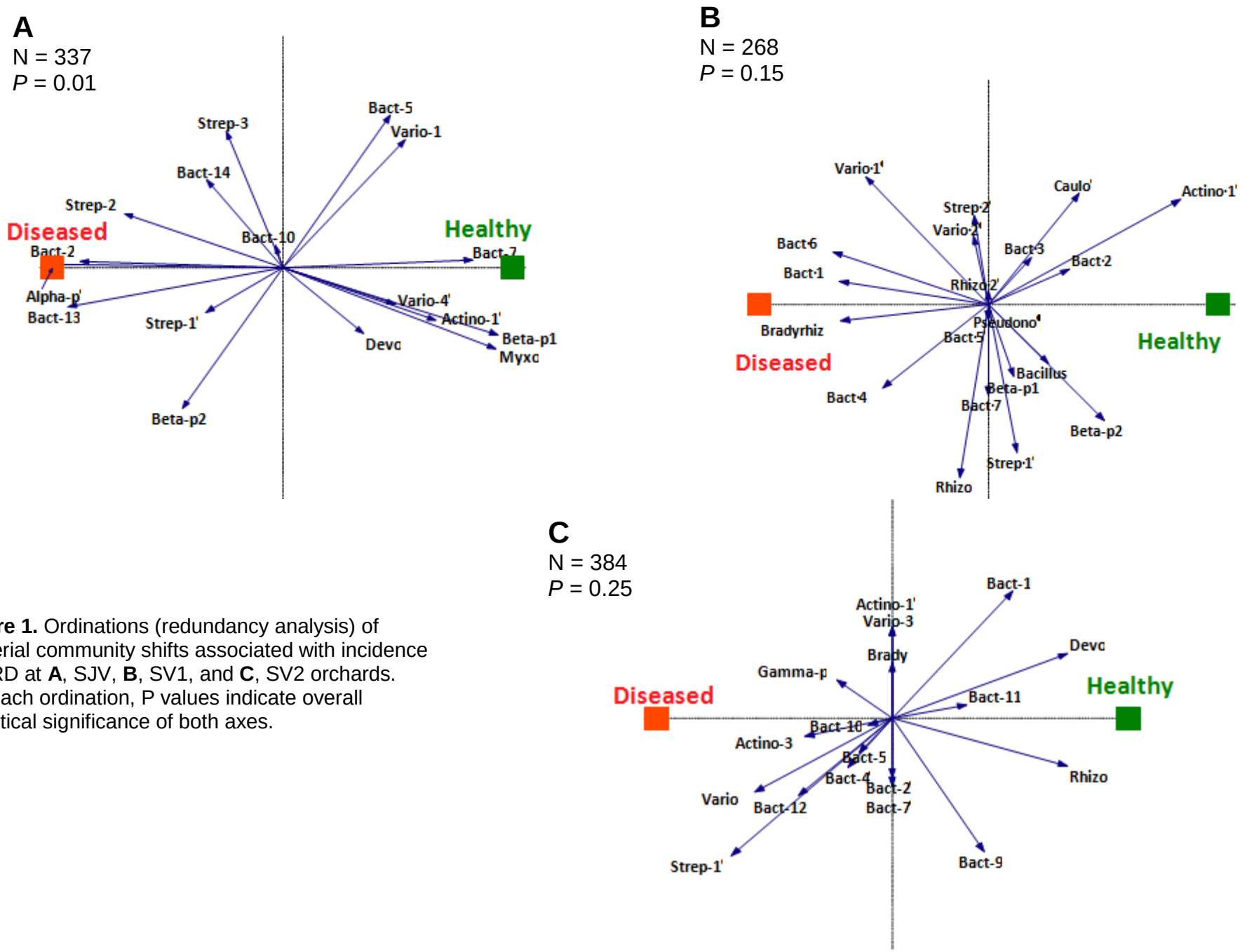
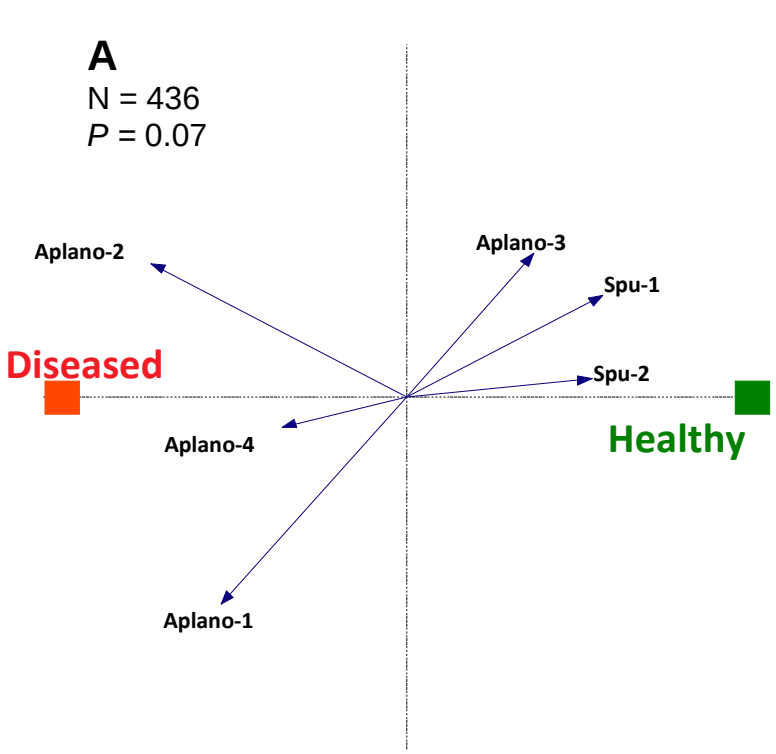
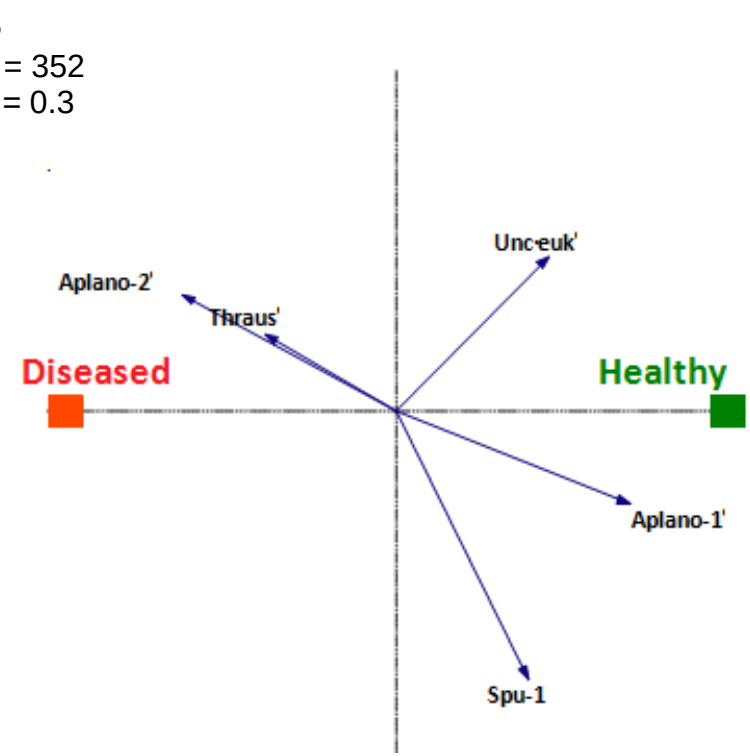


Figure 1. Ordinations (redundancy analysis) of bacterial community shifts associated with incidence of PRD at **A**, SJV, **B**, SV1, and **C**, SV2 orchards. For each ordination, P values indicate overall statistical significance of both axes.





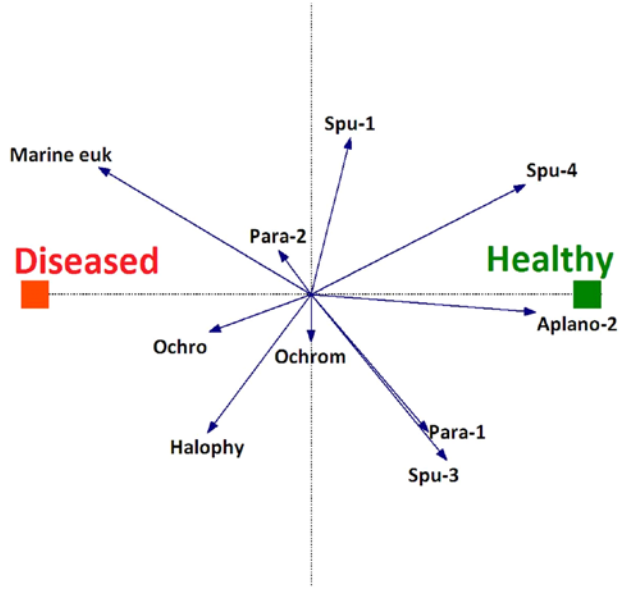
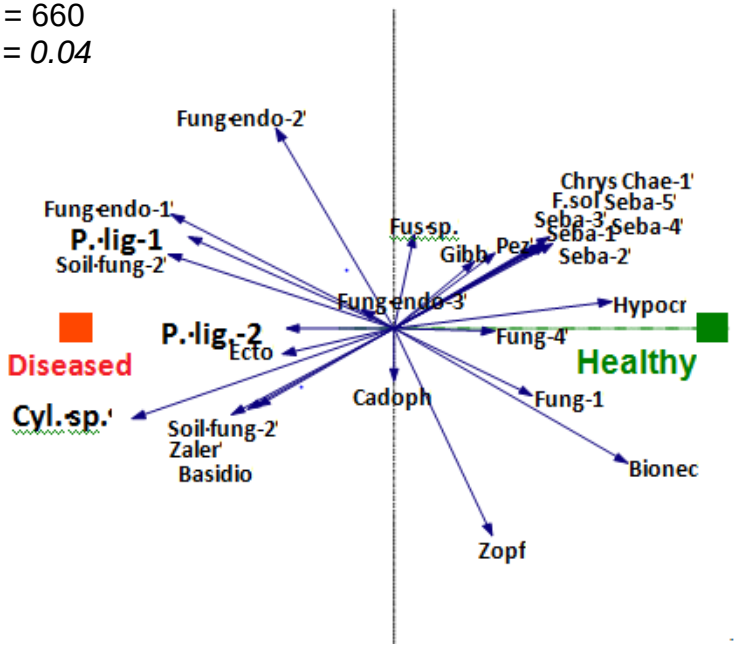
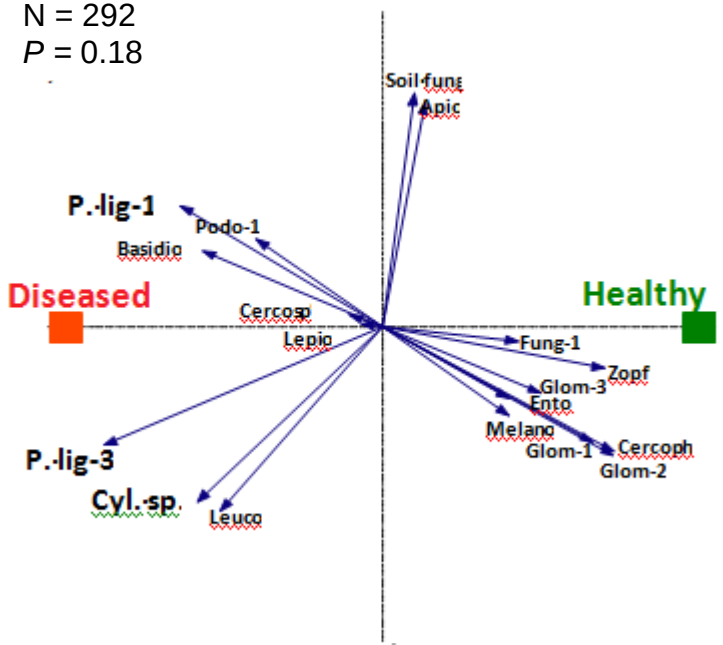


Figure 2. Ordinations (redundancy analysis) of stramenopile community shifts associated with incidence of PRD at **A**, SJV, **B**, SV1, and **C**, SV2 orchards. For each ordination, P values indicate overall statistical significance of both axes.

A
N = 660
P = 0.04



B
N = 292
P = 0.18



C
N = 286
P = 0.27

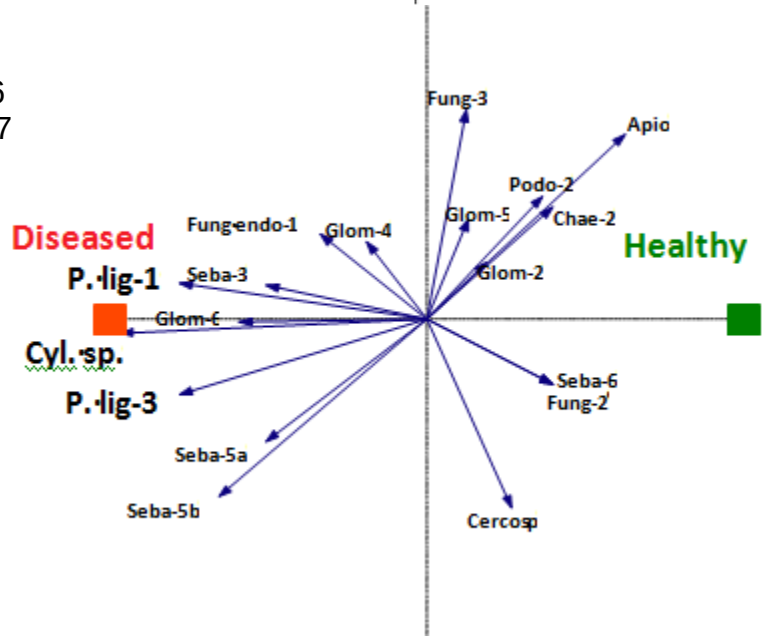


Figure 3. Ordinations (redundancy analysis)of fungal community shifts associated with incidence of PRD at **A**, SJV, **B**, SV1, and **C**, SV2 orchards. For each ordination, P values indicate overall statistical significance of both axes.