UNIVERSITY OF CALIFORNIA
RIVERSIDE

Investigations into Peach Replant Disease and Nematophagous Fungi

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Pathology

by

Jiue-in Yang

September 2012

Dissertation Committee:
Dr. James Borneman, Chairperson
Dr. Jörn Ole Becker
Dr. Michael D. Coffey

The Dissertation of Jiue-in Yang is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

ABSTRACT OF THE DISSERTATION

Investigations into Peach Replant Disease and Nematophagous Fungi

by

Jiue-in Yang

Doctor of Philosophy, Graduate Program in Plant Pathology
University of California, Riverside, September 2012
Dr. James Borneman, Chairperson

The research described in this dissertation examined peach replant disease and two nematode biological control systems using traditional plant pathology methods and molecular microbial ecology methods.

In Chapter 1, the experiments identified microbes associated with peach replant disease in soils with various levels of disease symptoms. To identify bacteria, fungi and oomycetes associated with the replant disease, culture and culture-independent analyses were performed on DNA extracted from plant roots. Among the most abundant bacterial operational taxonomic units, 27 were negatively correlated with peach top weights while 10 were positively correlated. Among the most abundant fungi and oomycetes, negative and positive associations were identified between *P. vexans* and *Trichoderma* spp. and peach top weights, respectively, and verified with sequence-selective quantitative PCR analyses.

In Chapter 2, the population dynamics between *Dactylella oviparasitica* and *Heterodera schachtii* were investigated. Higher initial *D. oviparasitica*

populations were associated with lower final *H. schachtii* populations. Regression models showed that the initial densities of *D. oviparasitica* were only significant when predicting the final densities of *H. schachtii* J2 and eggs as well as fungal egg parasitism, while the initial densities of J2 were significant for all final *H. schachtii* measurements. *H. schachtii*-associated *D. oviparasitica* populations were greatly reduced in nematodes collected from soil compared to nematodes collected from roots. Finally, phylogenetic analysis of rRNA genes suggested that *D. oviparasitica* belongs to a clade of nematophagous fungi with a large geographical distribution.

In Chapter 3, three strains of *Pochonia chlamydosporia* var. *chlamydosporia* were genetically characterized and examined for their biocontrol efficacies against *Meloidogyne incognita*. All strains exhibited different patterns with the enterobacterial repetitive intergenic consensus (ERIC) PCR analysis. Strains 1 and 4 were similar in the PCR analyses of ß-tubulin and the rRNA internal transcribed spacer. In greenhouse trials, all strains reduced the numbers of nematode egg masses. Strain 4 reduced almost 50% of the eggs, and reduced the numbers of J2 and root-galling. A newly developed small subunit rRNA-based PCR analysis differentiated strain 4 from the others, and could potentially be used as a screening tool for identifying other effective biocontrol strains of *P. chlamydosporia* var. *chlamydosporia*.

TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Chapter 1: Associations Between Bacteria, Fungi, Oomycetes and Peach Replant Disease Symptoms in a California Soil

Chapter 2: Population Dynamics of *Dactylella oviparasitica* and *Heterodera schachtii*: Toward a Sugar Beet Planting Decision Model

Chapter 3: Biocontrol Efficacy Among Strains of *Pochonia chlamydosporia* Obtained from a Root-Knot Nematode Suppressive Soil

LIST OF TABLES

Introduction

Plant-microbial interactions in soil are complicated and full of surprising and unpredictable outcomes. The structure and functionality of soil microbial communities depends on the chemical and physical characteristics of the soil, including soil texture, temperature, moisture, and pH (Morris and Blackwood, 2007). In agricultural systems, cropping strategies are also involved, including the farming machinery used, fertilizers, pesticides, watering, etc. As a plant pathologist and microbiologist, in this dissertation, I investigated microbial ecological phenomena, hoping to contribute knowledge to this fascinating microbial world.

A. Soil Suppression and Biological Control

A.1. Suppressive soils

The commonly accepted definition of suppressive soil was given by Baker and Cook, describing the following three conditions: “(A) soil in which the pathogen does not establish or persist (B) soil in which the pathogen establishes but causes little or no damage, or (C) soil in which the pathogen establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil (Baker and Cook, 1974).” Two types of soil suppression are known as “general” and “specific.” General suppression is related to the total microbial biomass in soil and no one microorganism is responsible for the suppression (Cook and Baker, 1983); the

suppression is not transferable between soils, and can often be enhanced by soil amendments (Cook and Rovira, 1976; Rovira and Wildermuth, 1981). Specific suppression, on the other hand, is due to the effects of individual or select groups of microorganisms on the life cycle of a pathogen and the transferability of the suppressiveness is the main characteristic of this type of suppression (Weller et al., 2002). Several specific suppressive phenomena are well studied: Fusarium wilt (Alabouvette, 1999), potato scab (Lorang et al., 1989; Menzies, 1959), and take-all decline (Cook, 2007).

A.2. Microbial activity, disease suppression and replant diseases

While some long-term monoculturing practices induce natural disease suppression, replant diseases can be described as disease suppressiveness diminishing because of continuous planting of a given crop in the same field (Weller et al., 2002). Microbial diversity in the rhizosphere can be related to disease suppressiveness in soil (Nitta, 1991; Workneh and van Bruggen, 1994). Soils with high microbial diversity and biomass can exhibit general disease suppression (Rovira and Wildermuth, 1981), and sometimes induce specific disease suppression later on. However, monoculture systems in agriculture can lead to the occurrence of lower microbial diversity and biomass in soil (Garbeva et al., 2004; van Elsas et al., 2002). A study examining an apple replant disease soil showed increases in several pathogens and the decreases of some beneficial microbes accompanied the diminishing of soil suppression against the disease (Mazzola, 1999).

A.3. Soil suppression on nematodes

Soils suppressive to nematodes including soybean cyst nematode (Chen, 2007; Liu and Wu, 1992; Carris et al., 1989), root knot nematode (Pyrowolakis et al., 2002), cereal cyst nematode (Kerry, Crump, and Mullen et al., 1980) and sugar beet cyst nematodes (Westphal and Becker, 1999, 2000) have been observed as well. Biological factors that drive the suppression against plant parasitic nematodes could be either nematode density-dependent or density-independent. Density dependent antagonists such as the obligate parasitic bacterium *Pasteuria penetrans* (Bishop, 2011) or the endoparasitic fungi, *Catenaria auxiliaries*, *Nematophthora gynophila* (Kerry and Crump, 1977)*, Hirsutella minnesotensis* (Mennan, Chen, and Melakeberhan 2006)*,* and *Hirsutella rhossiliensis* (Tedford et al., 1995; Zhang et al., 2008), impact host nematode populations. While other nematophagous fungi, polyphagous predatory nematodes, and micro-arthropods also limit nematode populations while not acting dependently with the nematode population in soil (Sikora, 1992; Gray, 1985). Physical factors like soil type, moisture, and temperature also affect population establishment and dynamics (Pyrowolakis et al., 2002).

A.4. Biological Control on Nematodes

Nematodes are economically important pathogens. The annual crop yield loss due to plant parasitic nematodes has been estimated to average over 10%, with major horticultural crops at 13.54%, and some other crops

close to 20% (Anwar and McKenry, 2012; Koenning et al., 1999; Sasser and Freckman, 1987). In monetary terms, each year the worldwide losses exceed $100 billion, with $19.37 billion for major horticultural crops (Bird and Kaloshian, 2003; Reddy, 2011; Anwar and McKenry, 2012). Chemical control is currently the major and most effective management method for nematodes. More than 100 million pounds of nematicide (active ingredient) were applied in the United States with a cost exceeding $1 billion per year (Bird, 2003). In the past decades, with public concerns about food safety and environmental toxicity associated with nematicides, biological control has been considered as an alternative management option (Sikora, 1992; Martin, 2003).

Biological control of nematodes has been described as action that involves one or more organisms resulting in the reduction of the population of the target nematode species, or in its capacity to feed on the plant or cause damage (Baker and Cook, 1974). This action could happen by introducing the antagonist(s) or by manipulating the environment, host plant or soil web (Stirling et al., 2011). The most studied natural control system is the decline of the cereal cyst nematode (*Heterodera avenae*) under monoculture in Europe caused by *Nematophora gynophila* and *Pochonia chlamydosprrium* (Kerry, 1982; Kerry et al., 1982). Though many nematophagous fungi have the ability to act antagonistically against nematodes (Moosavi and Zare, 2012), the development of practical, effective biological control strategies has been difficult due to their inconsistency, slow acting properties, and their tendencies to not persist (Kerry, 1997; Cook, 1993).

B. Peach Replant disease

B.1. Peach Replant disease

B.1.1. Replant Disease Terminology

Replant disease was first described by Worlidge in 1698 (Worlidge, 1698). Farmers of tree or vine crops often encounter uneven growth problems when they replant the same or similar crop on sites within several years of removing the previous crops (McKenry, 1999). “Replant diseases” and other terms, including soil sickness (Utkhede, 1987), replant problem (Koch, 1955), replant specific sickness (Hoestra and Oostenbrink, 1961), continuous cropping obstacle, rejection component (McKenry, 1999), and replant disorder (Eayre et al., 2000), have been used to describe these phenomena. Terms such as peach tree short life, or peach tree mortality have also been misused to describe the condition (Ritchie and Clayton, 1981). Different terms reflect the thoughts about the causal agent(s) of this disease, and of those who were studying it. Since the degree of replant crop specificity involved in the disease varies, the term “specific replant disease” is used to describe diseases that happen when the second crop is the same or a closely related species as the first (Savory, 1966). On the contrary, “non-specific replant disease” refers to the diseases that happen when the second crop is not the same or a closely related species as the first (Savory, 1966).

B.1.2. Symptoms of Specific Replant Disease

The aboveground symptoms of specific replant disease mostly include retarded growth, stunting, and various degrees of intercostal chlorosis (Koch, 1955). There is no reliable diagnostic leaf or stem symptom (Savory, 1966), as similar symptoms can also be observed on plants with other diseases. On the other hand, belowground symptoms are more consistent and are therefore a more reliable diagnostic. The root system of the diseased trees are small, dark, compact and feeble (Savory, 1966), and show varying degrees of discoloration and necrosis (Koch, 1955). Interestingly, diseased roots are often free of known pathogens, determined by microscopic observation (Savory, 1966). In severe cases, the diseased plants die (Koch, 1955). When diseased plants are transferred to fresh soil, which had no prior evidence of causing the same disease nor had been used to plant closely related crops, they exhibit a recovery in vigor (Savory, 1966). In addition, the causal agent(s) are also relatively persistent in the absence of the species they affect, as the disease cannot be remedied by short rest periods during which land is occupied by unrelated crops(Savory, 1966).

B.1.3. Regions and Crops Affected by Specific Replant Diseases

Specific replant disease has been observed around the world, and research has been performed in more than 27 countries (Figure 1), including United States, Canada, Australia, New Zealand China, Taiwan, Japan, India, United Kingdom of England, The Netherlands, Israel, France, Iraq, Germany,

Czechoslovakia, Italy, Czech Republic, Hungary, Belgium, Sweden, Poland, Belarus, Romania, Slovenia, Yugoslavia, South Africa, and Libya (Utkhede and Veghelyi, 1996; Utkhede and Smith, 1993; Utkhede, 1987). Specific replant diseases occur not only on annual crops, such as asparagus, cotton, and soybean, but also on perennial crops, such as peach, apple, cherry, and citrus (Utkhede and Veghelyi, 1996; Utkhede, 1987; Utkhede and Smith, 1993; Savory, 1966). However, the crops most affected have been citrus, apple, cherry and peach (Savory, 1966) (Figure 2).

B.1.4. Etiology of Specific Replant Diseases.

The causal agent(s) of specific replant diseases are still unknown. Numerous factors have been implicated in replant disease etiology. There have been many proposed causal factors yet the results are typically either inconclusive or controversial (Savory, 1966), and the specific factors implicated as the causal agents have varied considerably between geographic regions or between orchards in the same region. Abiotic factors such as imbalanced nutrients, soil acidity, soil structure and drainage, aeration on root growth, site deterioration and lack or excess of moisture and phytotoxic metabolites have been suggested to be involved (Mai and Abawi, 1981; Proebsting and Gilmore, 1941; Mizutaniet al., 1988 ; Patrick, 1955; Gur and Cohen, 1989; Utkhede and Smith, 1993; Rowe and Catlin, 1971). Factors contributing to poor soil structure and to decreased biological activity include compaction due to heavy agricultural machinery and decreases in the use of manure (Dumas, 1992;

Figure 1. Where replant disease research has occurred.

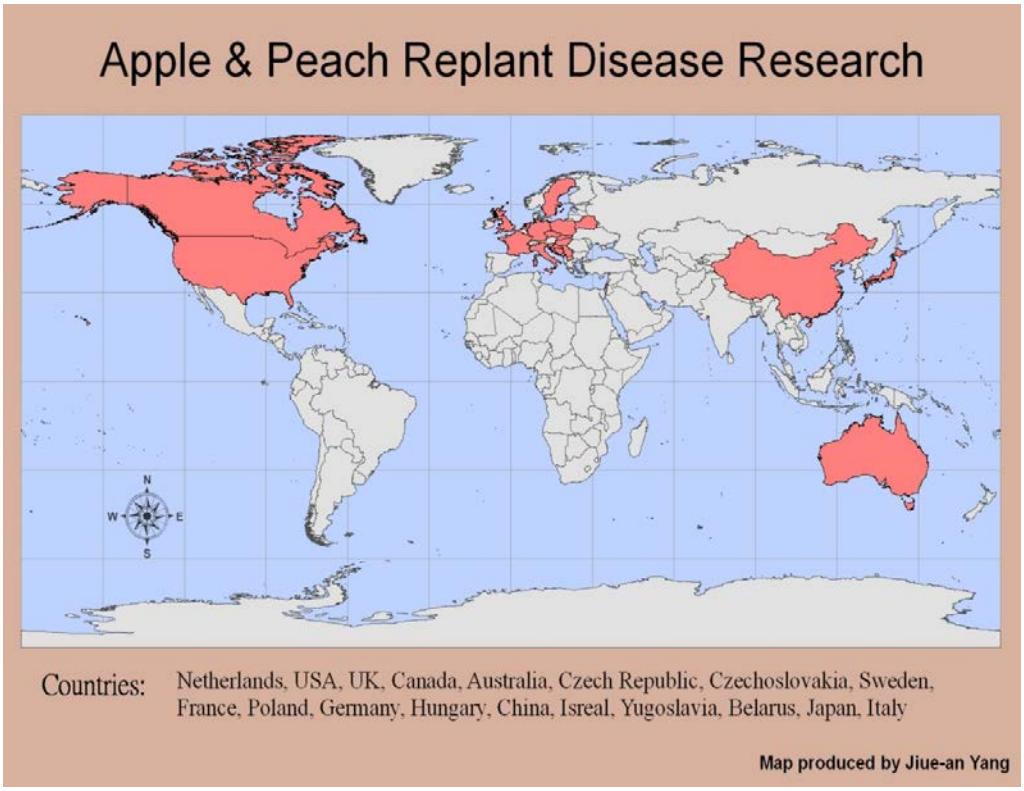


Figure 2. Where research on apple and peach replant diseases has occurred.

Benizri et al., 2005). Soil health decline is attributed to toxins, including hydrocyanic acid, benzaldehyde, and tannins, produced from the old root decomposition (Benizri et al., 2005; Gur and Cohen, 1989). Several studies suggested that the plant residues have reduction effects on the shoot and root of the second crops in peach orchids (Gur and Cohen, 1989; Tagliavini and Marangoni, 1992; Benizri et al., 2005).

Nevertheless, most studies point toward soil-borne organisms or complexes of soil-borne organisms as the most likely cause of specific replant disease. Previous investigations in our laboratory showed that replant disease symptoms were due to biological factors (Bent et al., 2009), with as little as 1% of the replant soil being able to transfer the plant growth decline symptoms. In addition, because specific temperature treatments (50-60C) produced a large effect in the symptoms, this suggests specific microorganism(s) may be causing this replant disease (Bent et al., 2009). In other labs, plant-parasitic nematodes, oomycetes, fungi, bacteria, and other microorganisms have all been implicated.

Nematodes: Root-lesion nematode (*Pratylenchus penetrans* Filipjev) has been reported to be responsible for the replant situation in apple orchards in the northeast United States (Merwin and Stiles, 1989). In a survey in Canada, orchards that had a previous history of the replant problem had three to four times greater soil populations of *P. penetrans* than those with no such history (Mountain and Boyce, 1958). Root knot nematodes were associated with apple replant disease in New York State (Mai and Abawi, 1981). Also, dagger

nematodes (*Xiphinema* spp.), ring nematodes (*Macroposthonia* spp.), and pin nematodes (*Paratylenchus* spp.) are frequently found associated with replant disease in pome and stone fruits in California and the southeastern United States (Mai and Abawi, 1981; Traquair, 1984).

Fungi and oomycetes: Many different fungi and oomycetes also have been associated with replant diseases. Replant disease in *Prunus* has been associated with higher populations of cyanogenic microorganisms (Benizri et al., 2005). Several investigations showed that *Fusarium* spp. (*F. equiseti*, *F. moniliforme*, *F. oxysporum*, and *F. solani*) were frequently isolated from peach orchards that showed replant symptoms in Canada (Wensley, 1956) and United States (Hine, 1961). Besides *Fusarium* spp., *Rhizoctonia solani* (Browne et al., 2006) and *Thielaviopsis basicola* have been implicated in cherry and plum replant disease in the United States (Fliegel et al., 1963), England (Sewell and Wilson, 1975; Pepin et al., 1975) and Holland (Hoestra, 1965). Other fungi such as complexes of *Cylindrocarpon destructans* (Mazzola, 1998; Mazzola et al., 2002), *Cylindrocarpon lucidum* (Mai and Abawi, 1981; Jaffee et al., 1982) are frequently associated with apple replant disease. Many *Pythium* spp. (Mircetich, 1971; Mulder, 1969; Sitepu and Wallace, 1974; Mazzola, 1998; Mazzola et al., 2002; Hendrix et al., 1966) were reported to be associated with replant diseases, and among them, *P. ultimum* (Bielenim, et al., 1976), *P. sylvaticum* (Sewell, 1981), *P. irregure* (Jaffee et al., 1982) and *P. vexans* (Mulder, 1969) are known root pathogens of various tree species. However, some studies also showed that *P. vexans* could enhance the growth of apple, wheat, and ryegrass in sterilized soils (Mazzola et al., 2002; Dewan

and Sivasithamparam, 1988). Another oomycete, *Phytophthora cactorum*, was associated with replant diseases of apple (Jones, 1971; Mazzola, 1998; Sutton et al., 1981), cherry (Mircetich and Matheron, 1976), peach (Hendrix and Powell, 1970), and apricot (Kouyeas, 1971; Traquair, 1984).

Bacteria: Replant disease in *Prunus* has been associated with an increase in rhizosphere bacilli (Benizri et al., 2005). Also, a variety of bacteria have been reported to be associated with the disease (Doll et al., 2008). Fluorescent pseudomonads in the rhizoplane were more abundant in grapevine replant soils than in non-replant soils (Waschkies, Schropp, and Marschner, 1994). Cyanide production by some rhizobacteria such as *Pseudomonas* spp. has been associated with apple and peach in replant conditions (Rumberger et al., 2007; McKenry, 1999). However, weather the chemical was the cause of replant disease symptom (Gur and Cohen, 1989) or whether it was produced by the bacteria group as a mechanism of protective antagonism against root pathogens (Blumer and Haas, 2000; Pal et al., 2000) has not been determined. In addition, *Bacillus* spp. have been reported to be positively associated with the replant disease (Utkhede, 1987; Benizri et al., 2005). In a study of replant of grape, the relative abundance of *Bacillus* sp. and *Flavobacterium* sp. decreased over several years (Guo et al., 2011).

Researchers have often concluded that specific replant diseases are caused by complexes of microbial interactions with their host crops. Utkhede and Li suggested *Bacillus subtilis*, *Pennicillium janthinellum, Costantinella*

terrestris and *Trichoderma* sp. all contributed to apple replant disease in British Columbia, Canada (Utkhede, 1987). In the peach replant research in Ontario, Canada, a series of studies concluded that the replant symptoms are caused by complexes of several fungi, nematodes, and toxic substances produced by microbes (Wensley, 1956; Patrick, 1955; Mountain and Patrick, 1959; Mountain and Boyce, 1958; Koch, 1955). Benizri et al. demonstrated a shift in the structure of bacterial communities with an increase of phytotoxic microorganisms in an artificial peach replant soil (Benizri et al., 2005). An investigation of apple replant orchards in South Africa suggested that the disease was caused by synergistic action of multiple microbes, including *Pythium* spp., *Phytophthora* spp., *Cylindrocarpon* spp., and *Pratylenchus* spp. (Tewoldemedhin et al., 2011).

B.1.5. Specific Replant Disease on Peach (Prunus persica) in California

California produces 65% of the peach crop in the USA (document 1994). Prunus species are considered highly sensitive to replant problems (Jiménez et al., 2011; Browne et al., 2006). Early studies suggested a 7% to 50% mortality rate (Traquair, 1984). Specific replant disease of *Prunus* results in poor growth, stunting, and delayed crop production, and in severe cases, tree death (Koch, 1955). Mortality rates are very high for some rootstocks, including PAC 960, HM-2 and PAC 9907-02, seven years after planting. Other rootstocks such as Evrica, PADAC 9907-23, ROOTPACR 40, and especially Tetra and PAC 9801-02 are less sensitive to replant conditions (Jiménez et al.,

2011; Browne, 2002). In addition, many other economically important crops in California that belong to the same genus *Prunus* are also susceptible to this disease, for example, almond, nectarine and plum.

B.1.6. Management Methods for Specific Replant Diseases

Because the etiology of specific replant disease is unknown, control methods for this disease are not targeted toward a specific factor, and are different from region to region and from crop to crop. Screening and development for resistant and tolerant rootstocks are difficult due to the gap of knowledge in its etiology. Management solutions involving selecting sites that have not been previously cropped to the crop of interest have been considered, yet are restricted by the lack of suitable cropping sites (Traquair, 1984). Prior research has determined that replant disease can only be temporarily controlled with fungicides, and changes in cultural practices do not reduce the disease symptoms (McKenry 1999; Browne, 2002). In California, soil profile modification (i.e. soil ripping, backhoeing of individual tree sites, soil trenching or slip plowing) coupled with soil fumigation has been practiced since 1960's, with methyl bromide being the most commonly used soil fumigant (McKenry, 1999; Mazzola, 1998). However, under the *Montreal Protocol on Substances that Deplete the Ozone Layer* (The Montreal Protocol on Substances that Deplete the Ozone Layer, 2000), and the Clean Air Act (The Clean Air Act Amendments of 1990, 2004), use of methyl bromide is being phased out. As the industry replants orchards with a newer generation of rootstocks better

adapted to adverse conditions, replant disease is becoming a major problem in peach production (Jiménez et al., 2011).

B.1.7. Methods Applied to Replant Disease Studies

Researchers have endeavored to identify the causal agent(s) of replant disease for centuries. Traditional methods to identify potential causal agents mainly rely on dilution-plating, coupled with the use of selective media, and microscopy to identify sporulating fungal bodies. These methodologies are simple and relatively low cost ways of identifying the dominant culturable taxa (Jeewon and Hyde, 2007). Molecular-based techniques, including both DNA- and PCR- based techniques, can provide a more comprehensive depiction of the diversity and composition of microbial communities. The utility of population-based approaches for identifying microorganisms involved in specific in situ processes was one effective method demonstrated by several laboratories (Benitez et al., 2007; Borneman and Becker, 2007; Borneman et al., 2007; Gardener et al., 2005; Rotenberg et al., 2007; Benitez and Gardener, 2008). Nevertheless, selecting the proper sampling timing and method for either type of study methodology has always been a challenge for microbiologists. Interactions of microbes in soil also complicate investigations. Environmental factors, such as drought, cycles of wetting and drying, and seasonal change can cause substantial changes in microbial community composition (Schimel and Bennett, 2004). In ecology, "landscape" stands for the particular spatial arrangement of components of the

environment that are important in some way to the population dynamics of a given species (Paul, 2007). Landscapes are different for different organisms, depending on the spatial scales over which the organisms interact with the environment (Wiens, 1997). It is desirable to be able to forecast population dynamics of plant pathogens or inoculant species for better disease management (Morris and Blackwood, 2007), yet the changes of early stage landscape succession in peach replant soils have not been studied.

B.2. Population-Based Method

In specific *in situ* processes, such as the soil suppressiveness, the utility of population-based approaches for identifying microorganisms involved has been demonstrated by several laboratories (Benitez et al., 2007; Gardener et al., 2005; Rotenberg et al., 2007; Benitez and Gardener, 2008; Lukow et al., 2000; McSpadden-Gardener and Weller, 2001; Weller et al., 2002). The main idea of this approach is to correlate the abundance of microbial populations with levels of the specific functional parameter (Borneman et al., 2007), such as soil suppressiveness (Borneman and Becker, 2007; Yin et al., 2003; Yin et al., 2003). Different levels of functional parameters could be obtained from naturally occurring soils, or they could be created by manipulating the microbial communities with physical, chemical, and biological methods such as heat treatments, antimicrobial agents, and nutritional or microbial amendments. The experiments can be performed by examining the microbial community compositions using a variety of culture or culture independent approaches

such as rRNA gene analysis. After associations of the relative abundance of each taxon with levels of functional parameters has been determined, the taxa exhibiting the strongest correlations represent organisms putatively involved in the defined *in situ* function, and can be further investigated in subsequent validation experiments.

The general approach includes three phases:

Phase I. Identify rRNA genes whose abundance correlates with the functional parameter.

Phase II. Validate the rRNA gene correlations identified in Phase I using an independent quantitative assay, such as sequence-selective qPCR.

Phase III. Isolate the microorganisms represented by the rRNA gene correlates and reintroduce them into the environment to assess their *in situ* functions.

C. Biological Control of Nematodes with Nematophagous Fungi

C.1. Fungus *Dactylella oviparasitica*

The fungus *D. oviparasitica* has been associated with nematode suppressive soils as potential suppressive agents against root knot nematodes and sugar beet cyst nematodes. The nematode-suppressive characteristic of *D. oviparasitica* (G.R. Stirling & R. Mankau) was first discovered in old peach orchards in San Joaqin Valley of California, where unexpectedly low

population densities of root knot nematode *Meloidogyne incognita* were observed in the soil despite the occurrence of susceptible rootstock and suitable environmental conditions (Ferris et al., 1976; Stirling and Mankau, 1978). Another suppressive soil that has been well studied is the 9E field at the Agricultural Experimental Research Station, University of California, Riverside. Here, suppressiveness against the sugarbeet cyst nematode *Heterodera schachtii* was developed by continuously cropping host plants and high initial *H. schachtii* populations (Westphal and Becker, 1999). Molecular population studies and Koch's postulates investigations showed that *D. oviparasitica* was the primary suppressive agent (Olatinwo et al., 2006; Yin et al., 2003).

Dactylella oviparasitica is an Ascomycete from the genus *Dactylella*. Type species *D. oviparasitica* (G.R. Stirling & R. Mankau) has described as with single sporogenous cells that are short, hyaline outgrowth from cells of aerial hyphae function as conidiophores; the length of the conidiophores are approximately the diameter of the hyphae bearing it, which is rarely more than 2 µm (Stirling and Mankau, 1978). Conidia usually form singly and apically as blown-out ends of the conidiophore apex, and are thin walled, hyaline, fusiform, 2.7 to 5.0 by 31 to 60-µm. In a taxonomy study based on morphology and rRNA ITS sequences, *D. oviparasitica* (G.R. Stirling & R. Mankau) is proposed to be the type species of the new Genus *Brachyphoris* J. Chen, L.L. Xu, B. Liu & Xing Z. Liu, gen. nov., with the proposed new species name *Brachyphoris oviparasitica* (G.R. Stirling & R. Mankau) J. Chen, L.L. Xu, B. Liu & Xing Z. Liu, comb. Nov. The word *Brachyphoris* refers to the very short conidiophores

(Chen et al., 2007). However, though the ITS regions are very similar, no conidia have been observed from *D. oviparasitica* strain 50 (Ole Becker, personal communication).

One of the characteristics of *D. oviparasitica* is its thin hyphae andslow growth on agar media. Its has also been described in the following manner: “Hyphae hyaline, septate, flexuous and frequently branched, varying from 1.5 to 3.5-µm in diameter (Chen et al. 2007).” The species forms compact, fluffy or thickly cottony colonies that reach 2 to 2.5-cm diameter on potato dextrose agar (PDA) after incubation at 25ºC for 15 days. It produces sparse aerial mycelium with the diameter of 5.5 to 6-cm when grown on corn meal agar (CMA). The fungus parasitizes later stages of developing juveniles as well as females and immature eggs with hyphae proliferating through egg masses; it does not form predacious organs, and thus is unable to capture nematodes after they hatch from eggs (Stirling and Mankau, 1978). The optimum radial growth temperature on agar for *D. oviparasitica* (G.R. Stirling & R. Mankau) is 24 to 27°C and 23 to 28˚C for strain 50 (Becker et al., 2011); however, the fungus was found to penetrate eggs of root knot nematodes better at a lower temperature ranging 12 to 27°C (Stirling, 1979).

C.2. Arkansas Fungus (ARF)

The fungus Arkansas Fungus 18 (ARF) was first reported and characterized as a biocontrol agent against the soybean cyst nematode (*Heterodera glycines*) by University of Arkansas researchers in the 1990s (Kim

and Riggs, 1991). ARF is a filamentous, non-sporulating fungus that produces sclerotium-like structures on CMA/2 medium and nematode cuticles. The hyphae are septate, frequently branched, and 3 to 4-µm in diameter. The fungus is capable of parasitizing *M. incognita* and several species of cyst nematodes, including *Cactodera betulae*, *Heterodera graminophila*, *H. lespedezae*, *H. leuceilyma*, *H. schacktii*, and *H. trifolii.* It infects more females than cysts. Females infected by ARF18 turn brown and lost turgor pressure. Variation in parasitism and biological control efficacies of different ARF strains has been reported with soybean cyst nematodes (Timper and Riggs, 1998). Parasitized eggs are dark and have attached fungal hyphae, and their surfaces become shrunken and wrinkled. Greater percentages of eggs are parasitized at 20 to 28˚C, yet the fungus grows better at 25 to 28˚C with pH at 7 to 9 (Kim and Riggs, 1991).

Dactylella oviparasitica shares many similarities with the ARF. Both are filamentous fungi with thin hyphae and similar growth rates on water agar and PDA. In addition, both *D. oviparasitica* and ARF are capable of parasitizing species of root knot nematodes and cyst nematodes (Kim and Riggs, 1991; Stirling, 1991). Smith-Becker at el. (2011) conducted further comparisons between the growth rates and infection capabilities of *D. oviparasitica* strain 50 and ARF strain L (Becker et al., 2011). Both fungal strains infected immature white *H. schachtii* females when plated together on water agar, but neither fungus was able to parasitize viable eggs *in vitro*. The growth rate of ARF strain L on PDA was approximately twice compared to the one of *D.*

oviparasitica at 28˚C. The later fungal strain grew fastest between 23 and 28˚C and was inhibited at higher temperatures, while the growth rate of ARF strain L increased up to 30˚C. The growth rate of *D. oviparasitica* was similar on water agar and PDA, while growth of ARF strain L was reduced by more than 50% on water agar. In a greenhouse experiment with sugar beet seedlings, *D. oviparasitica* reduced the number of white females of *H. schachtii* by 40% after one generation while ARF strain L had no effect. *D. oviparasitica* populations increased steadily in soil over a period of 6 weeks while ARF strain L population increased only slightly.

C.3. Fungus *Pochonia chlamydosporia*

Pochonia chlamydosporia var. *chlamydosporia* (syn. *Verticillium chlamydosporium*) (teleomorph = *Metacordyceps chlamydoaporia*), a ubiquitous facultative hyperparasitic fungus of plant-parasitic nematodes, was first reported associated with nematode-suppressive soils in the United Kingdom (Kerry et al., 1984). It is known to parasitize several economically important nematode species in the genera *Meloidogyne*, *Globodera* and *Heterodera* (Kerry, 1990). Different strains of *P. chlamydosporia* vary in their efficacy to control nematode populations (Bourne et al., 1994; Morton et al., 2003b; Mauchline et al., 2004). Strains differ in their virulence, ability to colonize root surfaces, and chlamydospore production. In a study of *Pochonia* species from Iranian soils, *in vitro* pathogenicity tests showed the fungal strains infected root-knot nematode eggs at varying rates between 39% and

95% (Moosavi et al., 2010). In a study of *P. chlamydosporium* strains from a Mexican soil, egg parasitism ratios ranged from 67% to 89% among 5 strains (Flores-Camacho et al., 2008). Different strains of the fungus might also occupy separate niches in soil and rhizosphere, possibly due to differences in their enzymatic activities (Segers et al., 1996; Mauchline et al., 2004). The ß-tubulin gene of *P. chlamydosporia* var. *chlamydosporia* contains an intron not present in other fungi, and thus provides considerable utility for *Pochonia*-selective assays (Hirsch et al., 2001; Kerry and Hirsch, 2011).

P. chlamydosporia has been shown to reduce root-knot nematode populations, either on its own or in combination with other agents. For example, *P. chlamydosporia* reduced *M. hapla* populations on tomato plants by more than 90%, and it also worked in various soil types. In addition, when combined with an aldicarb treatment, it was even more efficacious (Atkins et al., 2003). *Pochonia chlamydosporia* var. *catenulate* significantly reduced *M. incognita* populations in soil using a strategy that combined the fungus with crop rotation (Atkins et al., 2003). Application of *P. chlamydosporia* and *Pseudomonas aeruginosa* as a soil drench also resulted in enhanced growth of tomato plants (Siddiqui and Shaukat, 2003). *Pochonia chlamydosporia* and *Pasteuria penetrans* tended to complement each other when combined, and achieved up to 92% root-knot nematode population control at the second harvest (De Leij et al., 1992). When *P. chlamydosporia* was utilized with the cover plant Surinam grass, this combination significantly reduced *M. javanica*-induced galls by 72% under glasshouse conditions (Giaretta et al., 2011).

C.4. Sugar beet cyst nematodes

The sugarbeet cyst nematode (*Heterodera schachtii*) is a plant pathogen with wide host range covering over 200 plant species within 23 families (Amiri et al., 2002; Steele, 1965). The species has been observed in Europe, North and South America, the Middle East, Africa, and Australia (Baldwin and Mundo-Ocampo, 1991; Evans and Rowe, 1998). *Heterodera schachtii* is economically important to the sugar beet (*Beta vulgaris*) industry, especially in Europe and the United States (Heijbroek et al., 1983). The nematode decreases sugar content of sugar beets and reduces yields, with annual losses estimated at 90 million Euro in European countries (Muller, 1999). It belongs to the *H. schachtii sensu stricto* group (Subbotin et al., 2000), that also includes *H. betae* (=*H. trifolii* fsp. *Betae, H. trifolii* race *beet*) (Wouts et al., 2001) and *H. trifolii,* which are of economic importance for several western European countries (Amiri et al., 2002).

Pathogen management includes nematicides along with cultural methods such as sanitation (Smith et al., 2004). Recently, due to high costs, short term effectiveness, and environmental pollution concerns of chemical applications, crop rotations coupled with resistant sugar beet varieties have been led to profitable production of sugar beets (Niere, 2009; Msayleb and Ibrahim, 2011). In Europe, nematode resistant sugar beet varieties have been grown since 1996 (Plantard and Porte, 2004). Since no resistance genes against *H. schachtii* have been found in *Beta vulgaris* (Roberts, 1992), various

resistance genes, including *Hs1* $^{pro-1}$, *Hs1* $^{web-1}$ and *Hs2* $^{web-7}$, originating from closely related plants such as *B. procumbens* and *B. webbiana*, have been introgressed into sugar beets (Caromel and Gebhardt, 2011). In addition, planting *H. schachtii* resistant trap crops such as oil radish (*Raphanus sativus* L. ssp. *oleiferus* DC) and yellow mustard (Sinapis alba L.) have also been used for effective control (Smith et al., 2004).

C.5. Root knot nematodes

Root-knot nematodes (*Meloidogyne* spp.) are considered the most economically important plant-parasitic nematodes (Whitehead, 1998; Sasser and Freckman, 1987). They are responsible for more than half of the $100 billion annual crop losses caused by plant-parasitic nematodes worldwide (Bird and Kaloshian, 2003). The nematodes cause serious damage on a wide range of crops, especially on vegetables such as tomato, potato, eggplants, okra and pepper in tropical and subtropical agriculture (Sikora and Fernadez, 2005; Anamika et al., 2011). *Meloidogyne* spp. can infect plant roots at the early growing stage, and the invading populations develop as the root systems mature. The average annual yield loss for major crops due to root-knot nematodes is 12.3% (Sasser and Freckman, 1987), yet for some countries like India, it is as high as 27.2% for tomato (Jain et al., 2007) and up to 90% for bean (Anamika et al., 2011).

Besides proper cultural practices (Collange et al., 2011) and the

application of chemical nematicides, plant cultivars that are tolerant or resistant have been widely used for root knot nematode control (Taylor and Sasser, 1978). One of the resistant genes, the Mi gene, has been well studied in tomato (Williamson, 1998). Recently, the use of RNAi silencing has been discussed to achieved crop resistance (Huang et al., 2006). In addition, many possible biological control agents have been discovered and tested for their suppressive efficacy (Sharon et al., 2009; Spiegel, 2010; Singh and Mathur, 2010).

REFERENCE

The Montreal Protocol on Substances that Deplete the Ozone Layer. 2000. In *Secretariat for The Vienna Convention for the Protection of the Ozone Layer.*

Secretariat for The Montreal Protocol on Substances that Deplete the Ozone Layer. Nairobi, Kenya: United Nations Environment Programme.

The Clean Air Act Amendments of 1990. 2004. In *Title 42, Chapter 85.*, edited by U. S. E. P. Agency: 101st United States Congress.

Alabouvette, C. 1999. Fusarium wilt suppressive soils: an example of disease-suppressive soils. Australasian Plant Pathology 28 (1):57-64.

Amiri, S., Subbotin, S.A., and Moens, M. 2002. Identification of the beet cyst nematode *Heterodera schachtii* by PCR. European Journal of Plant Pathology 108 (6):497-506.

Anamika, S. S., Singh, K. P. and Ghosh, G. 2011. Distribution of root-knot nematode on major field crops in Uttar Pradesh (India). Archives of Phytopathology and Plant Protection 44 (2):191-197.

Anwar, S. A., and McKenry, M. V. 2012. Incidence and population density of plant-parasitic nematodes infecting vegetable crops and associated yield losses in Punjab, Pakistan. Pakistan J. Zool. 44 (2):327-333.

Baker, K. F., and Cook, R.J. 1974. Biological control of plant pathogens. San Francisco: Freeman.

Baldwin, J. G., and Mundo-Ocampo, M. 1991. Heteroderinae, cyst and non cyst forming nematodes. In *A Manual of Agricultural Nematology*, edited by W. Nickel. New York, NY, USA: Marcel Dekker Inc.

Becker, S. J., Yang, J., Borneman, J., Timper, P., Riggs, R.R., and Becker, J.O. 2011. Investigations into the relatedness of the nematophagous fungi Dactylella oviparasitica and ARF-L. Journal of Nematology 43:(in press).

Benitez, M., and Gardener, B.B.M.. 2008. Sequence-directed isolation of novel bacteria contributing to soil-borne disease suppression. Phytopathology 98:S21.

Benitez, M. S., Tustas, F.B., Rotenberg, D., Kleinhenz, M.D., Cardina, J., Stinner, D., Miller, S.A., and Gardener, B.B.M. 2007. Multiple statistical approaches of community fingerprint data reveal bacterial populations associated with general disease suppression arising from the application of different organic field management strategies. Soil Biol Biochem 39:2289-2301.

Benizri, E. , Piutti, S., Verger, S., Pages, L., Vercambre, G., Poessel, J. L. and Michelot, P. 2005. Replant diseases: bacterial co mmunity structure and diversity in peach rhizosphere as determined by metabolic and genetic fingerprinting. Soil Biol Biochem 37:1738-1746.

Bent, E., Loffredo, A., Yang, J., McKenry, M. V., Becker, J. O. and Borneman, J. 2009. Investigations into peach seedling stunting caused by a replant soil. *FEMS* Microbiology Ecology 68 (2):192-200.

Bielenim, A., Borecki, Z., andMillikan, D. F. 1976. Identification of *Pythium ultimum* in the collar rot complex of apple. Phytopathology 66:127-129.

Bird, D. M., and Kaloshian, I. 2003. Are roots special? Nematodes have their say. Physiological and Molecular Plant Pathology 62 (2):115-123.

Bishop, A. H. 2011. *Pasteuria penetrans* and its parasitic interaction with plant parasitic nematodes. In *Endospore-forming soil bacteria*, edited by N. A. Logan and P. Vos. Berlin Springer

Blumer, C., and Haas, D. 2000. Mechanism, regulation and ecological role of bacterial cyanide biosynthesis. Achievements in Microbiology 173:170-177.

Borneman, J., and Becker, J.O. 2007. Identifying microorganisms involved in specific pathogen suppression in soil. Annu Rev Phytopathol 45:153-172.

Borneman, J., Becker, J.O., Bent, E., Lanoil, B., Gardener, B.M., Olatinwo, R., Presley, L., Scupham, A.J., Valinsky, L., and Yin, B. 2007. Identifying microorganisms involved in specific in situ functions: experimental design considerations for rRNA genebased population studies and sequence-selective PCR assays. In *Manual of Environmental Microbiology*, edited by C. Hurst. Washington, DC.: ASM Press.

Browne, G. 2002. *Cultural control and etiology of replant disease of Prunus spp.* Davis, CA: University of California, Davis.

Browne, G.T., Connell, J.H., and Schneider, S.M. 2006. Almond replant disease and its management with alternative pre-plant soil fumigation treatments and rootstocks. Plant Disease 90:869-876.

Browne, G.T., Lee, R.C.M., McLaughlin, S.T., Connell, J.H., Schneider, S.M., and Bulluck, R. 2006. Investigating associations between culturable soilborne fungi and replant disease of stone fruits in California. Phytopathology 96 (suppl):S166.

Caromel, B., and Gebhardt, C. 2011. Breeding for nematode resistance: Use of genomic information. In *Genomics and Molecular Genetics of Plant-Nematode Interactions*, edited by J. Jones, G. Gheysen and C. Fenoll: Springer Netherlands.

Carris, L. M., Glawe, D. A., Smyth, C. A., and Edwards, D. I. 1989. Fungi Associated with Populations of Heterodera glycines in Two Illinois Soybean Fields. Mycologia 81 (1):66-75.

Chen, J., Xu, L. Liu, B., and Liu, X. 2007a. Taxonomy of *Dactylella* complex and *Vermispora*. I. Generic concepts based on morphology and ITS sequences data. Fungal diversity 26:73-83.

Chen, J., Xu, L. Liu, B., and Liu, X. 2007b. axonomy of *Dactylella* complex and *Vermispora*. III. A new genus *Brachyphoris* and revision of *Vermispora*. Fungal diversity 26:127-142.

Chen, S. 2007. Suppression of Heterodera glycines in soils from fields with long-term soybean monoculture. Biocontrol Science and Technology 17 (2):125-134.

Collange, B., Navarrete, M., Peyre, G., Mateille, T., and Tchamitchian, M. 2011. Root-knot nematode (*Meloidogyne*) management in vegetable crop production: The challenge of an agronomic system analysis. Crop Protection 30 (10):1251-1262.

Cook, R. J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. Annual Review of Phytopathology 31:53-80.

Cook, R. J., and Rovira, A. D. 1976. The role of bacteria in the biological control of *Gaeumannomyces graminis* by suppressive soils. Soil Biol. Biochem. 8:269-273.

Cook, R. J. 2007. Take-all decline: Model system in the science of biological control and clue to the success of intensive cropping. In *Biological control : a global perspective*, edited by C. Vincent, M. S. Goettel and G. Lazarovits. Cambridge, MA: CABI.

Cook, R.J., and Baker, K. F. *The nature and practice of biological coontrol of plant pathogens*. St. Paul, MN: Am. Phytopathol. Soc.

Dewan, M. M., and Sivasithamparam, . 1988. Pythium SPP in roots of wheat and rye-grass in Western Australia and their effect on root rot caused by *Gaeumannomyces graminis* var. *tritici*. Soil Biology and Biochemistry 20 (6):801-808.

Doll, D. A., Schmidt, L. S., Kluepfel, D. A. and Browne, G.T. 2008. Characterization of the rhizosphere bacterial community associated with *Prunus* replant disease in California. Phytopathology 98 (suppl):S47.

Dumas, Y. 1992. Re´flexions a` propos de la fatigue des sols en tomate d'industrie. Tomato News 4:11-19.

Eayre, C. G., Sims, J. J., Ohr, H. D. andMackey, B. 2000. Evaluation of Methyl Iodide for control of peach replant disorder. Plant Disease 84 (11):1177-1179.

Evans, K., and Rowe, J. A. 1998. Distribution and economic importance. In *The Cyst Nematodes*, edited by S. Sharma. London, UK: Kluwer Academic Publishers.

Ferris, H., McKenry, M. V. and McKinney, H. E. 1976. Spatial distribution of nematodes in peach orchards. Plant Disease Reporter 60:18-22.

Fliegel, P., Parker, K. C. and Mai, W. F. 1963. The fungus flora of non suberized roots of poorly growing cheery trees. Phytopathology 53:1368-1369.

Garbeva, P., van Veen, J. A. and van Elsas, J. D. 2004. Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review of Phytopathology 42:243-270.

Gardener, B. B. M., Gutierrez, L. J., Joshi, R., Edema, R. and Lutton, E. 2005. Distribution and biocontrol potential of phlD(1) pseudomonads in corn and soybean fields. Phytopathology 95:715-724.

Gray, N.F. 1985. Ecology of nematophagous fungi: Effect of soil moisture, organic matter, pH and nematode density on distribution. Soil Biology and Biochemistry 17 (4):499-507.

Guo, X., Li, K., Guo, Y., Xie, H., Sun, Y. and Hu, X. 2011. Effect of grape replant on the soil microbial community structure and diversity. Biotechnology & Biotechnological Equipment 25 (2):2334-2340.

Gur, A., and Cohen, Y. 1989. The peach replant problem - some causal agents. Soil Biol. Biochem. 21:829-834.

Heijbroek, W., Roelands, A. J., andJong, J. H. 1983. Transfer of resistance to beet cyst nematode from *Beta patellaris* to sugar beet. Euphytica 32 (2):287-298.

Hendrix, F. F., Powell, W. H. and Owen, J. H. 1966. Relation of root necrosis caused by *Pythium* species to peach decline. Phytopathology 56:1229-1232.

Hendrix, F. F. Jr., and Powell, W. M. 1970. Control of root pathogens in peach decline sites. Phytopathology 60:16-19.

Hine, R. B. 1961. The role of fungi in the peach replant problem. Plant Disese Report 45:462-465.

Hoestra, H. and Oostenbrink, M. 1961. Nematode damage and specific sickness in Rosa, Malus, and Laburnum. Tijdshr. Plantenziekten 67:264-272.

Hoestra, H. 1965. *Thielaviopsis basicola*, a factor in the cherry replant problem in the Netherlands. European Journal of Plant Pathology 71 (6):180-182.

Huang, G., Allen, R., Davis, E. L., Baum, T. J. and Hussey, R. S. 2006. Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. Proceedings of the National Academy of Sciences 103 (39):14302-14306.

Jaffee, B.A. , Abawi, G.S. and Mai, W.F. 1982. Fungi associated with roots of apple seedlings grown in soil from an apple replant site. Plant Disease 66:942-944.

Jain, R. K., Mathur, K. N. and Singh, R. V. 2007. Estimatimation of losses due to plant parasitic nematodes on different crops in India. Indian Journal of Nematology 37:219-221.

Jeewon, R., and Hyde, K. D. 2007. Detection and diversitv of funqi from environmental samples: Traditional versus molecular approaches In *Advanced techniques in soil microbiology*, edited by A. Varma and R. Oelmuller: Springer.

Jiménez, S., Pinochet, J., Romero, J., Gogorcena, Y., Moreno, M. A. and Espada, J. L. 2011. Performance of peach and plum based rootstocks of different vigour on a late peach cultivar in replant and calcareous conditions. Scientia Horticulturae 129 (1):58-63.

Jones, A. L. 1971. *Diseases of tree fruits in Michigan*, *Ext. Bull. E-714*. East Lansing: Coop. Ext. Serv., Mich. State Univ.

Kerry, B. R. 1982. The decline of *Heterodera avenae* populations. *EPPO Bulletin* 12 (4):491-496.

Kerry, B. R. 1997. Biological control of nematodes: prospects and opportunities. In *Expert Consultation on Plant Nematode Problems and their Control*. Near East Region, Karachi (Pakistan): FAO Plant Production and Protection Paper.

Kerry, B. R., and Crump, D. H. 1977. Observations on fungal parasites of females and eggs of the cereal cyst-nematode, *Heterodera Avenae*, and other cyst nematodes. Nematologica 23 (2):193-201.

Kerry, B. R., Crump, D. H. and Mullen, L. A. 1980. Parasitic fungi, soil moisture and multiplication of the cereal cyst nematode, Heterodera avenae. Nematologica 26 (1):57-68.

Kerry, B. R., Crump, D. H. and Mullen, L. A. 1982. Studies of the cereal cyst-nematode, *Heterodera avenae* under continuous cereals, 1975–1978. II. Fungal parasitism of nematode females and eggs. Annals of Applied Biology 100 (3):489-499.

Kim, D.G., and Riggs, R.D. 1991 Characteristics and efficacy of a sterile hyphomycete (ARF18), a new biocontrol agent for Heterodera glycines and other nematodes. J Nematol. 23 (3):275-282.

Koch, L. W. 1955. The peach replant problem in Ontario: I. Symptomatology and distribution. Canadian Journal of Botany 33 (5):450-460.

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O. and Fortnum, B. A. 1999. Survey of Crop Losses in Response to Phytoparasitic Nematodes in the United States for 1994. J Nematol. 31 (4S):587-618.

Kouyeas, H. 1971. On the apoplexy of fruit trees caused by *Phytophthora* spp. Annales Institut Phytopathologique Benaki 10:163.

Liu, X. Z. , and Wu, X. Y. 1992. Decline of soybean cyst nematode: A preliminary result. Paper read at The Second International Workshop on Plant Nematology, at Karachi, Pakistan.

Lorang, J. M., Anderson, N. A. , Lauer, F. I., and Wildung, D. K. 1989. Disease decline in a Minnesota potato scab plot. American journal of potato research 66:531.

Lukow, T., Dunfield, P. F. and Liesack, W. 2000. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol. Ecol. 32:241-247.

Mai, W. F., and Abawi, G. S. 1981. Controlling replant diseases of pome and stone fruits in Northeastern United States by preplant fumigation. Plant Disease 65:859-864.

Martin, F. N. 2003. Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. Annual Review of Phytopathology 41 (1):325-350.

Mazzola, M. 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. Phytopathology 88 (9):930-938.

Mazzola, M. 1999. Transformation of soil microbial community structure and *Rhizoctonia*-suppressive potential in response to apple roots. Phytopathology 89 (10):920-927.

Mazzola, M., Andrews, P. K., Reganold, J. P. and Lacvesque, C. A. 2002. Frequency, virulence, and metalaxyl sensitivity of *Pythium* spp. isolated from apple roots under conventional and organic production systems. Plant Disease 86 (6):669-675.

McKenry, M. V. 1999. *The Replant Problem and Its Management*. Fresno, CA: Catalina Publishing.

McSpadden-Gardener, B. B., and Weller, D. M. 2001. Changes in populations of rhizosphere bacteria associated with Take-All Disease of wheat. Appl. Environ. Microbiol. 67:4414-4425.

Mennan, S., Chen, S. and Melakeberhan, H. 2006. Suppression of *Meloidogyne hapla* populations by *Hirsutella minnesotensis*. Biocontrol Science and Technology 16 (2):181-193.

Menzies, J. D. 1959. Occurrence and transfer of a biological factor in soil that suppresses potato scab. Phytopathology 49:648-652.

Merwin, I. A., and Stiles, W. C. 1989. Root-lesion nematodes, potassium deficiency, and prior cover crops as factors in apple replant disease. Journal of the American Society for Horticultural Science 114 (5):728-732.

Mircetich, S. M. 1971. The role of *Pythium* in feeder roots of diseased and symptomless peach trees and in orchard soils in peach tree decline. Phytopathology 61:357-360.

Mircetich, S. M., and Matheron, M. E. 1976. *Phytophthora* root and crown rot of cherry trees. Phytopathology 66:549-558.

Mizutani, F., Hirota, R. and Kadoya, K. 1988 Growth inhibiting substances from peach roots and their possible involvement in peach replant problems . Acta Horticulturae 233:37-43.

Moosavi, M. R., and Zare, R. 2012. Fungi as biological control agents of plant-parasitic nematodes. In *Plant Defence: Biological Control*, edited by J. M. M. Mérillon and K. G. G. Ramawat: Springer Netherlands.

Morris, S. J., and Blackwood, C. B.. 2007. The ecology of soil organisms. In *Soil microbiology, ecology, and biochemistry*, edited by E. A. Paul. Burlington, MA: Elsevier.

Mountain, W. B., and Boyce, H. R. 1958. The peach replant problem in Ontario: V. The relation of parasitic nematodes to regional differences in severity of peach replant failure. Canadian Journal of Botany 36 (1):125-134.

Mountain, W. B., and Patrick, Z. A. 1959. The peach replant problem in Ontario: VII. The pathogenicity of Pratylenchus penetrans (Cobb, 1917) Filip. & Stek. 1941. Canadian Journal of Botany 37 (3):459-470.

Msayleb, N., and Ibrahim, S. 2011. Treatment of nematodes with ozone gas: A sustainable alternative to nematicides. Physics Procedia 21 (0):187-192.

Mulder, D. 1969. The pathogenicity of several *Pythium* species to rootlets of apple seedlings. European Journal of Plant Pathology 75 (1):178-181.

Muller, J. 1999. The economic importance of *Heterodera schachtii* in Europe. Helminthologia 36:205-213.

Niere, B. 2009. Principles of beet cyst nematode management. Sugar Industry / Zuckerindustrie 134 (3):186-192.

Nitta, T. 1991. Diversity of root fungal floras: its implications for soil-borne diseases and crop growth. Japan Agricultural Research Quarterly 25 (1):6-11.

Olatinwo, R., Yin, B., Becker, J. O. and Borneman, J. 2006. Suppression of the Plant-Parasitic Nematode Heterodera schachtii by the Fungus Dactylella oviparasitica. Phytopathology 96 (1):111-114.

Pal, K. K., Tilak, K.V.B.R., Saxena, A.K., Dey, R. and Singh, C.S. 2000. Antifungal characteristics of a fluorescent *Pseudomonas* strain involved in the biological control of *Rhizoctonia solani*. Microbiological Research 155:233-243.

Patrick, Z. A. 1955. The peach replant problem in Ontario. II. Toxic substances from microbial decomposition products of peach root residues. Can J Botany 33:461-486.

Paul, E. A. 2007. *Soil microbiology, ecology, and biochemistry*: Academic Press.

Pepin, H. S., Sewell, G. W. F. and Wilson, J. F. 1975. Soil populations of *Thielaviopsis basicola* associated with cherry rootstocks in relation to effects of the pathogen on their growth. Ann. Appl. Biol. 79:171-176.

Plantard, O., and Porte, C. 2004. Population genetic structure of the sugar beet cyst nematode *Heterodera schachtii*: a gonochoristic and amphimictic species with highly inbred but weakly differentiated populations. Molecular Ecology 13 (1):33-41.

Proebsting, E., and Gilmore, A. 1941. The relation of peach root toxicity to the re-establishing of peach orchards. Proceedings of American Society for Horticultural Science 38:21-26.

Pyrowolakis, A., Westphal, A., Sikora, R. A. and Becker, J. O. 2002. Identification of root-knot nematode suppressive soils. Applied Soil Ecology 19 (1):51-56.

Reddy, P. P. 2011. *Handbook of biological control in horticultural crops. Volume 3: Biomanagement of nematodes pests*. New Delhi: Studium Press (India) Pvt. Ltd.

Ritchie, D. F., and Clayton, C. N. 1981. Peach tree short life: A complex of. Plant Disease 65 (6):462-469.

Roberts, P. A. 1992. Current status of the availability, development, and use of host plant resistance to nematodes. J Nematol. 24 (2):213-227.

Rotenberg, D., Joshi, R., Benitez, M. S., Chapin, L.G., Camp, A., Zumpetta, C., Osborne, A., Dick, W. A. and Gardener, B. B. M. 2007. Farm management effects on rhizosphere colonization by native populations of 2,4-diacetylphloroglucinol-producing Pseudomonas spp. and their contributions to crop health. Phytopathology 97:756-766.

Rovira, A. D., and Wildermuth, G.B. 1981. The nature and mechanisms of suppression In *Biology and Control of Take-all*, edited by M. Asher and P. Shipton. London: Academic.

Rowe, RN, and Catlin, P. B. 1971. Differential sensitivity to waterlogging and cyanogenesis by peach, apricot, and plum roots. J. Am. Soc. Hort. Sci. 96:305-308.

Rumberger, A., Merwin, I. A. andThies, J. E. 2007. Microbial community development in the rhizosphere of apple trees at a replant disease site. Soil Biology and Biochemistry 39 (7):1645-1654.

Sasser, J. N., and Freckman, D. W. 1987. A world perspective on nematology: The role of the society. Edited by J. A. Veech and D. W. Dickson, *Vistas on Nematology, A Commemoration of the 25th Anniversary of the Society of Nematologists*. Hayattsville: Society of Nematologists, Inc.

Savory, B. M. 1966. *Specific Replant Diseases*. Kent: commonwealth agricultural bureaux.

Schimel, J. P., and Bennett, J. 2004. Nitrogen mineralization: challenges of a changing paradigm. Ecology 85:591-602.

Sewell, G. W. F. 1981. Effects of *Pythium* species on the growth of apple and their possible causal role in apple replant disease. Annals of Applied Biology 97 (1):31-42.

Sewell, G. W. F., and Wilson, J. F. 1975. The role of *Thielaviopsis basicola* in the specific replant disorders of cherry and plum. Annals of Applied Biology 79 (2):149-169.

Sharon, E., Chet, I., Bar-Eyal, M. andSpiegel, Y. 2009. Biocontrol of root-knot nematodes by Trichoderma - modes of action. IOBC/WPRS Bulletin 42:159-163.

Sikora, R A. 1992. Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Annual Review of Phytopathology 30 (1):245-270.

Sikora, R. A., and Fernadez, E. 2005. Nematode parasites of vegetables. In *Plant parasitic nematodes in subtropical and tropical agriculture*, edited by M. Luc, R. A. Sikora and J. Bridge. Wallingford, UK: CABI publishing.

Singh, S., and Mathur, N. 2010. Biological control of root-knot nematode, *Meloidogyne incognita* infesting tomato. Biocontrol Science and Technology 20 (8):865-874.

Sitepu, D., and Wallace, H.R. 1974. Diagnosis of retarded growth in an apple orchard. Austral. J. Expt. Agric. and Animal Husb. 14:577-584.

Smith, H. J., Gray, F. A. and Koch, D. W. 2004. Reproduction of *Heterodera schachtii* Schmidt on resistant mustard, radish, and sugar beet cultivars. J Nematol. 36 (2):123-130.

Spiegel, Y. 2010. Biological control of plant parasitic nematodes: From fantasy to reality. Acta Horticulturae (ISHS) 883:209-214.

Steele, A. E. 1965. The host range of the sugarbeet nematode *Heterodera schachtii* Schmidt. Journal of American Society of Sugar Beet Technology 13:573-603.

Stirling, G. R. 1979. Effect of temperature on parasitism of *Meloidogyne incognita* eggs by *Dactylella oviparasitica*. Nematologica 25:104-110.

Stirling, G. R., and Mankau, R. 1978. *Dactylella oviparasitica*, a new fungal parasite of *Meloidogyne* eggs. Mycologia 70 (4):774-783.

Stirling, G. R., Davies, K. and Spiegel, Y. 2011. Biological control of plant-parasitic nematodes: An ecological perspective, a review of progress and opportunities for further research. In *Biological Control of Plant-Parasitic Nematodes*, edited by K. Davies and Y. Spiegel: Springer Netherlands.

Subbotin, S. A., Waeyenberge, L. and Moens, M. 2000. Identification of cyst forming nematode of the genus Heterodera (Nematoda: Heteroderidae) based on the ribosomal DNA-RFLPs. Nematology 2:153-164.

Sutton, T. B., Wayne, D. W., Sullivan, W. T., Nardacci, J. F. and Klimstra, D. E. 1981. Causes of apple tree death in Henderson county, North Carolina. Plant Disease 65 (4):330-331.

Tagliavini, M., and Marangoni, B. 1992. Growth of peach as affected by decomposition of own root residues in soil. Plant and Soil 145 (2):253-260.

Taylor, A. L., and Sasser, J. N. 1978. *Biology, identification, and control of root-knot nematodes (Meloidogyne species)*. North Carolina State University Graphics.

Tedford, E.C., Jaffee, B. A. and Muldoon, A. E. 1995. Suppression of the nematode *Heterodera schachtii* by the fungus *Hirsutella rhossiliensis* as affected by fungus population density and nematode movement. Phytopathology 85:613-617.

Tewoldemedhin, Y. T., Mazzola, M., Labuschagne, I. and McLeod, A. 2011. A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. Soil Biology and Biochemistry 43 (9):1917-1927.

Traquair, J. 1984. Etiology and control of orchard replant problems: a review. Canadian Journal of Plant Pathology 6:54-62.

Utkhede, R. S., andSmith, E. M. 1993. Biotic and abiotic causes of replant problems of fruit trees. Paper read at Third International Symposium on Replant Problems, July 20-23, 1993, at Penticton, Canada.

Utkhede, R., and Veghelyi, K. 1996. Proceedings of the Fourth International Symposium on Replant Problems. Paper read at Fourth International

Symposium on Replant Problems, Aug 12-16, 1996, at Budapest, Hungary.

Utkhede, R. S. 1987a. The role of fungi, bacteria, and their interactions in apple replant disease complex in soils of British Columbia. Paper read at Workshop on Replant Problems with Fruit Trees, Aug 25-28, 1987, at Bonn, Germany.

Utkhede, R. S. 1987b. Workshop on replant problems with fruit trees. Bonn, germany: ISHS.

van Elsas, J. D., Garbeva, P. and Salles, J. 2002. Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. Biodegradation 13 (1):29-40.

Waschkies, C., Schropp, A. and Marschner, H. 1994. Relations between grapevine replant disease and root colonization of grapevine (*Vitis* sp.) by fluorescent pseudomonads and endomycorrhizal fungi. Plant and Soil 162 (2):219-227.

Weller, D. M., Raaijmakers, J. M., McSpadden-Gardener, B. B. and Thomashow, L. S. 2002. Microbial population responsible for specific soil suppressiveness to plant pathogens. Ann. Rev. Phytopathol. 40:309-348.

Wensley, R. N. 1956. The peach replant problem in Ontario:IV. Fungi associated with replant falure and their importance in fumigated and nonfumigated soils. Canadian Journal of Botany 34 (6):967-981.

Westphal, A., and Becker, J. O. 1999. Biological suppression and natural population decline of *Heterodera schachtii* in a California field. Phytopathology 89:434-440.

Westphal, A., and Becker, J. O. 2000. Transfer of biological soil suppressiveness against Heterodera schachtii. Phytopathology 90:401-406.

Whitehead, A. G. 1998. *Plant nematode control*. Wallingford, UK: CAB International.

Wiens, J. A. 1997. Metapopulation dynamics and landscape ecology. In *Metapopulation Biology: Ecology, Genetics, and Evolution*, edited by I. H. a. M. E. Gilpin. San Diego: Academic Press.

Williamson, V. M. 1998. Root-knot nematode resistance genes in tomato and their potential for future use. Annual Review of Phytopathology 36:277-293.

Workneh, F., and van Bruggen, A. H. C. 1994. Microbial density, composition, and diversity in organically and conventionally managed rhizosphere soil in relation to suppression of corky root of tomatoes. Applied Soil Ecology 1 (3):219-230.

Worlidge. 1698. Systema Agriculturae*. The Mystery of Husbandry Discovered*. London.

Wouts, W. M., Rumpenhorst, H. J. and Sturhan, D. 2001. *Heterodera betae* sp. nov., the yellow beet cyst nematode (Nematoda: Heteroderidae). Russian Journal of Nematology 9 (1):33-42.

Yin, B., Valinsky, L., Gao, X., Becker, J. O., and Borneman, J. 2003. Bacterial rRNA genes associated with soil suppressiveness against the plant-parasitic nematode *Heterodera schachtii*. Appl. Environ. Microbiol. 69:1573-1580.

Yin, B., Valinsky, L., Gao, X., Becker, J. O., and Borneman, J. 2003. Identification of fungal rDNA associated with soil suppressiveness against Heterodera schachtii using oligonucleotide fingerprinting. Phytopathology 93:1006-1013.

Zhang, L., Yang, E., Xiang, M., Liu, X. andChen, S. 2008. Population dynamics and biocontrol efficacy of the nematophagous fungus *Hirsutella rhossiliensis* as affected by stage of the soybean cyst nematode. Biological Control 47 (2):244-249.

Ch 1. Associations Between Bacteria, Fungi, Oomycetes and Peach Replant Disease Symptoms in a California Soil

ABSTRACT

The objective of this study was to identify bacteria, fungi and oomycetes associated with peach replant disease symptoms at a field location in California. Soil samples were subjected to treatments to create various levels of replant disease symptoms. Peach seedlings were grown in the treated soils in greenhouse trials. After 6 weeks, plant growth parameters were measured, and culture and culture-independent analyses were performed on DNA extracted from the plant roots to identify bacteria, fungi and oomycetes. A total of 9,320 bacterial operational taxonomic units (OTU) were identified. Among the 60 most abundant OTUs, 27 showed significant ($P < 0.05$) negative correlation with peach top weights while 10 were positively correlated. Most of these OTUs belonged to the bacterial phylum Proteobacteria (96%), including the classes Gammaproteobacteria (44.4%), Betaproteobacteria (33.3%) and Alphaproteobacteria (22.2%), and fell into the orders Pseudomonadales, Burkholderiales, Chromatiales, Rhodocyclales, and Sphingomonadales. The most abundant fungal taxa were *Trichoderma asperellum*, *Trichoderma virens, Fusarium oxysporum, Ceratocystis fimbriata* and *Fusarium solani.* The most abundenat oomycetes taxa were *Pythium vexans*, *Pythium violae* and an unidentified *Aplanochytrium* species. Sequence-selective quantitative PCR

analyses identified negative and positive associations between *P. vexans* and *Trichoderma* sp. and peach top weights, respectively.

INTRODUCTION

Replant disease was first described by Worlidge in 1698 (Worlidge, 1698). It has been subsequently observed in North America, Europe, Asia, Oceania and Africa (Utkhede, 1987). The crops most affected include citrus, apple, cherry and peach (Savory, 1966). Replant disease of *Prunus* species result in poor growth, delayed crop production, and, in severe cases, tree death (Koch, 1955). The disease can only be temporarily controlled with fungicides, and changes in cultural practices do not reduce the disease symptoms (Browne, 2002; McKenry, 1999). Since the 1960s, the practices for controlling replant disease in California have included soil profile modification coupled with soil fumigation (McKenry, 1999; Mazzola, 1998).

Numerous factors have been implicated in replant disease etiology. Specific factors implicated as the causal agents have varied considerably between geographic regions or between orchards in the same region. Abiotic factors such as nutrition, soil structure, and phytotoxic metabolites or remaining roots from previous crops have been suggested to be involved (Mai and Abawi, 1981; Proebsting and Gilmore, 1941; Mizutani et al., 1988 ; Patrick, 1955; Gur and Cohen, 1989; Benizri et al., 2005). Microorganisms including a variety of bacteria (Doll et al., 2008), complexes of fungi (Browne et al., 2006) and oomycetes (Jaffee et al., 1982; Mazzola, 1998; Hine, 1961; Hendrix et al., 1966; Mulder, 1969; Sewell, 1981; Sitepu and Wallace, 1974) have also been implicated. For example, replant disease in *Prunus* spp. have been associated

with an increase in rhizosphere bacilli and higher populations of cyanogenic microorganisms (Benizri et al., 2005).

In this study, we examined a peach replant disease soil in Kearny, CA. We first determined that there was a biological component to the replant disease symptoms. We then used a population-based approach to identify bacteria, fungi and oomycetes associated with replant disease symptoms. Finally, sequence-selective qPCR assays were used to validate the associations.

MATERIAL AND METHODS

Soil and peach seedlings. Soil was collected from the upper 30-cm in a field at the Kearney Agricultural Center in California, where replant disease symptoms were observed on Nemaguard rootstocks 10 weeks after planting (Michael McKenry, personal communication). Soil was passed through a metal sieve with 12-mm openings. Two-month-old clonal-Nemaguard-peach seedlings were obtained from Duarte Nursery, Hughson, CA.

Soil treatments. To confirm the biological nature of the replant disease symptoms and to establish various levels of the symptoms for microbial community analysis, soils were temperature-treated and diluted with various amounts of pasteurized soil. For the temperature treatments, soils were exposed to room temperature, 40°C, 50°C, 60°C and 70°C. Soil samples (~1 kg) were double-bagged and submerged in a water bath, and held for 30 minutes once the center of the sample reached the target temperature. The

bags were then cooled to room temperature under running tap water. All samples of the same treatment were pooled and mixed thoroughly. For the dilution treatments, soils were mixed with different percentages of pasteurized soil (121°C for 2 hours) at ratios of non-treated to pasteurized soil: 100:0, 10:90, 1:99, 0.1:99.9 and 0:100. Treated soils were incubated at room temperature for 2 days prior use.

Greenhouse trials. Plastic pots with drain holes were double-cupped and filled with 800-cm^3 of the treated soils described above. Each pot was planted with one Nemaguard peach seedling. Each pot was fertilized with 7-g of slow-release fertilizer (Sierra 17-6-10 plus Minors, Scotts-Sierra Horticultural Products Company, Marysville, OH) and watered as needed. Trials were arranged in a randomized complete design and incubated in a greenhouse with six replicates for each soil treatment. After 6 weeks, plant tops were cut off 10-cm above the soil level and weighed. Shoot lengths of each branch were measured from the main stem. Plant dry weights were measured after 3-days incubation in 125°C oven. Two hundred milligram root tip samples from each plant were collected and stored at -20°C for DNA extraction. Fine root tips were collected and stored in sterile tubes at room temperature for fungal and oomycetes isolation. Trials were performed twice.

Isolation of fungi and oomycetes. Pieces of fine root tips from each of the non-treated seedling replicate pots were collected at the end of the trials, stored at room temperature and processed for culturing within 24 hours after sampling. From each replicate pot, 12 pieces of 3-cm-long root tips were rinsed with ultrapure water for 15 seconds, dried by pressing between paper

towels, placed on 1% water agar, and incubated at room temperature. Fungi and oomycetes that emerged from the root surfaces during the first 36 hours were sub-cultured onto new 1% water agar plates. The hyphal-tip method was used to obtain pure cultures. All isolates were identified by rRNA gene sequence analysis.

DNA extraction. DNA was extracted from (1) root tip samples collected at the end of the green house trials and (2) fungi and oomycetes isolated from the roots. Two hundred milligrams of root tips or fungal hyphae were used for extraction. Genomic DNA of the isolates were extracted using FastDNA Spin Kit for Soil (Qbiogene, Carlsbad, CA) as described by the manufacturer using a 90 second bead-beating step in a FastPrep Instrument (Qbiogene) and a 5.5 setting. The extraction product was further purified by electrophoresis in 1% agarose gels. DNA larger than 3Kb was isolated by using a MinElute Gel Extraction Kit (Qiagen, Valencia, CA), without use of UV lighter or ethidium bromide.

Bacterial rRNA gene sequencing. To identify bacteria, we performed a high throughput sequencing analysis of the small-subunit rRNA genes using genomic DNAs extracted from the root samples collected at the end of the green house trials as template. One hundred microliter amplification reactions were performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Inc., Hercules, CA) and contained: 50 mM Tris (pH 8.3), 500 µg/ml bovine serum albumin (BSA), 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP), 400 nM of each primer, 4 µl of DNA template, and 2.5 units JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO). Primers used for PCR

were SSU-For (AATGATACGGCGACCACCGAGATCTACACTATCGCCGTTGTGTGCCAGC MGCCGCGGTAA) and SSU-Rev (CAAGCAGAAGACGGCATACGAGATCTAGCGTGCGTTAGTCAGTCAGCCG GACTACHVGGGTWTCTAAT) (Caporaso et al. 2011). Thermal cycling parameters were 94°C for 5 minutes; 35 cycles of 94°C for 45 seconds, 50°C for 60 seconds, and 72°C for 90 seconds, and followed by 72°C for 10 minutes. PCR products were purified using a MinElute Gel Extraction Kit (Qiagen) except PB buffer was substituted with QG buffer. PCR products were diluted to 20 ng/µl prior for sequencing. DNA sequencing was performed using an Illumina HiSeq2000 (Illumina, Inc., San Diego, CA). Primers and barcodes used for DNA sequencing are listed in Supplementary Table 1.

PCR amplification of oomycete and fungal rRNA genes. For the culture-independent analysis, root DNAs extracted from plants exhibiting a wide range of replant disease symptoms from greenhouse trials were used as templates. Ten microliter amplification reactions were performed in 10-µl glass capillary tubes using a RapidCycler (Idaho Technologies, Salt Lake City, UT) containing the following reagents: 50 mM Tris (pH 8.3), 500 mg/ml bovine serum albumin (BSA), 2.5 mM $MgCl_2$, 250 mM of each dNTP, 400 nM of each forward and reverse primer, 1-µl (~66 ng) of peach root DNA and 0.5 units *Taq* DNA polymerase. Fungi-selective primers were nu-SSU-0817-5 (TTAGCATGGAATAATRRAATAGGA) and nu-SSU-1536-3 (ATTGCAATGCYCTATCCCCA) (Borneman and Hartin, 2000) while oomycetes (examined using stramenopile-selective primers) were

StramenoSSUF1 (GATGATTAGATACCATCGTA) and StramenoSSUR2 (AAAGGGCAGGGACGT) (Bent et al., 2009), with PCR products being ~762 bp and ~638 bp, respectively. Thermal cycling parameters were 94°C for 5 minutes; 35 cycles of 94°C for 20 seconds, X°C for 30 seconds and 72°C for 40 seconds; followed by 72°C for 5 minutes, where X = 55 for fungi and 59 for stramenopiles.

For the culture-based analyses, fungi and oomycetes isolates were identified by analysis of DNAs extracted from pure cultures. rRNA gene primers ITS1FUSER (GGGAAAGUCTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4USER (TCCTCCGCTTATTGATATGC) (T.J. White et al. 1990) were used with the following conditions: 94°C for 5 minutes, followed by 40 cycles of 94°C for 20 seconds, 52°C for 20 seconds, 72°C for 40 seconds, and a final incubation at 72°C for 5 minutes. PCR master mixes were prepared as described above in this subsection.

To obtain the sequences of the internal transcribed spacer (ITS) region for *Pythium vexans*, *Ceratocystis fimbriata* and *Fusarium oxysporum* for quantitative PCR assay development, chromosome walking was conducted. PCR was performed on extracted peach root DNA using the following forward primers combined with ITS4 (TCCTCCGCTTATTGATATGC) (T.J. White et al. 1990) individually: PvexansSSUF3 (GGGACTTTTGGGTAATC), CfimbSSUF1 (AGGTCCAGACACAG), and FoxySSUF2 (TTCATTAATCAGGAACGA). The forward primers were designed using PRISE (Fu et al. 2008). The thermal cycling conditions were 94°C for 5 minutes; 40 cycles of 94°C for 20 seconds, 52°C for 30 seconds and 72 °C for 90 seconds; followed by 72°C for 10

minutes. Amplification products were gel isolated and cloned as described previously (Bent et al., 2009), and the nucleotide sequences were obtained as described below.

Quantitative PCR. *Pythium vexans, Ceratocystis fimbriata, Fusarium oxysporum, and Trichoderma* (targeting species *T. asperellum*, *T. harizianum* and *T. virens*) were quantified using real-time PCR assays performed in a Bio-Rad iCycler MyiQTM Real-Time Detection System (Bio-Rad Laboratories, Inc). The templates were genomic DNAs extracted from the root samples collected at the end of the green house trials. Sequence-selective primers developed in this study were designed using PRISE software (Fu et al., 2008). The selective primers for *Pythium vexans* were VexansITSF31 (GCTGCTGGCGCTTGAT) and VexansITSR31 (TTCGTCCCCACAGTATACTT). The primers for *C. fimbriata* were CfimbITSF2 (TCTTCCTTGACAGAGATG) and CfimbITSR9 (TCACTGAGCCATCCAA). The primers for *F. oxysporum* were FOSITSF1 (ATATGTAACTTCTGAGTA) and FOITSR11 (GTTCAAAGATTCGATG). The primers for *Trichoderma* species were TricoITSF9 (TCCGAGCGTCATTTCAA) and TricoITSR3 (GTGCAAACTACTGCGC). The targets were fragments of the ITS rRNA gene with sizes of 131-bp, 181-bp, 140-bp and 126-bp, respectively. The thermal cycling conditions were 94°C for 5 minutes; X cycles of 94°C for 20 seconds, Y°C for 30 seconds and 72°C for Z seconds; followed by 72°C for 10 minutes; where (X, Y, Z) = (44, 69.5, 40) for *P. vexans*, (38, 66.5, 30) for *C. fimbriata*, (40, 62.1, 30) for *F. oxysporum*, and (42, 65, 30) for *Trichoderma*. The amplification reactions were performed in iCycler iQ PCR Plates with Optical Flat 8-Cap

Strips (Bio-Rad Laboratories Inc.). PCR amplifications were performed in 25-µl reactions contained the following reagents: 50 mM Tris (pH 8.3), 500 ug/ml bovine serum albumin (BSA), 2.5 mM $MgCl_2$, 250 mM of each dNTP, 400 nM of each primer, 1-µl of template DNA (~176 ng), 2-µl of 10X SYBR Green I (Invitrogen, Carlsbad, CA) and 1.25 units *Taq* DNA polymerase. rRNA gene levels in the root DNAs were quantified by interpolation from a standard curve comprised of a dilution series of cloned rRNA genes.

Nucleotide sequence analysis of rRNA gene clones. Nucleotide sequences of fungi and oomycetes rRNA gene fragments were determined using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence identities were determined by an analyses using BLAST (NCBI) (Altschul et al. 1997).

Sequence and statistical analyses. Plant growth parameters obtained from the green house trials were subjected to ANOVA and two-tailed student t-tests using Microsoft Excel 2007 (Microsoft, Redmond, WA). Bacterial rRNA gene sequence data were analyzed using QIIME (Caporaso et al., 2010) with OTUs binned at 97% identity.

RESULTS

Replant disease soil. A series of investigations were performed on a soil exhibiting replant disease symptoms. This soil was collected from a field located in Kearny, California. When peach seedlings are grown in this soil in a

field situation, they show retarded growth in height and trunk-width ten weeks after planting. In addition, the root systems have less developed feeder roots and are slightly darker in color (Michael McKenry, personal communication).

To further examine the nature of this phenomenon, we performed greenhouse trials comparing plant growth parameters of peach seedlings grown in autoclaved and non-autoclaved portions of this soil (Table 1). After 10 weeks, root weights, top weights and shoot length were measured. In all cases, plant growth was better in the autoclaved portions, indicating a biological component in this replant disease.

Bacterial associations. To identify bacteria associated with the replant disease symptoms, an Illumina-based, high throughput sequencing analysis of the small-subunit rRNA gene was performed. A total of 9,320 bacterial operational taxonomic units (OTU) were identified. Among the 60 most abundant OTUs, 27 showed significant ($P < 0.05$) negative correlation with peach top weights (Table 2) while 10 were positively correlated (Table 3). Most of these OTUs belonged to the bacterial phylum Proteobacteria (96%), including the classes Gammaproteobacteria (44.4%), Betaproteobacteria (33.3%) and Alphaproteobacteria (22.2%), and the orders Pseudomonadales, Burkholderiales, Chromatiales, Rhodocyclales, and Sphingomonadales (Figure 1)

Fungal and oomycete associations. To identify fungi and oomycetes associated with replant disease symptoms, both culture and culture-independent analyses were performed. For the culture-based studies, 295 fungal and oomycetes isolates were obtained from fine roots, and

identified by sequence analysis of the rRNA internal transcribed spacer (ITS). For the culture-independent analysis, 192 small-subunit rRNA gene clones were analyzed.

The most abundant fungi isolated roots collected from the replant soil were *Trichoderma asperellum* (54%), *Fusarium oxysporum* (19%) and *Trichoderma virens* (15%) (Figure 2A). In contrast, the most abundant fungi obtained from the culture-independent analysis of roots collected from the replant soil were *Ceratocystis fimbriata* (33%), *Fusarium solani* (14%), and *F. oxysporum* (9%) (Figure 2B). A culture-independent analysis of roots from pasteurized portions of the replant soil was dominated by *F. oxysporum* (67%) (Figure 2C).

The most abundant oomycetes isolated roots collected from the replant soil were *Pythium vexans* (65%), *Pythium violae* (19%) and *Pythium irregulare* (8%) (Figure 3A). The most abundant oomycetes obtained from the culture-independent analysis of roots collected from the replant soil were *Pythium vexans* (46%), unidentified *Pythium* species (13%), and unidentified *Aplanochytrium* species (13%) (Figure 3B). A culture-independent analysis of roots from pasteurized portions of the replant soil identified *Pythium vexans* (54%) and an unidentified chrysophyte (11%) (Figure 3C).

The most abundant fungal and oomycetes species and phylotypes were subjected to further analysis using sequence-selective qPCR assays targeting the ITS region. For the phylotypes identified by the small-subunit rRNA gene analyses, chromosomal walking procedures were used to obtain the ITS sequences. Using an assay targeting both of the *Trichoderma* species, a positive association ($P = 0.012$) was detected with plant top weights (X = 10.7

+ 1.01Y, where X plant top weight and Y = rRNA copy number/gram of root). Using an assay targeting *P. vexans*, a negative association (*P* = 0.008) was detected with plant top weights (Y= 5.01 - 0.09X). No significant associations were found between *F. oxysporum, C. fimbriata, P. violae, P. ultimum* var. *ultimum*, or *P. irregulare* and plant top weights.

DISCUSSION

This study identified bacteria, fungi and oomycetes associated with peach replant disease symptoms in a Californian soil. Such associations point toward organisms that could be either protective or causal. Subsequent follow-on investigations that assess cause and effect, such as Koch's postulates experimentation, will be needed to further define the roles of these organisms.

Deleterious rhizobacteria, that have long been associated with replant disease symptoms, inhibit root and shoot growth while causing no other obvious visual symptoms (Fredrickson and Elliott, 1985). In our bacterial analysis, several OTUs exhibiting negative associations with peach top weights were from the genus *Pseudomonas*, including those with high sequence identities to *P. pachastrellae, P. putida, P. fluorescens, P. straminea, P. fulva, P. taiwanensis,* and *P. monteilii* (Table 2, and example in Figure 4A). In grapevine investigations, fluorescent pseudomonads were more abundant in the rhizoplane of plants grown in replant disease soils (Waschkies, Schropp, and Marschner 1994). Cyanide production by *Pseudomonas* spp. has been implicated in apple and peach replant diseases (Rumberger et al., 2007).

However, whether the chemical was the cause of replant disease symptoms (Gur and Cohen, 1989) or it was produced by the bacteria as a mechanism of protective antagonism against root pathogens (Blumer and Haas, 2000; Pal et al., 2000), has not been determined. We also identified several *Pseudomonas* strains showing positive correlation with the peach top weights (Table 3). Some *Pseudomonas* strains isolated from soil can be antagonists of plant pathogens. For example, in soils where wheat is continuously cropped, *Pseudomonas putida* populations are high (Mazzola and Gu, 2000), and they can exhibit antagonistic activity against *Verticillium dahlia* (Berg et al., 2002), *Cylindrocarpon destructans*, *Pythium ultimum* and *Rhizoctonia solani* (Gu and Mazzola, 2003). As has been suggested by Mazzola et al. (Mazzola, 2007), understanding the roles of these putatively beneficial rhizobacteria could lead to strategies to better manage replant disease symptoms.

It is surprising and unexpected to found out that Xanthomonadaceae is one of the dominant taxa exhibiting a positive association with the plant top weights in our trials. These bacteria, and specifically pathovars in the order Xanthomonas, have been shown to cause diseases on at least 124 monocotyledons and 268 dicotyledons (Leyns et al., 1984). This finding from our data implies a possible beneficial role of the bacteria group, which might interact with the plants or other microbes directly or indirectly on plant growth promoting. Furthermore, among the identified *Xanthomonas* species, one of our OTU sequences has 100% identity to the 16S rRNA gene of *Rhodanobacter lindaniclasticus* (Figure 4B), which has shown lindane-degrading activity under aerobic conditions (Nalin et al., 1999).

Lindane is an organochlorine chemical variant of hexachlorocyclohexane that has been used as agricultural insecticide, and it is harmful to higher level animals (Mougin et al., 1996). Future isolation and green house experiments will provide us more information on the interaction between the Xanthomonadaceae species and peach seedlings.

Several oomycetes have been implicated in replants diseases (Mircetich, 1971; Mulder, 1969; Sitepu and Wallace, 1974; Mazzola, 1998; Mazzola et al., 2002; Hendrix et al., 1966; Jones, 1971; Sutton et al., 1981; Mircetich and Matheron, 1976; Hendrix and Powell, 1970; Kouyeas, 1971; Traquair, 1984). In our study, *P. vexans* was frequently detected in both culture-based and culture-independent analysis, and qPCR analysis revealed its negative correlation with the peach top weights. However, P. vexans was not significantly correlated with plant biomass in another peach replant soil (Bent et al., 2009). This soil dependent variation has also been observed in apple replant disease soils. P. vexans was a common isolate from apple replant soils, and it was shown to be pathogenic to apple seedlings (Mulder, 1969); however, other studies have showed that it enhanced plant growth parameters (Mazzola et al., 2002; Dewan and Sivasithamparam, 1988). This phenomenon could be due to virulence differences among *P. vexans* strains, or the result of the microbial community interactions in soils with different biotic and abiotic characteristics (Cantrell and Dowler, 1971; Gardner and Hendrix Jr., 1973).

Many fungi have been associated or implicated in peach replant disease. *Fusarium equiseti*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium*

solani, *Alternaria tenuis*, *Myrothecium verrucariae*, and *Myceilia sterilia* were frequently isolated from replant peach soils in Ontario, Canada, while *Papularia spaerosperma*, *Gliomastix convoluta*, and *Coniothyrium* sp., *Curvularia* sp., *Humicola* sp., *Peyronellaea* sp., and *Truncatella* spp. were also recovered but less common (Wensley, 1956). In Kent, England, *Thielaviopsis basicola* was implicated in cherry and plum replant disease (Yadava and Doud, 1980; Sewell and Wilson, 1975; Hoestra, 1965). *Armillaria* and *Verticillum* were associated with peach and almond replant symptoms in California (Yadava and Doud, 1980; Doll, 2010). In Italy, several species of *Fusarium*, *Penicillium*, *Aspergillus*, and *Trichoderma* were common isolates from the peach replant soils (Manici and Caputo, 2010).

In this study, *Trichoderma asperellum*a and *Trichoderma virens* were frequently isolated from peach roots. qPCR analysis showed that they were positively associated with plant top weights, suggesting that they may be inhibiting the replant disease symptoms or acting as a plant growth promoter. *Trichoderma spp*. are free-living fungi in roots, soil and foliar environments (Harman et al., 2004). The genera is well known as plant growth promoters, acting through its competitive abilities and antagonism mechanisms against pathogens (Sharma et al., 2011). *Trichoderma sp.* produces several lytic enzymes and antibiotics against plant pathogens, and many products made from the fungi have been commercially marketed as biopesticides, biofertilizers and soil amendments (Vinale et al., 2008). *T. virens* is one of the well-studied species, which exhibits mycoparasitic characteristics and the

ability to produce several potent epithiodiketopiperazine antibiotics that inhibit oomycetes such as *Pythium* and *Phytophthora* spp.. It also produces a mixture of peptaibols, which is a linear peptide antibiotic that might control bacteria and other fungi (Howell, 2006). Some strains of *T. asperellum* and *T. harzianum* are capable of activating plant defense responses (Yedidia et al., 1999; Yedida et al., 2003). Strains of *T. asperellum* have also been shown to suppress important plant pathogens including, *Pythophthora megakarya* (Tondje et al., 2007), *Fusarium oxysporum* f. sp. *lycopersici* (Cotxarrera et al., 2002), *Rizoctonia solani* (Trillas et al., 2006) and *Meloidogyne javanica* (Sharon et al., 2007). Further studies will be needed determine the role our two *Trichoderma* species play peach replant disease in this soil.

ACKNOWLEDGEMENTS

The authors thank John Darsow for his technical assistance, and the Duarte Nursery (Hughson, CA) for their generous donation of the peach seedlings. This project was supported Cooperative State Research, Education, and Extension Service, US Department of Agriculture, under Award No. 2005-51102-02340, and by the University of California Agricultural Experiment Station.

FIGURE LEGEND

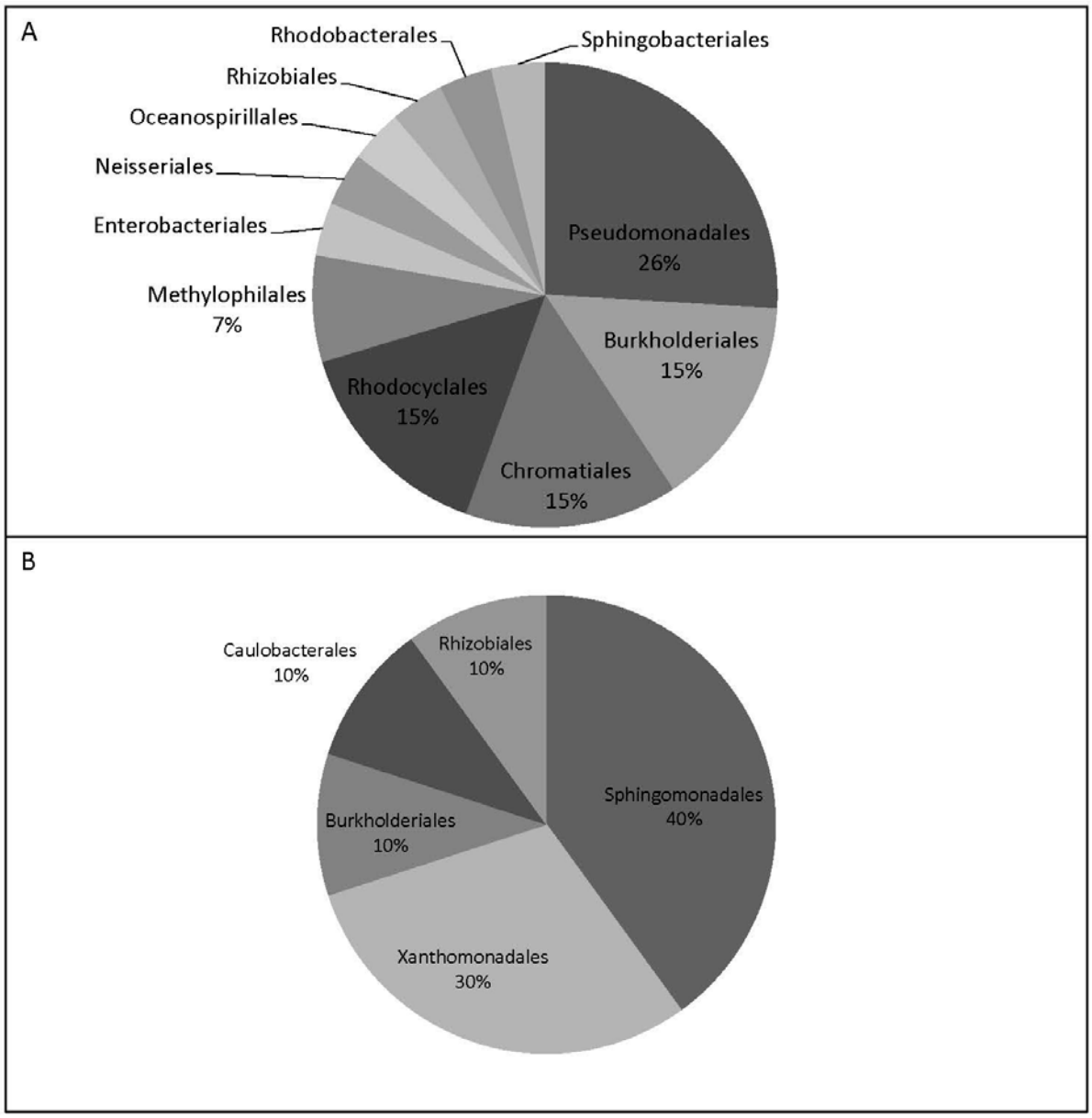
Figure 1. The most abundant bacterial orders (A) positively or (B) negatively associated ($P < 0.05$) with fresh peach top weights.

Figure 2. Fungi identified from peach seedling roots grown in soil exhibiting peach replant disease symptoms. A. Cultured isolates from plants grown in the replant soil; values are % of 269 isolates. B. Culture-independent analysis of plants grown in the replant soil; values are % of 48 sequences. C. Culture-independent analysis of plants grown in pasteurized replant soil; values are % of 48 sequences.

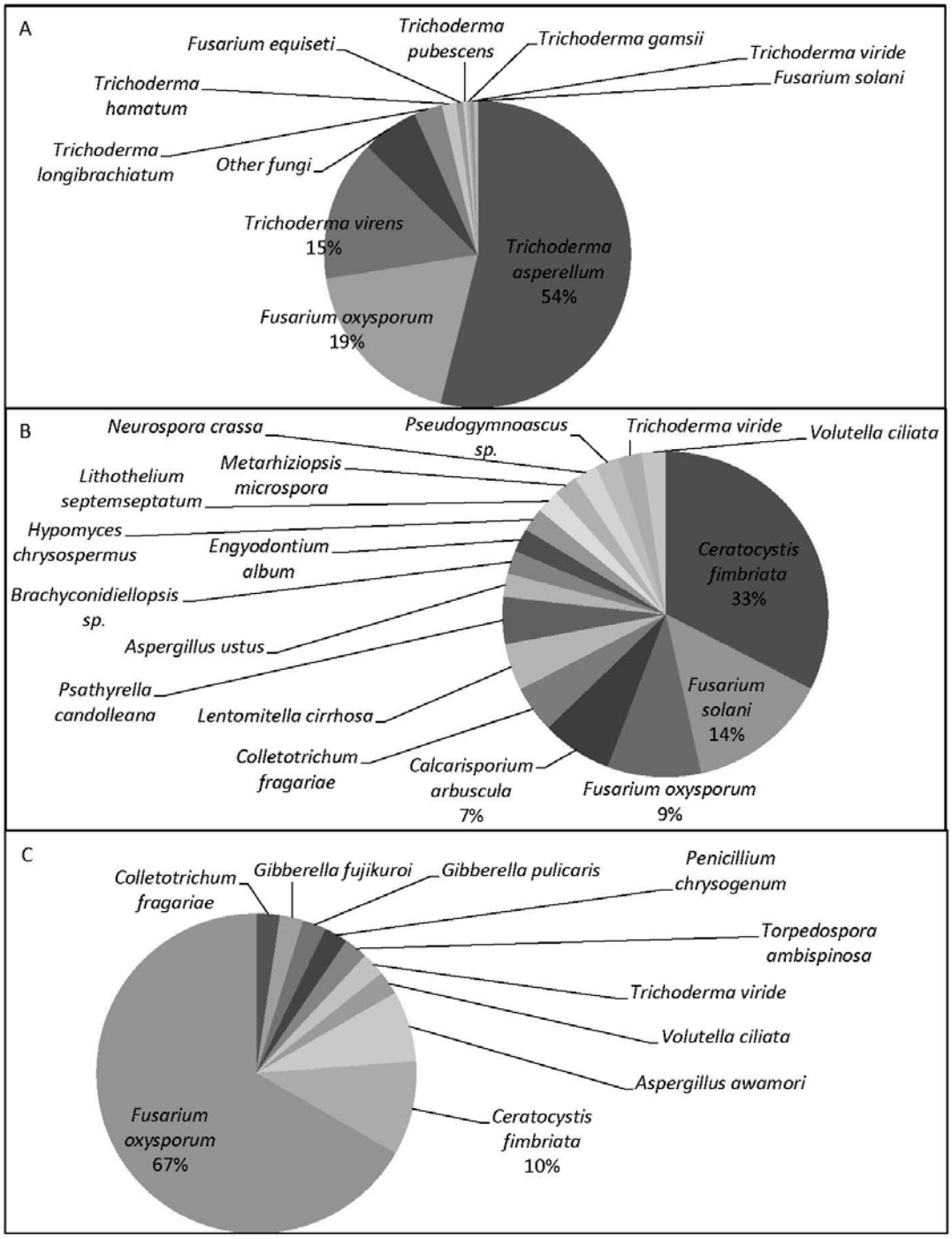
Figure 3. Stramenoplies identified from peach seedling roots grown in soil exhibiting peach replant disease symptoms. A. Cultured isolates from plants grown in the replant soil; values are % of 26 isolates. B. Culture-independent analysis of plants grown in the replant soil; values are % of 48 sequences. C. Culture-independent analysis of plants grown in pasteurized replant soil; values are % of 48 sequences.

Figure 4. Relationships between (A) *Pseudomonas fluorescens* and (B) *Rhodanobacter lindaniclasticus* and fresh peach top weights.

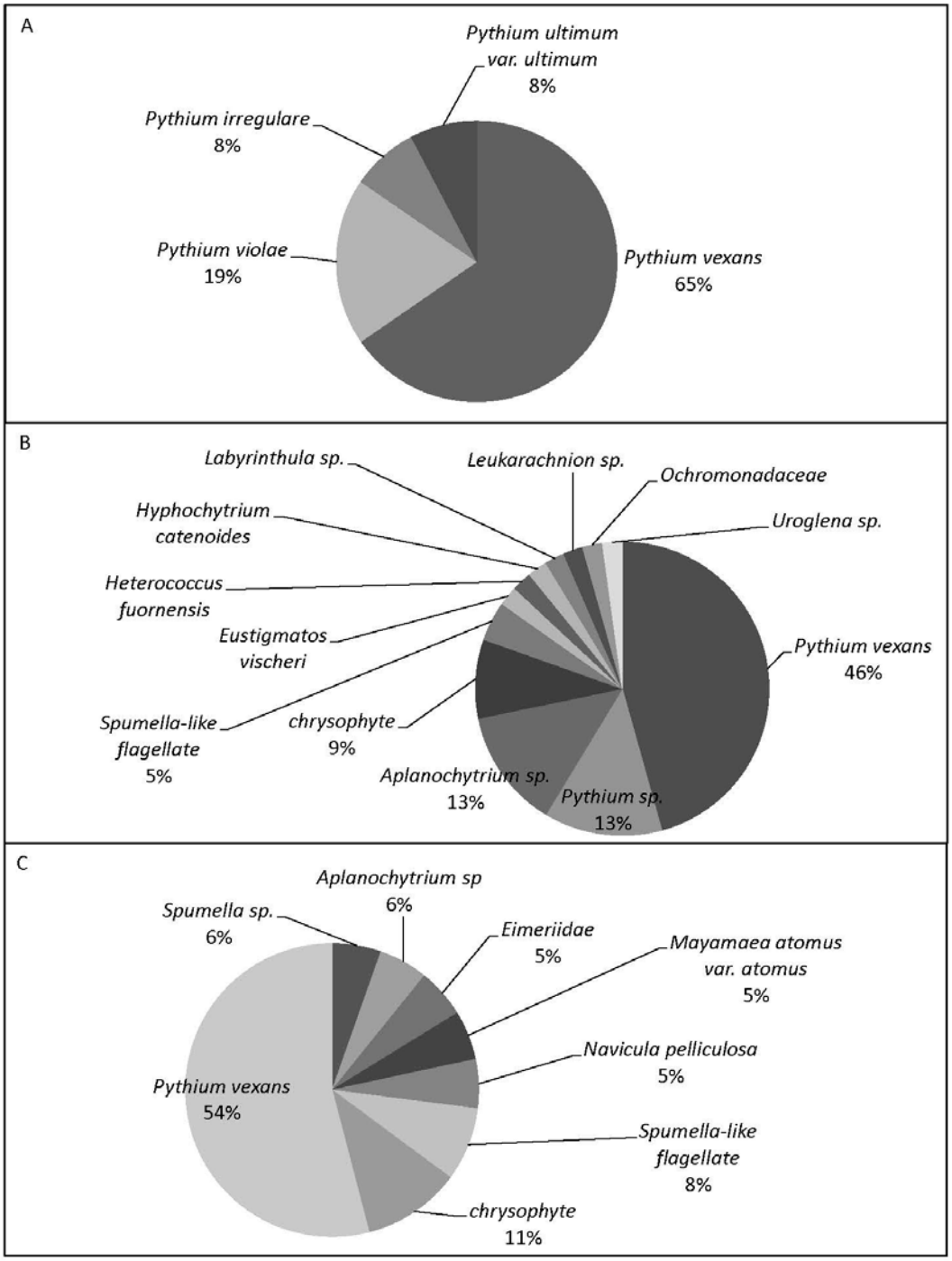
Figure 1. The most abundant bacterial orders (A) positively or (B) negatively associated ($P < 0.05$) with fresh peach top weights. Percentage lower than 5 are not specified in figure.



Figures 2. Fungi identified from peach seedling roots grown in soil exhibiting peach replant disease symptoms. A. Cultured isolates from plants grown in the replant soil; values are % of 269 isolates. B. Culture-independent analysis of plants grown in the replant soil; values are % of 48 sequences. C. Culture-independent analysis of plants grown in pasteurized replant soil; values are % of 48 sequences.



Figures 3. Stramenopiles identified from peach seedling roots grown in soil exhibiting peach replant disease symptoms. A. Cultured isolates from plants grown in the replant soil; values are % of 26 isolates. B. Culture-independent analysis of plants grown in the replant soil; values are % of 48 sequences. C. Culture-independent analysis of plants grown in pasteurized replant soil; values are % of 48 sequences.



Figures 4. Relationships between (A) *Pseudomonas fluorescens* and (B) *Rhodanobacter lindaniclasticus* and fresh peach top weights.

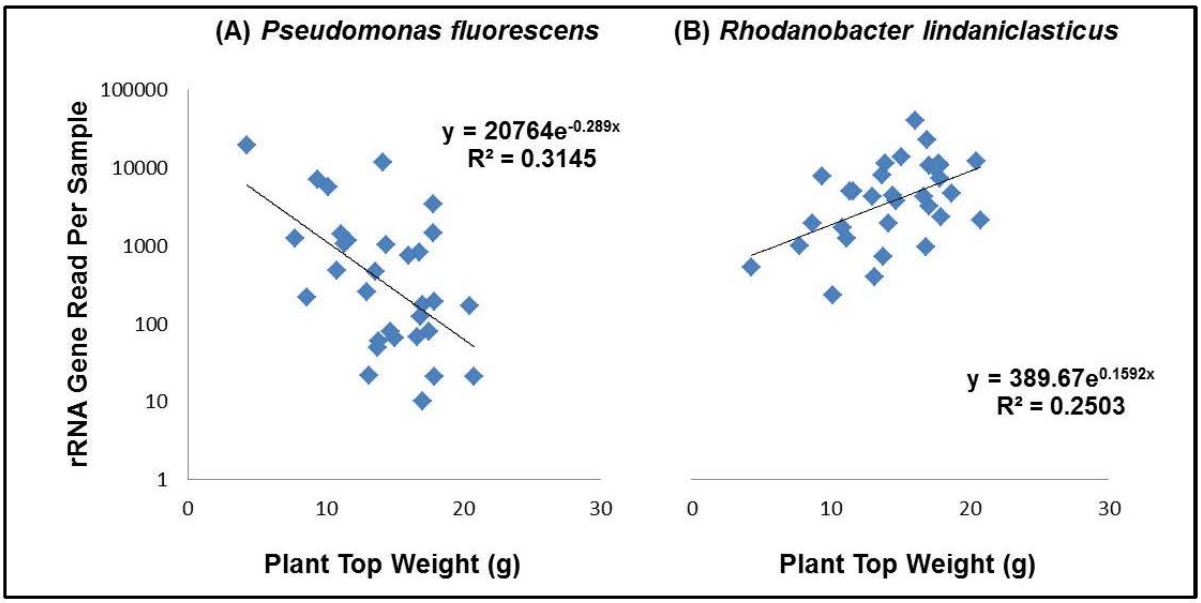


TABLE 1. Plant growth parameters of peach seedlings grown in pasteurized and non-pasteurized portions of the replant disease soil

Soil type	Plant growth parameters[a]				
	Fresh weight (g)		Dry weight (g)		Length (cm)
	Roots	Tops	Roots	Tops	Tops
Replant soil	11.17 X	12.33 X	2.82 X	4.36 X	98.56 X
Pasteurized replant soil	15.69 Y	22.41 Y	3.61 Y	8.28 Y	126.98 Y

[a]Results from two trials were similar and were combined. Values in the table are the means of 6 replicates pots. Values in columns followed by the same letter are not statistically significant ($P < 0.05$).

TABLE 2. Bacterial OTUs negatively associated with fresh peach top weights

Phylotype designation (OTU No)	Nearest cultured relative (accession) (% identity)	Nearest uncultured relative accession (% identity)[a]	Correlation values	
			r[b]	*P*[c]
278666	*Hydrogenophaga flava* (AB681848) (98%)	HQ120802 (98%)	-0.687	$1.41*10^{-5}$
243054	*Aquabacterium* sp. (FN692032) (98%)	HE583131 (98%)	-0.686	$1.48*10^{-5}$
61	*Cupriavidus* sp. (AB681843) (100%)	HQ783640 (100%)	-0.626	$1.26*10^{-4}$
35800	*Pseudogulbenkiania* sp. (AP012224) (98%)	AB657767 (98%)	-0.611	$2.06*10^{-4}$
26781	*Pseudomonas pachastrellae* (HQ425676) (94%)	FJ568592 (100%)	-0.567	$7.32*10^{-4}$
129755	*Bacterium* MI-37 (AB529705) (95%)	FJ568592 (97%)	-0.561	$8.42*10^{-4}$
172482	*Azoarcus* sp. (AP012304) (100%)	JN825463 (100%)	-0.554	$1.01*10^{-3}$
234080	*Azoarcus* sp. (AP012304) (96%)	JN825463 (96%)	-0.551	$1.09*10^{-3}$
250441	*Thiocystis violacea* (FN293059) (95%)	JF990363 (98%)	-0.530	$1.79*10^{-3}$
115618	*Pseudomonas fluorescens* (JN411289) (98%)	AB579016 (98%)	-0.520	$2.27*10^{-3}$
273727	*Pseudomonas putida* (JN411453) (96%)	AB579016 (96%)	-0.503	$3.35*10^{-3}$
210082	*Dechloromonas* sp. (GU202936) (100%)	GU179639 (100%)	-0.493	$4.15*10^{-3}$
193280	*Pseudomonas* sp. (HE586886) (100%)	JQ032435 (100%)	-0.484	$5.00*10^{-3}$
236351	*Rahnella aquatilis* (JQ014185) (100%)	JN998890 (100%)	-0.481	$5.35*10^{-3}$
288392	*Ramlibacter* sp. (HQ323427) (98%)	FQ690103 (98%)	-0.468	$6.94*10^{-3}$
184527	*Rhizobacter* sp. (HE616175) (100%)	FQ659876 (100%)	-0.458	$8.37*10^{-3}$
244218	*Methylophaga thalassica* (AB681780) (95%)	HQ697540 (100%)	-0.427	$1.47*10^{-2}$
207860	*Pseudomonas taiwanensis* (JQ014182) (100%)	HE650703 (100%)	-0.415	$1.82*10^{-2}$
273656	*Methylobacillus* sp. (EU194898) (97%)	FQ659555 (98%)	-0.405	$2.16*10^{-2}$
17162	*Bradyrhizobium* sp. (HQ836187) (98%)	JN540015 (98%)	-0.387	$2.88*10^{-2}$
167695	*Methylophilus leisingeri* (NR_041258) (100%)	AB635923 (100%)	-0.387	$2.89*10^{-2}$

TABLE 2. Bacterial OTUs negatively associated with fresh peach top weights (continue)

Phylotype designation (OTU No)	Nearest cultured relative (accession) (% identity)	Nearest uncultured relative accession (% identity)[a]	Correlation values	
			r[b]	*P*[c]
246943	*Pseudomonas* sp. (FN995250) (94%)	FQ659619 (97%)	-0.374	$3.48*10^{-2}$
166091	*Woodsholea maritima* (FM886859) (97%)	HE614733 (99%)	-0.368	$3.84*10^{-2}$
234039	*Terrimonas lutea* (NR_041250) (100%)	FQ706675 (100%)	-0.367	$3.91*10^{-2}$
11757	*Thiocystis violacea* (FN293059) (97%)	FR853185 (99%)	-0.361	$4.24*10^{-2}$
164910	*Pseudomonas* sp. (FN995250) (92%)	FQ659619 (95%)	-0.361	$4.26*10^{-2}$
32731	*Cellvibrio japonicus* (CP000934) (99%)	HQ691969 (98%)	-0.360	$4.32*10^{-2}$

[a]% identity results are >96% coverage of the query sequences in NCBI BLAST.

[b]r is the Pearsons correlation coefficient.

[c]*P* is the probability value.

TABLE 3. Bacterial OTUs positively associated with fresh peach top weights

Phylotype designation (OTU No)	Nearest cultured relative (accession) (% identity)	Nearest uncultured relative accession (% identity)[a]	Correlation values	
			r^b	P^c
30925	*Rhodanobacter lindaniclasticus* (L76222) (100%)	JF341837 (100%)	0.366	$3.91*10^{-2}$
275502	*Dyella* sp. (GQ369135) (100%)	JF341880 (100%)	0.394	$2.56*10^{-2}$
162892	*Novosphingobium subterraneum* (HM032869) (98%)	FQ741870 (98%)	0.398	$2.41*10^{-2}$
233081	*Rhodopseudomonas palustris* (AB689796) (98%)	JN863157 (98%)	0.422	$1.61*10^{-2}$
259461	*Sphingopyxis* sp. (JF297627) (98%)	HQ118566 (98%)	0.423	$1.58*10^{-2}$
173712	*Novosphingobium naphthalenivorans* (AB681685) (98%)	HQ754243 (98%)	0.431	$1.39*10^{-2}$
66648	*Novosphingobium naphthalenivorans* (AB681685) (99%)	HQ754243 (99%)	0.442	$1.13*10^{-2}$
286079	*Sphingopyxis* sp. (JF297627) (99%)	HQ118566 (99%)	0.447	$1.03*10^{-2}$
101298	*Thermomonas haemolytica* (GU195191) (98%)	FQ680347 (98%)	0.532	$1.72*10^{-3}$
164017	*Massilia aerilata* (HQ406763) (98%)	JN590660 (98%)	0.546	$1.24*10^{-3}$

[a]% identity results are >96% coverage of the query sequences in NCBI BLAST.

[b]r is the Pearsons correlation coefficient.

[c]*P* is the probability value.

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25 (17):3389-3402.

Benizri, E., Piutti, S., Verger, S., Page`s, L., Vercambre, G., Poessel, J. L. and Michelot, P. 2005. Replant diseases: bacterial community structure and diversity in peach rhizosphere as determined by metabolic and genetic fingerprinting. Soil Biol Biochem 37:1738-1746.

Bent, E., Loffredo, A., Yang, J., McKenry, M. V., Becker, J. O. and Borneman, J. 2009. Investigations into peach seedling stunting caused by a replant soil. FEMS Microbiology Ecology 68 (2):192-200.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A. and Smalla, K. 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic *Rhizobacteria* isolated from different *Verticillium* host plants. Applied and Environmental Microbiology 68 (7):3328-3338.

Blumer, C. and Haas, D. 2000. Mechanism, regulation and ecological role of bacterial cyanide biosynthesis. Achievements in Microbiology 173:170-177.

Borneman, J., and Hartin, R. J. 2000. PCR Primers That Amplify Fungal rRNA Genes from Environmental Samples. Applied and Environmental Microbiology 66 (10):4356-4360.

Browne, G. 2002. *Cultural control and etiology of replant disease of Prunus spp.* Davis, CA: University of California, Davis.

Browne, G.T., Lee, R. C. M., McLaughlin, S. T., Connell, J.H., Schneider, S. M. and Bulluck, R. 2006. Investigating associations between culturable soilborne fungi and replant disease of stone fruits in California. Phytopathology 96 (suppl):S166.

Cantrell, H. F. and Dowler, W. M. 1971. Effects of temperature and pH on growth and composition of *Pythium irregulare* and *Pythium vexans*. Mycologia 63 (1):31-37.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Meth 7 (5):335-336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N. and Knight, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108 (Supplement 1):4516-4522.

Cotxarrera, L., Trillas-Gay, M. I., Steinberg, C. and Alabouvette, C. 2002. Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress *Fusarium* wilt of tomato. Soil Biology and Biochemistry 34 (4):467-476.

Dewan, M. M. and Sivasithamparam, K. 1988. *Pythium* spp. in roots of wheat and rye-grass in western Australia and their effect on root rot caused by *Gaeumannomyces graminis* var. *tritici*. Soil Biology and Biochemistry 20 (6):801-808.

Doll, D.A., Schmidt, L.S., Kluepfel, D.A. and Browne, G.T. 2008. Characterization of the rhizosphere bacterial community associated with *Prunus* replant disease in California. Phytopathology 98 (suppl):S47.

Doll, D. 2010. Present and future replant considerations and strategies. Paper read at 38th Annunal Almond Industry Conference, at Modesto, California.

Fredrickson, J. K. and Elliott, L. F. 1985. Colonization of winter wheat roots by inhibitory *Rhizobacteria*. Soil Sci. Soc. Am. J. 49 (5):1172-1177.

Fu, Q., Ruegger, P., Bent, E., Chrobak, M. and Borneman, J. 2008. PRISE (PRImer SElector): Software for designing sequence-selective PCR primers. Journal of Microbiological Methods 72 (3):263-267.

Gardes, M. and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2 (2):113-118.

Gardner, D. E. and Hendrix Jr., F. F. 1973. Carbon dioxide and oxygen concentrations in relation to survival and saprophytic growth of *Pythium irregulare* and *Pythium vexans* in soil. Canadian Journal of Botany 51 (9):1593-1598.

Gu, Y. and Mazzola, M.. 2003. Modification of fluorescent pseudomonad community and control of apple replant disease induced in a wheat cultivar-specific manner. Applied Soil Ecology 24 (1):57-72.

Gur, A. and Cohen, Y. 1989. The peach replant problem - some causal agents. Soil Biol. Biochem. 21:829-834.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. and Lorito, M. 2004. *Trichoderma* species - opportunistic, avirulent plant symbionts. Nat Rev Micro 2 (1):43-56.

Hendrix, F.F., Powell, W.H. and Owen, J.H. 1966. Relation of root necrosis caused by *Pythium* species to peach decline. Phytopathology 56:1229-1232.

Hendrix Jr., F. F. and Powell, W. M. 1970. Control of root pathogens in peach decline sites. Phytopathology 60:16-19.

Hine, R.B. 1961. Role of fungi in peach replant problem. *Plant Dis Rep* 45:462-465.

Hoestra, H. 1965. *Thielaviopsis basicola*, a factor in the cherry replant problem in the Netherlands. European Journal of Plant Pathology 71 (6):180-182.

Howell, C. R. 2006. Understanding the mechanisms employed by *Trichoderma virens* to effect biological control of cotton diseases. Phytopathology 96 (2):178-180.

Jaffee, B. A. , Abawi, G.S. and Mai, W.F. 1982. Fungi associated with roots of apple seedlings grown in soil from an apple replant site. Plant Dis. 66:942-944.

Jones, A. L. 1971. *Diseases of tree fruits in Michigan*, *Ext. Bull. E-714*. East Lansing: Coop. Ext. Serv., Mich. State Univ.

Koch, L. W. 1955. The peach replant problem in Ontario: I. Symptomatology and distribution. Canadian Journal of Botany 33 (5):450-460.

Kouyeas, H. 1971. On the apoplexy of fruit trees caused by *Phytophthora* spp. Annales Institut Phytopathologique Benaki 10:163.

Leyns, F., De Cleene, M., Swings, J. and De Ley, J. 1984. The host range of the genus *Xanthomonas*. The Botanical Review 50 (3):308-356.

Mai, W.F., and Abawi, G.S. 1981. Controlling replant diseases of pome and stone fruits in Northeastern United States by preplant fumigation. Plant Disease 65:859-864.

Manici, L. M. and Caputo, F. 2010. Soil fungal communities as indicators for replanting new peach orchards in intensively cultivated areas. European Journal of Agronomy 33 (3):188-196.

Mazzola, M. 1998. Elucidation of the microbial complex having a causal role in the development of apple replant disease in Washington. Phytopathology 88 (9):930-938.

Mazzola, M. 2007. Manipulation of rhizosphere bacterial communities to induce suppressive soils. Journal of Nematolology 39 (3):213-220.

Mazzola, M., Andrews, P. K., Reganold, J. P. and Lacvesque, C. A. 2002. Frequency, virulence, and metalaxyl sensitivity of *Pythium* spp. isolated from apple roots under conventional and organic production systems. Plant Disease 86 (6):669-675.

Mazzola, M. and Gu, Y. 2000. Impact of wheat cultivation on microbial communities from replant soils and apple growth in greenhouse trials. Phytopathology 90 (2):114-119.

McKenry, M. V. 1999. *The replant problem and its management.* Fresno, CA: Catalina Publishing.

Mircetich, S. M. 1971. The role of *Pythium* in feeder roots of diseased and symptomless peach trees and in orchard soils in peach tree decline. Phytopathology 61:357-360.

Mircetich, S. M. and Matheron, M. E. 1976. *Phytophthora* root and crown rot of cherry trees. Phytopathology 66:549-558.

Mizutani, F. , Hirota, R. and Kadoya, K. 1988 Growth inhibiting substances from peach roots and their possible involvement in peach replant problems. Acta Horticulturae 233:37-43.

Mougin, C., Pericaud, C., Malosse, C., Laugero, C. and Asther, M. 1996. Biotransformation of the Insecticide Lindane by the White Rot Basidiomycete Phanerochaetechrysosporium. Pesticide Science 47 (1):51-59.

Mulder, D. 1969. The pathogenicity of several *Pythium* species to rootlets of apple seedlings. European Journal of Plant Pathology 75 (1):178-181.

Nalin, R., Simonet, P., Vogel, T. M. and Normand, P. 1999. *Rhodanobacter lindaniclasticus* gen. nov., sp. nov., a lindane-degrading bacterium. International Journal of Systematic Bacteriology 49 (1):19-23.

Pal, K. K., Tilak, K. V. B. R., Saxena, A. K., Dey, R. and Singh, C. S. 2000. Antifungal characteristics of a fluorescent *Pseudomonas* strain involved in the biological control of *Rhizoctonia solani*. Microbiological Research 155:233-243.

Patrick, Z. A. 1955. The peach replant problem in Ontario. II. Toxic substances from microbial decomposition products of peach root residues. Can J Botany 33:461-486.

Proebsting, E. and Gilmore, A. 1941. The relation of peach root toxicity to the re-establishing of peach orchards. Proceedings of American Society for Horticultural Science 38:21-26.

Rumberger, A., Merwin, I. A. and Thies, J. E. 2007. Microbial community development in the rhizosphere of apple trees at a replant disease site. Soil Biology and Biochemistry 39 (7):1645-1654.

Savory, B. M. 1966. *Specific replant diseases*, *Research Review No. 1.* East Malling, Kent: Commonwealth Bureau of Horticulture and Plantation Crops.

Sewell, G. W. F. 1981. Effects of *Pythium* species on the growth of apple and their possible causal role in apple replant disease. Annals of Applied Biology 97 (1):31-42.

Sewell, G. W. F. and Wilson, J. F. 1975. The role of *Thielaviopsis basicola* in the specific replant disorders of cherry and plum. Annals of Applied Biology 79 (2):149-169.

Sharma, P., Kumar, P. V., Ramesh, R., Saravanan, K., Deep, S., Sharma, M., Mahesh, S. and Dinesh, S. 2011. Biocontrol genes from *Trichoderma* species: A review. African Journal of Biotechnology 10 (86):19898-19907.

Sharon, E., Chet, I., Viterbo, A., Bar-Eyal, M., Nagan, H., Samuels, G. and Spiegel, Y. 2007. Parasitism of Trichoderma on *Meloidogyne javanica* and role of the gelatinous matrix. European Journal of Plant Pathology 118 (3):247-258.

Sitepu, D. and Wallace, H.R. 1974. Diagnosis of retarded growth in an apple orchard. Austral. J. Expt. Agric. and Animal Husb. 14:577-584.

Sutton, T. B., Wayne, D.W., Sullivan, W.T., Nardacci, J. F. and Klimstra, D.E. 1981. Causes of apple tree death in Henderson county, North Carolina. Plant Disease 65 (4):330-331.

White, T. J., Bruns, T. D., Lee, S. B. and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols - A Guide to Methods and Applications*, edited by D. H. G. M.A. Innis, J.J. Sninsky and T.J. White. San Diego, CA: Academic Press.

Tondje, P. R., Roberts, D. P., Bon, M. C., Widmer, T., Samuels, G. J., Ismaiel, A., Begoude, A. D., Tchana, T., Nyemb-Tshomb, E., Ndoumbe-Nkeng, M., Bateman, R., Fontem, D. and Hebbar, K. P. 2007. Isolation and identification of mycoparasitic isolates of *Trichoderma asperellum* with potential for suppression of black pod disease of cacao in Cameroon. Biological Control 43 (2):202-212.

Traquair, J. 1984. Etiology and control of orchard replant problems: a review. Canadian Journal of Plant Pathology 6:54-62.

Trillas, M. I., Casanova, E., Cotxarrera, L., Ordovás, J., Borrero, C. and Avilés, M. 2006. Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. Biological Control 39 (1):32-38.

Utkhede, R. S. 1987. Workshop on replant problems with fruit trees. bonn, germany: ISHS.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L. and Lorito, M. 2008. *Trichoderma*–plant–pathogen interactions. Soil Biology and Biochemistry 40 (1):1-10.

Waschkies, C., Schropp, A. and Marschner, H. 1994. Relations between grapevine replant disease and root colonization of grapevine (*Vitis* sp.) by fluorescent pseudomonads and endomycorrhizal fungi. Plant and Soil 162 (2):219-227.

Wensley, R. N. 1956. The peach replant problem in Ontario:IV. Fungi associated with replant falure and their importance in fumigated and nonfumigated soils. Canadian Journal of Botany 34 (6):967-981.

Worlidge, J. 1698. *Systema Agriculturae; The Mystery of Husbandry Discovered*. London.

Yadava, U. L. and Doud, S. L.. 1980. The short life and replant problems of deciduous fruit trees. In *Horticultural Reviews*: John Wiley & Sons, Inc.

Yedida, I., Shorest, M., Karem, Z., Benhamou, N., Kapulnik, Y. and Chet, I. 2003. Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lacrymans* in cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. Appl. Environ. Microbiol. 69:7343-7353.

Yedidia, I., Benhamou, N. and Chet, I. 1999. Induction of defense response in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Applied Environmental Microbiology 653:1061-1070.

Ch2. Population Dynamics of *Dactylella oviparasitica* and *Heterodera schachtii*: Toward a Sugar Beet Planting Decision Model

ABSTRACT

A series of investigations were performed to examine the population dynamics of the sugarbeet cyst nematode, *Heterodera schachtii*, and the nematophagus fungus *Dactylella oviparasitica*. After two nematode generations, the population densities of *H. schachtii* were measured in relation to various initial infestation densities of both *D. oviparasitica* and *H. schachtii*. In general, higher initial levels of *D. oviparasitica* were associated with lower final levels of *H. schachtii*. Regression models showed that the initial densities of *D. oviparasitica* were only significant when predicting the final densities of *H. schachtii* J2 and eggs as well as fungal egg parasitism, while the initial densities of J2 were significant for all final *H. schachtii* population density measurements. We also showed that the densities of *H. schachtii*-associated *D. oviparasitica* fluctuate greatly, with rRNA gene numbers going from zero in most field-soil-collected cysts to an average of 4.24×10^8 in cysts isolated directly from root surfaces. Finally, phylogenetic analysis of rRNA genes suggested that *D. oviparasitica* belongs to a clade of nematophagous fungi with a wide geographical distribution. We posit that these findings will provide foundational data facilitating the development of more effective sugar beet planting decision models.

INTRODUCTION

The sugarbeet cyst nematode (*Heterodera schachtii*) is an economically important plant parasitic nematode that affects a wide range of crop plants including, sugar beet, broccoli, cabbage, cauliflower, spinach, Brussels sprouts, rapini, radish, mustard, kale, canola, Swiss chard and others (Whitehead, 1998). In California, the nematode is widespread in the Imperial Valley where the state's sugar beet production is concentrated. To reduce crop damage due to *H. schachtii*, in the 1960s, representatives of the local sugar beet factory, growers, the County Agricultural Commissioner's Office and Nematologists of the University of California designed a cropping scheme based on a cyst nematode dump-sample survey (Roberts and Thomason, 1981). A dump sample is a 600-g representative soil sample from a ~5 acre area that is taken when harvested sugar beets are processed at the sugar factory. Fields are considered infested if three or more cysts are found in a sample. Non-infested fields cannot be cropped to sugar beets more than two years in a row and not more than four out of ten years. In infested fields, sugar beets can be grown only once every four years. This cropping program has been used effectively for half a century. The reason for the success is the natural decline in the population density of *H. schachtii* in the absence of host plants. For example, in the Imperial Valley, annual population decline rates of more than 50% were reported. Egg density of the sugarbeet cyst nematode in four different fields dropped below the detection level during the fourth year under continuous non-host alfalfa (Roberts et al., 1981). The authors suggested that

previously reported egg parasitism by *Fusarium oxysporum*, *Acremonium strictum* and other fungi (Nigh et al., 1980) may be a major cause of destruction of *H. schachtii* eggs and consequently contribute to the decline of the nematode population.

The nematophagous fungus *Dactylella oviparasitica* was previously identified in an *H. schachtii*-suppressive field soil (9E) at the Agricultural Experimental Station, University of California, Riverside (Westphal and Becker, 2001). Molecular population studies and Koch's postulates investigations showed that *D. oviparasitica* was a primary suppressive agent in this soil (Yin et al., 2003; Olatinwo et al., 2006c). This fungus was earlier described as a parasite of root-knot nematode eggs (Stirling and Mankau, 1978). In field experiments, the addition of *D. oviparasitica* strain 50 to conductive soil reduced *H. schachtii* population densities to those found in the suppressive 9E soil (Olatinwo et al., 2006b). In addition, *D. oviparasitica* was capable of suppressing *H. schachtii* in soils with a variety of physicochemical characteristics (Olatinwo, 2006a). In preliminary surveys, we found that *D. oviparasitica* is widespread in Californian soils (unpublished data). We therefore posit that *D. oviparasitica* affects *H. schachtii* populations throughout sugar beet growing regions, and that adding information about *D. oviparasitica* density to planting decision models will make them more effective.

Toward this goal, in this study, we performed a series of investigations examining the population dynamics of *H. schachtii* and *D. oviparasitica*. We determined the relationships between various initial population densities and those

occurring after two nematode generations. We showed that the density of *D. oviparasitica* in *H. schachtii* cysts differ greatly depending on the age of the cysts, and we discuss this in relation to the development of planting decision models. Finally, we showed that *D. oviparasitica* belongs to a clade of other nematophagous fungi with a large geographical distribution.

MATERIAL AND METHODS

Soil preparation. The soil used in this study was from field 9E, located at the Agricultural Experiment Station, University of California, Riverside. This soil is a Hanford fine sandy loam (60.8% sand, 29.8% silt and 9.4% clay, 0.7% OM, pH 7.8). The soil was sieved through a 12-mm metal mesh sieve and amended with 20% steam-pasteurized plaster sand to improve physical characteristics before use in root box and greenhouse experiments.

Fungal culture. *Dactylella oviparasitica* strain 50 (Yin et al., 2003) was cultured on potato dextrose agar medium (PDA) at 23 ± 2^{o}C for 21 days prior to being amended to soil and prior to DNA extraction. Arkansas Fungus strain L (ARF-L) (Timper and Riggs, 1998) was cultured on PDA medium for 21 days prior to DNA extraction. Fungal cultures were blended (Sunbeam 6 Speed Blender (Model 4142; Sunbeam Products Inc., Boca Raton, FL) with 25-ml of sterile water for 30 s. One milliliter of the fungal suspensions was used to determine the colony forming units (CFU) from a dilution series plated on PDA.

Nematode preparation*.* Sugar beets (*Beta vulgaris* L.) seedlings were infested with second-stage *H. schachtii* and maintained in greenhouse pot cultures for approximately 3 months. Cysts were extracted from the soil using a Fenwick flotation can method (Caswell et al., 1985). Soil samples were placed on modified Baermann funnels (Flegg and Hooper, 1970) containing 0.4% $ZnCl_2$ to stimulate juvenile hatching. Second-stage juveniles (J2) of *H. schachtii* were collected daily and stored in aerated water at 15 ± 1^{o}C for approximately 48 hours before soil infestation. The numbers of J2 in the suspensions were determined under a stereo microscope.

Greenhouse experiments. Twenty-four milliliters of the fungal suspensions for each dilution were added into plastic bags containing 1600-cm^3 of methyl iodide-fumigated 9E soil (Becker et al., 1998). Soils were thoroughly mixed by tumbling the plastic bags, and then transferred to 15-cm-diameter pulp pots. The treatments consisted of a factorial design of 4 population levels of *H. schachtii* (25, 50, 100 and 200 J2/100-cm^3 soil) and 4 CFU levels of *D. oviparasitica* strain 50 (Table 1). Soil without fungal amendment served as a control. Several Swiss chard seeds (*Beta vulgaris* L. cv. Large White Ribbed, Lockhart Seeds Inc., Stockton, CA) were planted in each pot. The trial was arranged in a randomized complete block design with five replicates per treatment. The plants were maintained in a greenhouse under ambient light at 25 ± 3^{o}C. Soil temperature was monitored for degree-day determination using HOBO Temperature Data Loggers (Onset Computer Corporation, Bourne, MA) buried in

an additional pot. After emergence, the seedlings were thinned to one per pot and fertilized with 16-g slow-release fertilizer (Osmocote 14-14-14, Scotts Co., Marysville, OH). Four weeks after seeding, pots were infested with the appropriate amounts of nematodes (25, 50, 100 or 200 *H. schachtii* J2 per 100-cm^3 soil) by pipetting aqueous suspensions of freshly hatched J2 into the three holes (~5-cm deep and 1.5-cm wide) in the soil near the base of each plant. The control pots (non-suppressive and suppressive) were infested with 100 J2/100-cm^3 soil. Two replicate greenhouse trials were conducted, separated in time and space, between the months of February and June.

The greenhouse trials were terminated 13 weeks (~946 degree-days for trial 1 and 957 degree-days for trial 2, base temperature 8^{o}C (Curi and Zmoray, 1966)) after nematode infestation. The aboveground parts of the plants were cut at soil level and the root systems were removed from soil. Shoot and root weights were determined. Cysts were extracted from 350-g sub-samples of soil using a Fenwick flotation method (Caswell et al., 1985). Cysts were counted and then broken in a tissue homogenizer to enumerate the eggs. Parasitism was assessed by examination of 100 randomly selected eggs per sample under light microscope (Olatinwo, 2006a). Cysts from additional 350-g sub-samples were extracted for J2 enumeration. These cysts were placed on modified Baerman funnels containing 0.4% zinc chloride to stimulate juvenile hatching. After 14 days of incubation at 26^{o}C, the total number of collected *H. schachtii* J2 was determined. Another 100-cm^3 sub-sample of soil was processed with a centrifuge flotation technique

for extracting *H. schachtii* J2 (Jenkins, 1964). Eggs, J2 and parasitized eggs were observed and enumerated under an inverted microscope.

Field 9E soil survey. Field 9E was divided into 16 sections of identical size. Approximately 20 soil cores from each section were collected with an Oakfield sampler (2.5-cm x 10-cm). The samples from each section were pooled, thoroughly mixed and passed through a 12-mm metal mesh sieve. DNA was extracted and *D. oviparasitica* rRNA genes were PCR amplified as described below.

Root box experiments. Root boxes (27-cm x 23.5-cm x 2.5-cm) enabled collection of females and cysts from root surfaces because one side of the box was transparent and removable. Soils were added to the root boxes and seeded with Swiss chard (*Beta vulgaris* subsp. *cicla* (L.) W. Koch 'Large White Ribbed'; Lockhart Seeds Inc., Stockton, CA) as the host crop. The soil was infested three weeks after planting with approximately 1000 J2 per root box. The first- and second-generation white females and cysts that became visible on the root surface were collected and stored at -80°C before DNA extraction.

DNA extraction. DNA was extracted from 200-mg samples of soil, fungi, *H. schachtii* females and *H. schachtii* cysts using the FastDNA Spin Kit for Soil (Qbiogene, Carlsbad, CA) as described by the manufacturer; a 90 s bead-beating step (5.5 setting) was used for soil and hyphae and 150 s for cysts using a FastPrep Instrument (Qbiogene). DNA extracts were further purified by subjecting them to electrophoresis on 1% agarose gel, and isolating the DNA above 3Kb

using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) without exposing the DNA to UV or ethidium bromide.

PCR of rRNA genes. PCR primers were ITS1Fuser (GGGAAAGUCTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns, 1993) and ITS4user (TCCTCCGCTTATTGATATGC) (White et al., 1990) for the internal transcribed spacer region (ITS) and LR0R (ACCCGCTGAACTTAAGC) and LR5 (TCCTGAGGGAAACTTCG) (Vilgalys and Hester, 1990) for the large subunit (LSU) rRNA gene. Thermal cycling conditions were: 94^{o}C for 5 minutes, followed by X cycles of 94^{o}C for 20 s, 52^{o}C for 20 s, 72^{o}C for Y s, and a final incubation at 72^{o}C for 5 min; for ITS, X = 40 and Y = 40, for LSU, X = 35 and Y = 50. For the ITS region, 10-µl amplification reactions were performed in 10-µL glass capillary tubes using a RapidCycler (Idaho Technologies, Salt Lake City, UT) containing the following reagents: 50 mM Tris (pH 8.3), 500 µg/ml bovine serum albumin (BSA), 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP), 400 nM of each primer, 1-µl DNA template, and 0.5 U *Taq* DNA polymerase. For the LSU region, PCRs were performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) in 25-µl amplification reactions containing the reagents described above. Amplification products were isolated, cloned and sequenced as previously described (Bent et al., 2009). Sequence identities were determined by analyses using BLAST (NCBI) (Altschul et al., 1997).

***Dactylella oviparasitica* qPCR.** Sequence-selective PCR primers targeting the rRNA ITS region of *D. oviparasitica* 50 were designed using the

PRISE software (Fu et al., 2008). These primers were DacITSF5 (GGGCTTGTCTGGGTTT) and Dac50ITSR5 (GTGCTGTTACAACCTATAAAT), and amplified a 100-bp fragment. Twenty-five-µl reactions contained the following reagents: 50 mM Tris (pH 8.3), 500 µg/ml BSA, 2.5 mM $MgCl_2$, 250 mM of each dNTP, 400 nM of each primer, 1-µl DNA template, 2-µl 10X SYBR Green I (Invitrogen, Carlsbad, CA) and 1.25 U *Taq* DNA polymerase. Thermal cycling parameters were: 94°C for 5 min; 42 cycles of 94°C for 20 s, 63°C for 30 s, and 72°C for 30 s; followed by 72°C for 10 min. Real-time PCR assays were performed in Bio-Rad iCycler MyiQ Real-Time Detection System (Bio-Rad Laboratories Inc.).

Phylogenetic tree construction. LSU and ITS rRNA gene sequences obtained in this study, and their closest relatives determined by analyses using BLAST, were aligned separately using the ClustalW algorithm (Thompson et al., 1994). The alignments were 847-910 bp (LSU) and 334-418 bp (ITS), after highly variable regions were removed. Phylogenetic trees were generated using the Geneious Tree Builder (Geneious Pro 5.1.7, Biomatters Ltd, Auckland, New Zealand) using the UPGMA method (Sneath and Sokay, 1973) and the Tamura-Nei genetic distance model (Tamura and Nei, 1993) with 1000 bootstrap samplings (Efron, 1979).

Regression analyses. The goal of this analysis was to determine if the initial population densities of *D. oviparasitica* and *H. schachtii* J2 have any predictive power in the final population densities of *H. schachtii* cysts, eggs, J2, fungal egg parasitism and plant weights, 9 weeks after nematode infestation. A

linear regression analysis was used to find the appropriate model for predicting each of the dependent variables from the initial densities of *D. oviparasitica* and *H. schachtii* J2 (Table 2). A total of eight linear regression models were tested, one for each dependent variable. For the model assumptions to be valid, identical and independent normally distributed residuals were need. To achieve this, Box-Cox power transformations were performed on both the dependent and independent variables in some cases. A summary of the model for each dependent variable and the transformations, if applicable, is also provided.

RESULTS AND DISCUSSION

Relationships between the population densities of *D. oviparasitica* and *H. schachtii*. In greenhouse trials, we examined the relationships between the initial population densities of *D. oviparasitica* and *H. schachtii* and the final densities of *H. schachtii*, measured two nematode generations after the initial infestations. As expected, higher initial densities of *D. oviparasitica* were associated with lower final densities of *H. schachtii* (Table 1), and regression models for each of the dependent variables were generated (Table 2). The initial densities of *D. oviparasitica* were only significant when predicting the final densities of *H. schachtii* J2 and eggs as well as fungal egg parasitism, while the initial densities of *H. schachtii* J2 were significant for each of the dependent variables. In the models that included only the initial J2 densities, there was a

negative relationship when the various plant measurements were the dependent variables; in other words, as the initial J2 densities were increased, the final plant weights decreased. In the other two models that included only the initial J2 densities, the final densities of both cysts and J2s exhibited positive relationships with the initial J2 densities.

The fitted models that included both the initial population densities of *D. oviparasitica* and *H. schachtii* J2 are shown in response surface plots (Figs. 1-3). The darker points in the plot are observed values that are above the predicted response surface while the lighter points are the observed values that are below the predicted response surface. The plots show the effect that a change in one of the independent variables has on the prediction value of the dependent variable. For example, when predicting the final densities of eggs (Fig. 1) or J2 (Fig. 2), these values increase as the initial density of J2 increases, and vice versa. In the case of predicting fungal egg parasitism, the reverse is true (Fig. 3).

***Dactylella oviparasitica* in *H. schachtii cysts*.** Implementing more effective planting decision models also will require biologically meaningful measurements of *D. oviparasitica* population densities. In this study, we detected huge differences in the content of *D. oviparasitica* in field-collected and root box-obtained *H. schachtii* cysts. Using a sequence-selective qPCR assay, the population densities of *D. oviparasitica* were enumerated in *H. schachtii* cysts collected from the suppressive 9E field soil. This analysis detected *D. oviparasitica* in only 2 of 16 regions in this field, and the amounts measured in

these regions were low: 18.3 and 5.15 rRNA genes per cyst. Conversely, *D. oviparasitica* was detected in all *H. schachtii* cysts and females that were obtained from Swiss chard roots grown in these same soils but in root boxes. In addition, the amounts of *D. oviparasitica* in these rootbox females and cysts were much higher than in field soil-obtained cysts, with average rRNA genes per cyst being 6.14×10^8 and 2.34×10^8 after one and two nematode generations, respectively, and average rRNA genes per female being 7.35×10^2 and 2.10×10^5 after one and two nematode generations, respectively. We posit that fungal parasitism is initiated in young *H. schachtii* females that break through the root surface (with their posterior end) and become exposed to the rhizosphere. When the females develop into cysts filled with eggs, *D. oviparasitica* populations increase because of the increased availability of food, in the form of the nematode eggs. During or after the consumption of this nutritional source, other microorganisms compete with *D. oviparasitica* and eventually replace this fungus, resulting in a drop of its detectable rRNA gene levels. Consequently, we find *D. oviparasitica* at very low levels in older cysts from the suppressive field soil. In the context of taking measurements for a cropping system model, these results suggest that *D. oviparasitica* populations will need to be measured in a bioassay in which females and/or young cysts serve as semi-selective baits.

Relationship of *Dactylella oviparasitica* strain 50 to ARF-L.

Phylogenetic analysis of *D. oviparasitica* and its closest relatives showed that they form a clade of fungi comprised of at least one other nematophagous fungus –

Arkansas Fungus strain L (ARF-L). An rRNA ITS analysis showed that these two fungi belong to an assemblage of organisms (see DO Clade) with broad geographical distribution, including Austria, China, France, Germany, United States, and Norway (Figure 4). Analysis of the LSU rRNA gene confirmed the association between *D. oviparasitica* 50 and ARF-L (Figure 5); LSU sequences for the other *D. oviparasitica*-clade members (DO Clade, Figure 4) from the ITS tree were not in GenBank (NCBI), and thus were not included in the LSU tree. Analysis of the 5.8S rRNA gene showed that *D. oviparasitica* strain 50 and ARF-L are 97% similar.

Dactylella oviparasitica strain *50* shares many similarities with ARF-L, which was identified as a nematophagous fungus of the soybean cyst nematode (*Heterodera glycines*) by University of Arkansas researchers in the 1990s (Kim and Riggs, 1991). Both *D. oviparasitica* and ARF-L are capable of parasitizing species of root-knot nematodes and cyst nematodes (Kim and Riggs, 1991; Stirling, 1991). Variation in parasitism and biological control efficacies of different ARF strains have been reported with soybean cyst nematodes (Timper and Riggs, 1998). Both *D. oviparasitica* strain 50 and ARF-L are filamentous, non-sporulating fungi with thin hyphae. Both fungi infect immature white *H. schachtii* females when combined on water agar, yet neither is able to parasitize viable eggs *in vitro* (Smith Becker et. al, 2011).

Conclusions and future research. A greater understanding of the distribution and population dynamics between *H. schachtii* and *D. oviparasitica*

might result in improved sugar beet planting models, which we posit will lead to more effective cropping decisions. Earlier research on *H. schachtii* population dynamics in the Imperial Valley produced an interaction matrix of major factors and environmental parameters that affect the nematode's egg decline (Roberts et al., 1981). The authors speculated that those factors, together with sugar beet management tools and practices could be manipulated to maximize rotation efficiency and improve crop production in *H. schachtii*-infested fields. We support that view, although we attribute the cyst nematode population decline primarily to parasitism and ultimately to the destruction of the late-stages of developing juveniles and females (Smith Becker et al., 2011). Future research will endeavor to assess the abilities of the different fungal subtypes (DO Clade, Figure 4) to reduce populations of various cyst and root-knot nematodes. As was done in this study for *D. oviparasitica* strain 50, we will develop sequence-selective qPCR assays to track and enumerate the population densities of each of the different fungal subtypes and incorporate this information into the planting models. More broadly, given that *D. oviparasitica* and related organisms comprise a clade of fungi containing effective biological control agents targeting several economically important nematodes, and that similar fungi have been identified on several continents, this overall approach may prove to be useful for a wide range of crops in other geographical locations.

ACKNOWLEDGEMENT

The authors thank John Darsow for his technical assistance. This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (CSREES), grant 2007-35302-18164 and by the University of California Agricultural Experiment Station.

Table 1. Plant growth parameters and *Heterodera schachtii* population densities after two nematode generations in relation to various initial amounts of *Dactylella oviparasitica* and *H. schachtii*.

Treatment number	Treatments[a] Population densities at beginning of trials		Values measured at the end of the greenhouse trials, two nematode generations after *H. schachtii* infestation[b]						
			Plant weights (g)		Counts in 350-g soil			J2/100-cm^3 soil	Parasitized eggs (%)
	Dactylella oviparasitica CFU/1,600-cm^3[c]	J2/100-cm^3 soil	Dry shoot	Fresh root	Cysts	Eggs	J2 hatched from eggs		
1	3.3×10^7	25	23.5	176.9	71	194	7,450	61	25.7
2	3.3×10^7	50	19.7	125.0	83	560	6,146	121	19
3	3.3×10^7	100	22.4	137.5	81	614	8,049	160	19.7
4	3.3×10^7	200	20.6	117.2	118	1,518	10,480	300	14.5
5	3.3×10^6	25	19.5	152.2	62	274	5,536	111	19.6
6	3.3×10^6	50	22.1	152.0	70	506	6,580	132	15.9
7	3.3×10^6	100	20.5	146.2	74	737	17,318	220	15.7
8	3.3×10^6	200	19.1	122.3	94	859	19,740	340	12.5
9	3.3×10^5	25	22.3	166.2	67	353	5,714	110	18.1
10	3.3×10^5	50	19.0	126.4	97	1,101	9,095	408	14.8
11	3.3×10^5	100	18.9	136.8	87	891	8,748	318	10.5
12	3.3×10^5	200	19.3	146.6	134	1,791	11,746	478	9.4
13	3.3×10^4	25	20.5	172.0	79	623	11,714	142	14.8
14	3.3×10^4	50	21.1	119.5	88	618	7,599	227	15.6
15	3.3×10^4	100	21.4	136.9	87	1,090	12,939	330	12.3
16	3.3×10^4	200	17.7	110.7	101	1,195	10,443	468	9.8
17	0	100	5.8	8.3	103	359	209	11	21.5
18	0	100	18.8	138.9	73	600	6,593	253	5.8

[a]Treatments (1-18) were a factorial design of 4 cfu levels of *Dactylella oviparasitica* strain 50 (10^7, 10^6, 10^5, and 10^4 CFU/1,600-cm^3) and 4 population levels of *Heterodera schachtii* (25, 50, 100 and 200 J2/100-cm^3 of soil); 9E soil (Treatment 17) and fumigated 9E soil without *D. oviparasitica* amendment (Treatment 18) were the positive and negative controls, respectively.

[b]Results from two trials were similar and were combined. Values in the table are the means of 10 replicates pots.

[c]CFU densities presented in the table are the average from both trials (values were 3.16 and 3.43 in trials 1 and 2, respectively).

Table 2. A summary of the model for each dependent variable and the transformations used in the linear regression analyses.

Regression model	Coefficient	Coefficient estimate	Coefficient standard error	P-value
$\log(fJ2) = \beta_0 + \beta_1\log(iJ2) + \beta_2\log(iDac)$	β_0	2.91	0.41	4.13e-11
	β_1	0.64	0.087	3.54e-08
	β_2	-0.085	0.026	0.001
$(fegg)^{0.2} = \beta_0 + \beta_1(iJ2)^{0.2} + \beta_2(iDac)^{0.2}$	β_0	1.72	0.42	6.90e-05
	β_1	0.85	0.17	9.77e-07
	β_2	-0.071	0.030	0.018
$\log(par/(1-par)) = \beta_0 + \beta_1\log(iJ2) + \beta_2\log(iDac)$	β_0	-1.069	0.25	0.00053
	β_1	-.29	0.053	4.59e-08
	β_2	0.075	0.016	6.12e-06
$\log(fcys) = \beta_0 + \beta_1 iJ2$	β_0	4.18	0.061	<2e-16
	β_1	0.0019	0.00053	0.0004
$\sqrt{(frweight)} = \beta_0 + \beta_1 iJ2$	β_0	12.079	0.32	2e-16
	β_1	-0.0059	0.0028	0.033
$ftweight = \beta_0 + \beta_1 iJ2$	β_0	203.17	6.32	2e-16
	β_1	-0.13	0.055	0.024
$\sqrt{(dtweight)} = \beta_0 + \beta_1 iJ2$	β_0	4.61	0.079	2e-16
	β_1	-0.0015	0.00069	0.032
$(fhatch)^{0.2} = \beta_0 + \beta_1 iJ2$	β_0	5.14	0.22	2e-16
	β_1	0.0039	0.0019	0.041

iJ2 = Initial numbers of *Heterodera schachtii* J2/100-cm^3 soil.
iDac = Initial amount of *Dactylella oviparasitica* (CFU/cm^3 soil).
fJ2 = Final numbers of *H. schachtii* J2/100-cm^3 soil.
fegg = Final numbers of *H. schachtii* eggs/350-g soil.
Par = Parasitism (%).
Fcys = Final numbers of *H. schachtii* cysts/350-g soil.
frweight = Fresh root weight (g).
ftweigth = Fresh top weight (g).
tdtweigh = Dry top weight (g).
fhatch = Final numbers of *H. schachtii* J2 hatched from eggs/350-g soil.

Fig. 1. Response surface plot predicting final *Heterodera schachtii* J2 densities. iJ2 represents the initial numbers of J2/100-cm^3 soil, iDac is the initial amount of *Dactylella oviparasitica* (CFU/ cm^3 soil) and Par is the percentage of parasitism. The dark points above the plane are greater than the predicted value and the lighter points below the plane are smaller than the predicted value.

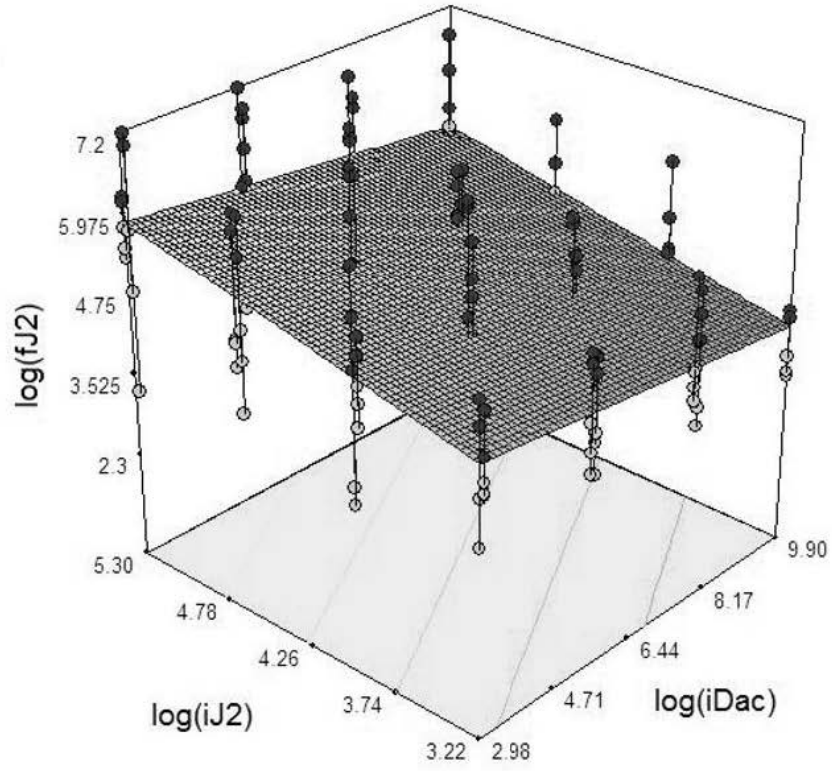


Fig. 2. Response surface plot predicting final *Heterodera schachtii* eggs densities. iJ2 represents the initial numbers J2/100-cm^3 soil, iDac is the initial amount of *Dactylella oviparasitica* (CFU/ cm^3 soil) and fegg is the number of final *H. schachtii* eggs/350-g soil. The dark points above the plane are greater than the predicted value and the lighter points below the plane are smaller than the predicted value.

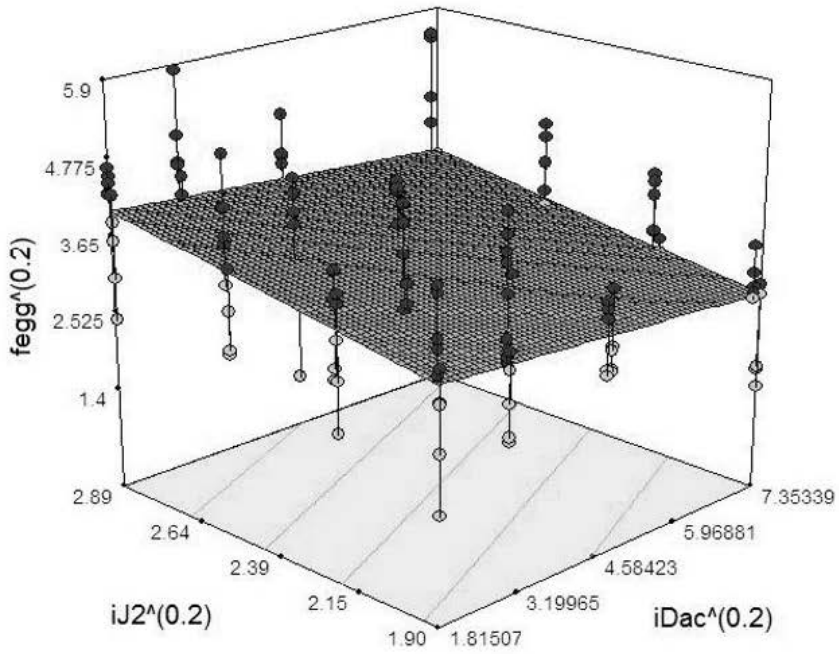


Fig. 3. Response surface plots predicting fungal egg parasitism. iJ2 represents the initial numbers *Heterodera schachtii* J2/100-cm^3 soil, iDac is the initial amount of Dactylella *oviparasitica* (CFU/ cm^3 soil) and Par is the percentage of parasitism. The dark points above the plane are greater than the predicted value and the lighter points below the plane are smaller than the predicted value.

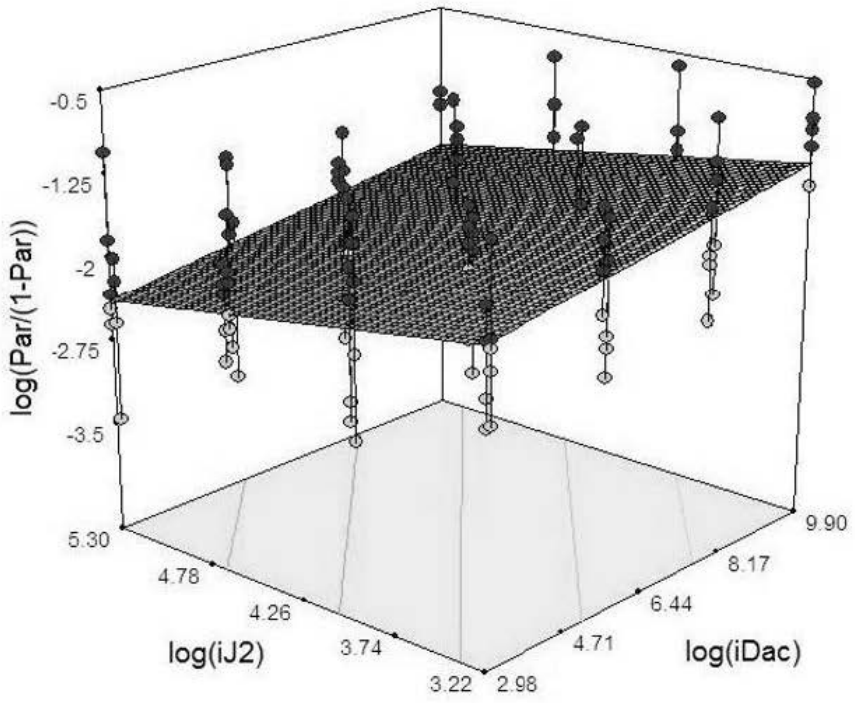


Fig. 4. Phylogenetic analysis of *Dactylella oviparasitica* and related fungi using the rRNA internal transcribed spacer (ITS) region. The analysis includes 47 sequences between 847-910 bp in length. The tree was constructed using the UPGMA method and the Tamura-Nei genetic distance model with 1000 bootstrap samplings.

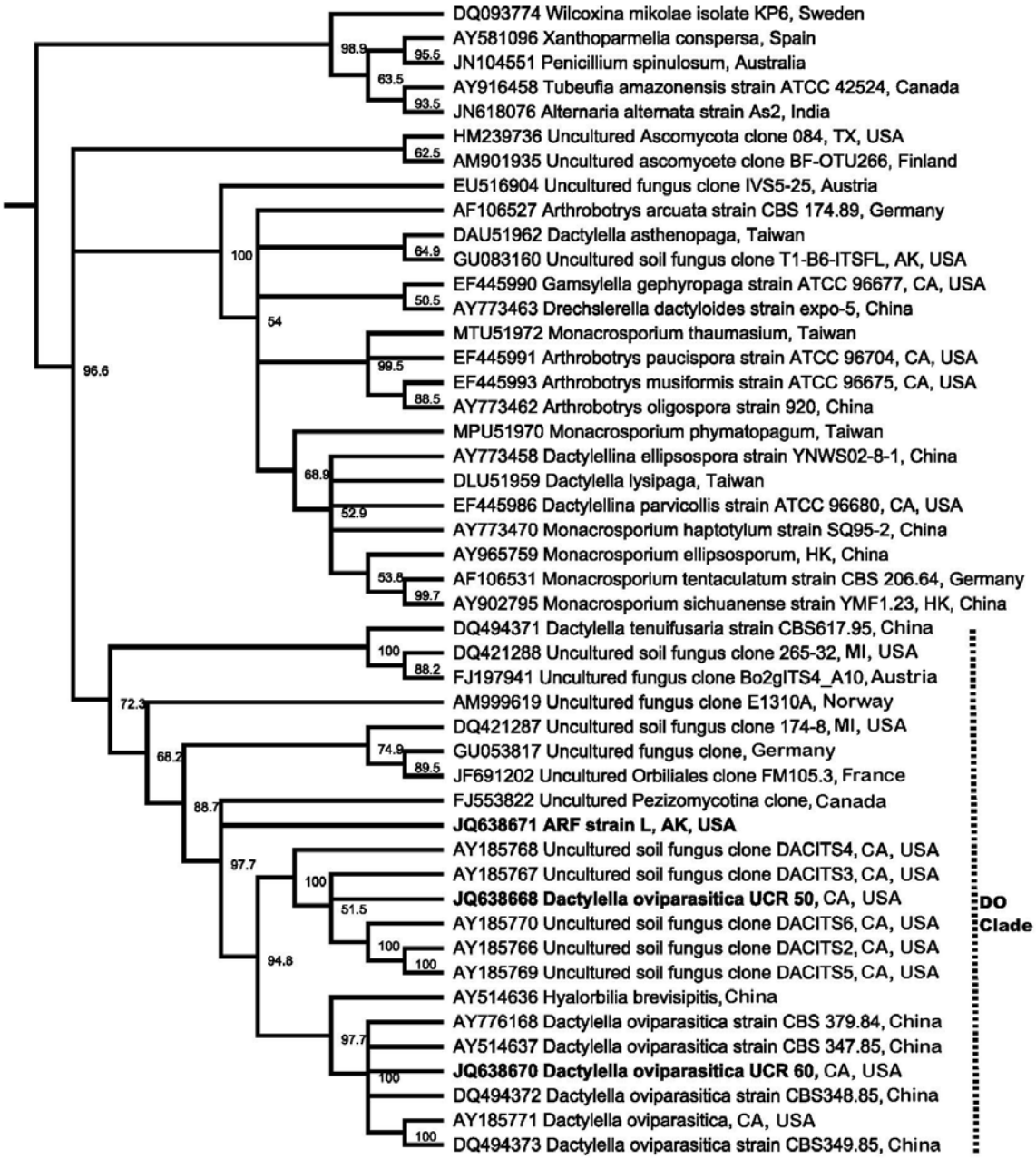
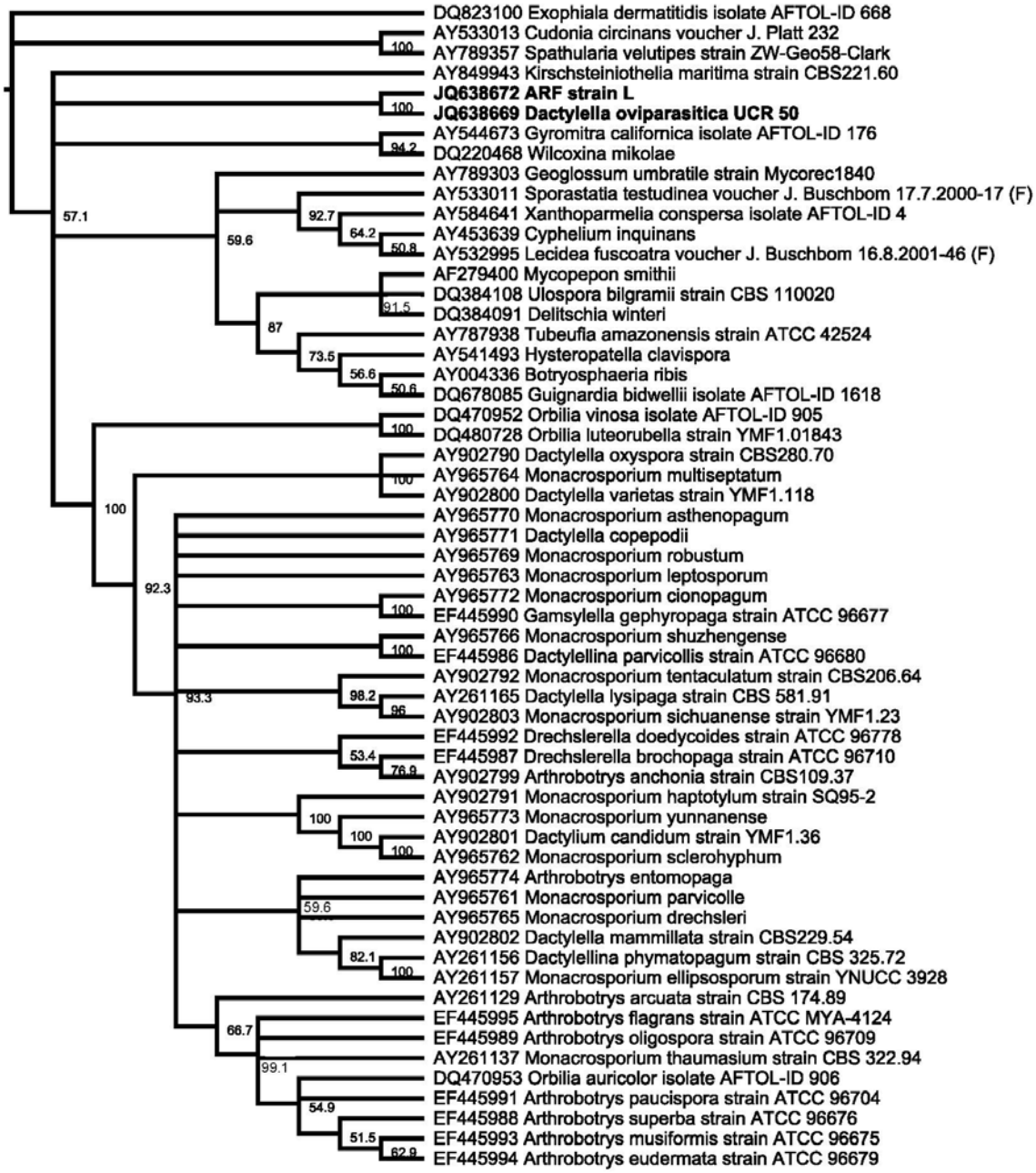


Fig. 5. Phylogenetic analysis of *Dactylella oviparasitica* and related fungi using the rRNA large subunit (LSU) gene. The analysis includes 58 sequences between 334-418 bp in length. The tree was constructed using the UPGMA method and the Tamura-Nei genetic distance model with 1000 bootstrap samplings.



REFERENCE

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Becker, J. O., Ohr, H. D., Grech, N. M., McGiffen, M. E., Jr., and Sims, J. J. 1998. Evaluations of methyl iodide as a soil fumigant in container and small field plot studies. Pesticide Science 52:58-62.

Bent, E., Loffredo, A., Yang, J., McKenry, M. V., Becker, J. O., and Borneman, J. 2009. Investigations into peach seedling stunting caused by a replant soil. FEMS Microbiology Ecology 68:192-200.

Caswell, E. P., Thomason, I. J., and McKinney, H. E. 1985. Extraction of cysts and eggs of *Heterodera schachtii* from soil with an assessment of extraction efficiency. Journal of Nematology 17:337-340.

Curi, J., and Zmoray, I., 1966. Beziehung klimatischer Faktoren zur Entwicklungsdauer von Heterodera schachtii in der Slowakei (CSSR). Helminthologia (Bratisl.) 7:49-63.

Efron, B. 1979. Bootstrap methods: Another look at the jackknife. Annals of Statistics 7:1-26.

Flegg, J. J. M., and Hooper, D. J. 1970. Extratction for free-living stages from soil. Pp. 5-22 *in* J. F. Southey, Ed. Laboratory methods for working with plant and soil nematodes. London: Her Majesty's stationery office.

Fu, Q., Ruegger, P., Bent, E., Chrobak, M., and Borneman, J. 2008. PRISE (PRImer SElector): Software for designing sequence-selective PCR primers. Journal of Microbiological Methods 72:263-267.

Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2:113-118.

Jenkins, W. R. 1964. A rapid centrifuge flotation technique for separating nematodes from soil. Plant Disease Report 48:692.

Kim, D. G., and Riggs, R. D. 1991. Characteristics and efficacy of a sterile hyphomycete (ARF18), a new biocontrol agent for *Heterodera glycines* and other nematodes. Journal of Nematology 23:275-282.

Nigh, E. A., Thomason, I. J., and Van Gundy, S. D. 1980. Identification and distribution of fungal parasites of *Heterodera schachtii* eggs in California. Phytopathology 70:884-889.

Olatinwo, R., Becker, J. O., and Borneman, J. 2006a. Suppression of *Heterodera schachtii* populations by *Dactylella oviparasitica* in four soils. Journal of Nematology 38:345-348.

Olatinwo, R., Borneman, J., and Becker, J. O. 2006b. Induction of beet-cyst nematode suppressiveness by the fungi *Dactylella oviparasitica* and *Fusarium oxysporum* in field microplots. Phytopathology 96:855-859.

Olatinwo, R., Yin, B., Becker, J. O., and Borneman, J. 2006c. Suppression of the plant-parasitic nematode *Heterodera schachtii* by the fungus *Dactylella oviparasitica*. Phytopathology 96:111-114.

Roberts, P. A., and Thomason, I. J. 1981. Sugarbeet Pest Management: Nematodes. Special Publication 3272, UC ANR Publications, Oakland, CA 30 pp.

Roberts, P. A., Thomason, I. J., and McKinney, H. E. 1981. Influence of nonhosts, crucifers, and fungal parasites on field populations of *Heterodera schachtii*. Journal of Nematology 13: 164-171.

Smith Becker, J., Yang, J., Borneman, J., Timper, P., Riggs, R. R., and Becker, J.O. 2011. Investigations into the relatedness of the nematophagous fungi *Dactylella oviparasitica* and ARF-L. Journal of Nematology 43: (in press).

Sneath, P. H. A., and Sokay, R. R. 1973. Numerical Taxonomy. San Francisco: W. H. Freeman.

Stirling, G. R. 1991. Biological control of plant parasitic nematodes: progress, problems and prospects. Wallingford, Oxon, UK: C.A.B International.

Stirling, G. R., and Mankau, R. 1978. *Dactylella oviparasitica*, a new fungal parasite of *Meloidogyne* eggs. Mycologia 70:774-783.

Tamura, K., and Nei, M. 1993. Estimation of the number of nucleotides substitutions in the control region of mitochondrial DNA in humans in chimpazees. Molecular Biological and Evolution 10:512-526.

Thompson, J., Higgins, D., and Gibson, T. 1994. Clustal-w - improving the sensitivity of progressive multiple sequence alignment through sequence

weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680.

Timper, P., and Riggs, R. D. 1998. Variation in efficacy of isolates of the fungus ARF against the soybean cyst nematode *Heterodera glycines*. Journal of Nematology 30:461- 467.

Vilgalys, R., and Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172:4238-4246.

Westphal, A., and Becker, J. O. 2001. Components of soil suppressiveness against *Heterodera schachtii*. Soil Biology and Biochemistry 33:9-16.

White, T. J., Bruns, T. D., Lee, S. B., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 *in* M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White Eds. PCR protocols - A guide to methods and applications. New York: Academic Press.

Whitehead, A. G. 1998. Plant nematode control. CAB International Wallingford, UK.

Yin, B., Valinsky, L., Gao, X., Becker, J. O., and Borneman, J. 2003. Identification of fungal rDNA associated with soil suppressiveness against *Heterodera schachtii* using oligonucleotide fingerprinting. Phytopathology 93:1006-1013.

Ch3. Biocontrol Efficacy among Strains of *Pochonia chlamydosporia* Obtained from a Root-Knot Nematode Suppressive Soil

ABSTRACT

Three *Pochonia chlamydosporia* var. *chlamydosporia* strains were isolated from a *Meloidogyne incognita*-suppressive soil, and then genetically characterized with multiple *Pochonia*-selective typing methods based on analysis of ß-tubulin, rRNA internal transcribed spacer (ITS), rRNA small subunit (SSU), and enterobacterial repetitive intergenic consensus (ERIC) PCR. All strains exhibited different patterns with the ERIC analysis. Strains 1 and 4 were similar with PCR analysis of ß-tubulin and ITS. The strains' potential as biological control agents against root-knot nematodes were examined in greenhouse trials. All three *P. chlamydosporia* strains significantly reduced the numbers of nematode egg masses. When chlamydospores were used as inoculum, strain 4 reduced egg numbers on tomato roots by almost 50%, and showed effects on the numbers of J2 and on nematode-caused root-galling. A newly developed SSU-based PCR analysis differentiated strain 4 from the others, and could therefore potentially be used as a screening tool for identifying other effective biocontrol strains of *P. chlamydosporia* var. *chlamydosporia*.

INTRODUCTION

Biological control is one alternative management strategy addressing the potential environmental problems associated with chemical control of plant-parasitic nematodes. Certain fungi and bacteria can suppress plant-parasitic nematode populations directly through parasitism or indirectly by toxic metabolites (Dong and Zhang, 2006). *Pochonia chlamydosporia* var. *chlamydosporia* (syn. *Verticillium chlamydosporium*) (teleomorph = *Metacordyceps chlamydoaporia*), a ubiquitous facultative hyperparasitic fungus of plant-parasitic nematodes, was first reported from the United Kingdom associated with nematode-suppressive soils (Kerry et al., 1984). It is known to parasitize several economically important nematode species in the genera *Meloidogyne*, *Globodera* and *Heterodera* (Kerry, 1990). However, strains of *P. chlamydosporia* vary in their efficacy to control nematode populations (Bourne et al., 1994; Morton et al., 2003b; Mauchline et al., 2004). Strains differ in their virulence, ability to colonize root surfaces, and chlamydospore production. Different strains of the fungus might also occupy separate niches in soil and rhizosphere possibly due to differences in their enzymatic activities (Segers et al., 1996; Mauchline et al., 2004).

Molecular biotyping techniques are useful tools for identifying and screening potential biocontrol organisms (Gil-Lamaignere et al., 2003). These methods enable the study of microorganisms at the genome level. They are reasonably rapid and economically feasible to perform, and they allow evaluation of large numbers of candidates (Gil-Lamaignere et al., 2003).

Methods commonly applied for fungal typing include restriction fragment length polymorphism (RFLP) (Diguta et al., 2011), various PCR-based techniques (Cogliat et al., 2000), electrophoretic karyotyping (EK) (Usami et al., 2008), and multilocus enzyme electrophoresis (MLEE) (Tibayrenc, 2009). Application of enterobacterial repetitive intragenic consensus (ERIC) PCR on nematophagous fungi has been useful for delineating strains that are not differentiated by rRNA ITS analyses (Arora et al., 1996; Morton et al., 2003a; Manzanilla-López et al., 2009; Kerry and Hirsch, 2011). More specifically, the ß-tubulin gene of *P. chlamydosporia* var. *chlamydosporia* contains an intron not present in other fungi, and thus provides considerable utility for *Pochonia*-selective assays (Hirsch et al., 2001; Kerry and Hirsch, 2011).

Three strains of *Pochonia chlamydosporia* var. *chlamydosporia* were isolated from a *Meloidogyne incognita*-suppressive soil (Bent et al., 2008). The objectives of this study were to genetically characterize the three strains with multiple *Pochonia*-selective typing methods based on analysis of ß-tubulin, rRNA ITS, rRNA SSU, and ERIC-PCR. Furthermore, we determined the potential of the strains to act as biological control agents against the Southern root-knot nematode (*M. incognita*) in greenhouse trials.

MATERIAL AND METHODS

Characterization of test soil. The soil used was obtained from the University of California Kearney Research and Extension Center, Parlier, CA.

This soil was previously selected among six California soils for its abilities to biologically suppress a *M. incognita* population on two different crops under greenhouse conditions (Bent et al., 2008). The test soil was a sandy loam (66% sand, 23% silt, 11% clay; 0.5% organic matter; pH 7.3) mixed with pasteurized silica sand (4:1) to facilitate water drainage and aeration during the greenhouse tests. The soil was pasteurized by submerging samples in plastic bags into a 60^{o}C water bath. Once the center of the sample reached 60^{o}C, the soil was left immersed for 30 minutes at this temperature. The bags were then quickly cooled to room temperature under running tap water. All pasteurized soil samples were pooled and thoroughly mixed.

***Pochonia chlamydosporia* phylotyping.** The fungal strains were grown at 22˚C on a sterilized moist mixture of two parts sandy loam, one part sand and one part compost in capped and parafilm-sealed test tubes (modified after Schneider, 1958). After the substrate had dried out, the tubes were stored at 16˚C. For new starter cultures, a few crumbs of the fungal-colonized substrate were sprinkled aseptically onto PDA. Multiple *Pochonia*-selective phylotyping methods were employed based on analysis of ß-tubulin, rRNA ITS, rRNA SSU, and ERIC. The ß-tubulin, rRNA ITS and ERIC methods were used as previously described (Arora et al., 1996; Hirsch et al., 2000). The rRNA SSU method was developed in this study, and it was performed using 10-ul PCR reactions in a RapidCycler (Idaho Technologies, Salt Lake City, UT) containing 50 mM Tris (pH 8.3), 500 ug mL-1 bovine serum albumin (BSA), 2.5 mM MgCl2, 250 mM of each dNTP, 400 nM of each primer, 1 mL (c. 66 ng) of template DNA (agarose gel purified) and 0.5 unit *Taq* DNA polymerase.

Pochonia-selective PCR primers PochSSUF5 (TGCTTTGGCAGTACGCC) and PochSSUR4 5'- CTTCCGGCCAAGGG - 3' were used with the following thermal cycling conditions: 94^{o}C for 5 min, followed by 42 cycles of 94^{o}C for 20 sec, 64^{o}C for 30 sec, 72^{o}C for 30 sec, and a final incubation at 72^{o}C for 2 min. The amplification product size was 149 bp. Primers used for obtaining ITS sequences were ITS1FUSER (GGGAAAGUCTTGGTCATTTAGAGGAAGTAA) and ITS4USER (TCCTCCGCTTATTGATATGC) with the following thermal cycling conditions: 94^{o}C for 5 min, followed by 40 cycles of 94^{o}C for 20 sec, 52^{o}C for 20 sec, 72^{o}C for 40 sec, and a final incubation at 72^{o}C for 5 min. PCR amplification products were isolated and cloned as previously described using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) (Bent et al., 2008). Sequence identities were determined by BLAST analysis (Altschul et al., 1997).

Inoculum production. For the production of chlamydospores, 250-ml flasks were prepared with 20 g of barley immersed in 40 ml of deionized water. After overnight incubation, excess water was discarded. The flasks were autoclaved at 121^{o}C for 20 min and cooled to room temperature before use. Each flask was inoculated with a different strain of *P. chlamydosporia* and closed with a sterile cotton plug. Chlamydospores were harvested from the flasks after four wk of incubation at 25°C. The content of each flask was blended separately with 50 ml water in a mixer for 1 min. The suspension was poured and washed with a fine jet spray through several sieves (300-µm-pore to 50-µm-pore). After a final rinse the chlamydospores were retained on a 10-µm-pore sieve. The concentration of chlamydospores was determined

using a hemotocytometer (De Leij et al., 1993). To check the viability of chlamydospores, a known amount of inoculum was diluted in series onto 1.7% corn meal agar (CMA) with antibiotics (50 mg per liter streptomycin sulfate, chloramphenicol and chlortetracycline). The percentage of germination was determined after incubation at 25^{o}C for 2 d. For the production of hyphae, *P. chlamydosporia* was cultured on 20 ml potato dextrose agar medium (PDA) in 90 mm petri dishes at 23 ± 2^{o}C for 21 d prior to inoculum preparation. Each plate of fungal culture was mixed with 50 ml sterile water with a Sunbeam 6 Speed Blender (Model 4142; Sunbeam Products Inc., Boca Raton, FL) for 1 min using the “blend” setting. The number of CFUs in each mixture was determined from a dilution series. The remaining portions of the fungal mixtures were added to soil (within 1 hr of blending the fungi) as described below.

Greenhouse trials. The efficacy trials were conducted in a greenhouse of the Department of Nematology, University of California, Riverside, CA. Tomatoes (*Solanum lycopersicum*) cv. UC 82 were sown in seedling trays filled with Sunshine mix #5 (Sun Gro Horticulture Canada Ltd) and incubated in a greenhouse at 26 ± 2^{o}C and ambient light. After three wk the seedlings were transplanted into 800 cm^{3} cups with pasteurized, infested test soil. The soil contained 5,000 chlamydospores/cm^{3} or 50 ml of blended hyphae of *P. chlamydosporia* and 600 eggs/100 cm^{3} of *M. incognita*. Each of the *Pochonia* strains were tested with chlamydospores as inoculum while strain 4 was also evaluated by amending the soil with a blended hyphae suspension. *Pochonia*-free soil served as a control treatment. The cups were arranged in a

randomized complete block design with 5 treatments and six replications. The plants were fertilized with 2 g slow-release fertilizer (Osmocote 17-6-10, Scotts, Marysville, OH) and watered as needed. After 6 wk incubation, the plants were cut off at the soil level and dry weights of shoots were determined. The roots were removed from the soil, rinsed with water and blotted dry before weighing. Root galling was rated on a scale of 0-10 (Zeck, 1971). The roots were immersed in erioglaucine solution overnight and the stained egg masses of root-knot nematodes were counted (Omwega et al., 1988). The eggs from the roots were extracted and counted (Hussey and Barker, 1973). A soil subsample (50 cm^3) from each cup was incubated on a Baermann funnel for 5 days at 26° C. The collected J2 were counted under low power magnification (x30 - 40 magnification). The trial was repeated once with the tomato cultivar Red Gnome. Population and gall rating data were transformed $\log_{10}(x + 1)$ and by arcsin ($\sqrt{x}$), respectively before statistical analysis. The data were subjected to ANOVA and, if appropriate, mean separation by Fisher's LSD test ($P \leq 0.05$). Nontransformed data are shown.

RESULTS

***Pochonia chlamydosporia* phylotyping.** Molecular phylotyping of the *P. chlamydosporia* strains used in this study showed that they were all distinct (Fig. 1). Multiple assays based on analysis of ß-tubulin (panel A), rRNA SSU (panel B), rRNA ITS (panel C), and ERIC (panel D) were employed. Strains 1 and 4 were similar for the ß-tubulin and rRNA ITS analyses. All three strains

exhibited different ERIC patterns. Analysis of the ITS sequences showed that strains 3 and 4 had greater than 98% sequence identity (GenBank accession numbers: JQ433952-433954). Strain 4 was most similar to strain Vc10 (Hirsch et al., 2000), based on the ITS sequences and the ß-tubulin and rRNA ITS PCR analyses. The ITS sequence of strain 4 is 99% identical to *P. chlamydosporia* var. *chlamydosporia.* The rRNA SSU method differentiated strain 4 from the others.

Greenhouse trials*.* When chlamydospores were used as inoculum, all *P. chlamydosporia* strains significantly reduced the numbers of nematode egg masses when compared with the control plants (Table 1, 2). Egg numbers were reduced to almost 50% by *P. chlamydosporia* strain 4. The numbers of J2 were equally reduced by both inocula forms of *P. chlamydosporia* strain 4. The inoculation method did not influence the efficacy; inocula based on hyphae or chlamydospores produced similar results. As for root galling index, only the chlamydospore inoculum of *P. chlamydosporia* strain 4 showed a consistent reduction effect. In Trial 1, shoot dry weights were greatest following inoculation with chlamydospores of strain 4. However, there were no differences in shoot dry weights among treatments in Trial 2.

DISCUSSION

Pathogen-suppressive soils are a potential source of effective biological control agents. In prior research, we identified a *M. incognita*-suppressive soil, and with a series of biocidal soil treatments, we demonstrated the biological

nature of the suppressiveness (Loffredo et al., 2010). Subsequent molecular population-based studies identified negative associations between the amounts of *P. chlamydosporia* and *M. incognita* through the use of a fungal rRNA gene analysis (oligonucleotide fingerprinting of rRNA genes) and qPCR validation experiments (Bent et al., 2008). We isolated and identified three genetically different *P. chlamydosporia* strains from parasitized *M. incognita* eggs and demonstrated the nematode-suppressing abilities of the fungal strains in greenhouse trials. In particular *P. chlamydosporia* strain 4 reduced nematode eggs by more than 50% and negatively affected the number of J2 extracted from the soil. Since reducing or damaging nematode females and/or eggs will be reflected in lower J2 populations, these results suggest that this strain is a contributor to the suppressiveness.

The three *P. chlamydosporium* strains possessed varying abilities to impact *M. incognita* populations, a result that has been observed in other studies. In a study of *Pochonia* species from Iranian soils, *in vitro* pathogenicity tests showed the fungal strains infected root-knot nematode eggs at varying rates between 39% and 95% (Moosavi et al., 2010). Variation in the efficacies of *P. chlamydosporium* strains are likely caused by differing traits of the fungi, including their abilities to grow rapidly in the environment, to produce chlamydospores, and to possess effective virulence factors. Varying *P. chlamydosporia* population dynamics in soil have been previously reported. In a microplot experiment, *P. chlamydosporium* survived in loamy sand and sand but did not multiply after 8 wk after infestationwith root-knot nematodes (De Leij et al., 1993). After being applied to soil, *P. chlamydosporia* var. *catenulate*

populations increased over a 5-month period (Atkins et al., 2003). Some studies have shown that a one-time application of *P. chlamydosporia* was able to reduce *M. javanica* population densities for at least 5-7 months compared to the control (Van Damme et al., 2005), while others have observed that the fungus persisted 2 (Crump, 2004) to 5 years (Atkins et al., 2003). Strains of *P. chlamydosporia* also differed in the amount of chlamydospores produced, and the amount decreased as the culture time increased (Kerry et al., 1986). Though several strains have shown good efficacy in lab, greenhouse or field trials (Müller, 1982; De Leij and Kerry, 1991; Crump and Irving, 1992; De Leij et al., 1993, Hay and Skipp, 1993; Siddiqui and Mahmood, 1996), protease studies indicated host-related genetic variation among strains of *P. chlamydosporia* might contribute to the host preference at the infra species level (Morton et al., 2003b; Mauchline et al, 2004). The extracellular enzymes produced by *P. chlamydosporia* species include chitinases, esterases, and lipases. Strain VCP1 was shown to be influenced by culture medium amendments and culture time, and significant differences were found among strains and the amount of enzymes produced (Esteves et al., 2009).

Co-evolution phenomena between hosts and pathogens is commonly accepted in evolutionary and ecological functional genomics studies (Feder and Mitchell-Olds, 2003). The Red Queen Hypothesis was proposed to explain the evolutionary arms race between host pathogen populations, whereby one needs to evolve continuously to avoid being overwhelmed by the other (Clay and Kover, 1996). Virulence genes have the tendency to be kept and modified in the pathogen during gene selection and inheritance, for it to be at a selective

advantage against particular host genotypes. Further studies would be needed to determine whether the differences among our *Pochonia* strains were driven by Red Queen dynamics.

Our study is concordant with others that have demonstrated the utility of molecular phylotyping for differentiating *P. chlamydosporia* strains possessing varying biological control efficacies. The greenhouse trials showed that among the test strains, only strain 4 reduced egg masses, eggs, J2, and root galling (Table 1, 2). Based on the analysis of ITS sequence, as well as the ß-tubulin and ITS PCR assays (Fig. 1), *P. chlamydosporia* strain 4 was most similar to *P. chlamydosporia* var. *chlamydosporia* strain Vc10 (Hirsch et al., 2000). Strain Vc10 was originally isolated from *M. incognita* eggs. Among several *P. chlamydosporia* strains tested for their ability to multiply in the rhizosphere of root-knot infested tomato plants, Vc10 had the highest proliferation rate. Among 10 tested plant species, Vc10 populations increased the most in the rhizosphere of tomato (Bourne et al., 1994). Furthermore, strain Vc10 exhibited maximum in vitro proteolytic activity among 5 tested *P. chlamydosporia* strains, which is a trait that might play a key role in nutrient acquisition as well as in its biocontrol activity against nematodes by degrading the outer layer of nematode eggs (Segers et al., 1994). The phylotyping method developed in this study, which targets the small subunit ribosomal gene, was able to differentiate *P. chlamydosporia* strain 4 from the other strains, making it a potentially useful screening tool for similar strains with superior biological control attributes.

ACKNOWLEDGEMENT

The authors thank John Darsow for his technical assistance. This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (CSREES), grant 2005-35302-16150 and by the University of California Agricultural Experiment Station.

TABLE 1. Effects of different *Pochonia chlamydosporia* strains on tomato (cv. UC82) plant vigor and on *Meloidogyne incognita* populations.

Strain (inoculum)	number of egg masses	number of eggs	J2/50 cm^3 soil	root galling index	shoot dry weight (g)	root fresh weight (g)
P. chlamydosporia strain 1 (chlamydospores)	421 a	646,666 b	752 c	4.7 b	12.7 ab	43.9 a
P. chlamydosporia strain 3 (chlamydospores)	420 a	652,500 b	582 bc	4.8 b	14.4 bc	46.4 a
P. chlamydosporia strain 4 (chlamydospores)	400 a	383,333 a	417 b	4.0 a	15.7 c	45.4 a
P. chlamydosporia strain 4 (hyphae)	423 a	359,166 a	156 a	4.3 ab	13.3 ab	46.4 a
Non-inoculated control	581 b	635,000 b	683 c	4.8 b	14.0 b	50.2 a

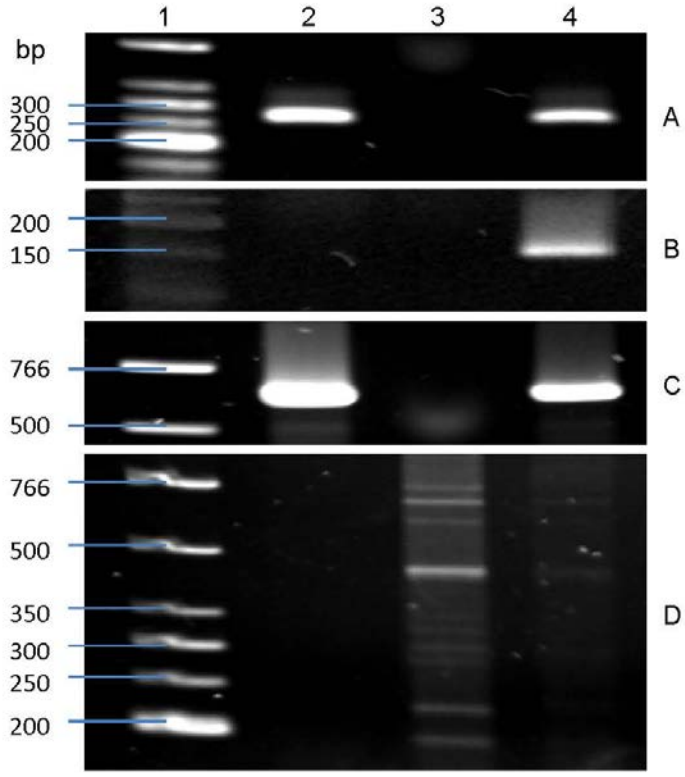
*Values means of 6 replicates. Number with same letter are significantly different according to Fisher's protected (LSD) test at $P \leq 0.05$.

TABLE 2. Effects of different *Pochonia chlamydosporia* strains on tomato (cv. Red Gnome) plant vigor and on *Meloidogyne incognita* populations.

Strain (inoculum)	number of egg masses	number of eggs	J2/50 cm^3 soil	root galling index	shoot dry weight (g)	root fresh weight (g)
P. chlamydosporia strain 1 (chlamydospores)	485 c	605,833 b	860 a	5.6 c	9.5 a	38.0 a
P. chlamydosporia strain 3 (chlamydospores)	406 ab	537,000 b	848 a	3.6 a	11.3 a	32.8 a
P. chlamydosporia strain 4 (chlamydospores)	362 a	391,000 a	814 a	4.1 ab	10.4 a	32.4 a
P. chlamydosporia strain 4 (hyphae)	496 c	605,833 b	869 a	6.0 c	10.7 a	36.6 a
Non-inoculated control	630 d	826,250 c	1288 b	5.7 c	10.2 a	36.1 a

* Values means of 6 replicates. Number with same letter are significantly different according to Fisher's protected (LSD) test at $P \leq 0.05$.

Fig. 1. Molecular phylotyping of *Pochonia chlamydosporia* strains used in this study. *Pochonia*-selective PCR-based assays targeted: A, ß-tubulin; B, rRNA SSU; C, rRNA ITS; and D, ERIC. Lanes were: Lane 1, Low molecular weight DNA ladder (New England Biolabs, Inc., Beverly, MA); Lane 2, *P. chlamydosporia* strain 1; Lane 3, *P. chlamydosporia* strain 3; Lane 4, *P. chlamydosporia* strain 4.



REFERENCE

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Arora, D.K., Hirsch, P.R., and Kerry, B. R. 1996. PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. Mycological Research 100:801-809.

Atkins, S. D., Hidalgo-Diaz, L., Kalisz, H., Mauchline, T. H., Hirsch, P. R., and Kerry, B.R. 2003. Development of a new management strategy for the control of root-knot nematodes (*Meloidogyne* spp) in organic vegetable production. Pest Management Science 59:183-189.

Bent, E., Loffredo, A., McKenry, M. V., Becker, J. O., and Borneman, J. 2008. Detection and investigation of soil biological activity against *Meloidogyne incognita*. Journal of Nematology 40:109-118.

Bourne, J.M., Kerry, B. R., and de Leij, F. A. A. M. 1994. Methods for the study of *Verticillium chlamydosporium* in the rhizosphere. Journal of Nematology 26:587-591.

Clay, K., and Kover, P. X. 1996. The red queen hypothesis and plant/pathogen interactions. Annual Review of Phytopathology 34:29-50.

Cogliati, M., Allaria, M., Tortorano, A. M., and Viviani, M. A. 2000. Genotyping *Cryptococcus neoformans* var. *neoformans* with specific primers designed from PCR-fingerprinting bands sequenced using a modified PCR-based strategy. Medical Mycology 38:97-103.

Crump, D. H. 2004. Biocontrol- a route to market. Pp. 165-174 *in* R. H. Cook, and D. J. Hunt, eds. Proceedings of the Fourth International Congress of Nematology. Tenerife, Spain. Nematology Monographs and Perspectives 2. Leiden, The Netherlands: Brill.

Crump, D. H., and Irving, F. 1992. Selection of isolates and methods of culturing *Verticillium chlamydosporium* and its efficacy as a biological control agent of beet and potato cyst nematodes. Nematologica 38:367-74

De Leij, F. A. M. M., and Kerry, B. R. 1991. The nematophagus fungus *Verticillium chlamydosporium* as a potential biological control agent for *Meloidogyne arenaria*. Revue de Nematologia 14:157-164.

De Leij, F. A. A. M., Kerry, B. R., and Dennehy, J. A. 1993. *Verticillium chlamydosporium* as a biological control agent for *Meloidogyne incognita* and *M. hapla* in pot and microplot tests. Nematologica 39:115-126.

Diguta, C. F., Vincent, B., Guilloux-Benatier, M., Alexandre, H., Rousseaux, S. 2011. PCR ITS-RFLP: A useful method for identifying filamentous fungi isolates on grapes. Food Microbiology 28:1145-1154.

Dong, L. Q., and Zhang, K. Q. 2006. Microbial control of plant-parasitic nematodes: a five-party interaction. Plant and Soil 288:31-45.

Esteves, I., Peteira, B., Atkins, S. D., Magan, N., and Kerry, B. 2009. Production of extracellular enzymes by different isolates of *Pochonia chlamydosporia*. Mycological Research 113:867-876.

Feder, M. E., and Mitchell-Olds, T. 2003. Evolutionary and ecological functional genomics. Nature Reviews Genetics 4:649-655.

Gil-Lamaignere, C., Roilides, E., Hacker, J., and Müller, F. M. C. 2003. Molecular typing for fungi- a critical review of the possibilities and limitations of currently and future methods. Clinical Microbiology and Infection 9:172-185.

Hay, F. S., and Skipp, R. A. 1993. Fungi and actinomycete associated with cysts of *Heterodera trifolii* Goffart (Nematoda: *Tylenchida*) in pasture soils in New Zealand. Nematologica 39:376-384.

Hirsch, P. R., Atkins, S. D., Mauchline, T. H., Morton, C. O., Davies, K. G., and Kerry, B. R. 2001. Methods for studying the nematophagous fungus *Verticillium chlamydosporium* in the root environment. Plant and Soil 232:21-30.

Hirsch, P. R., Mauchline, T. H., Mendum, T. A., and Kerry, B. R. 2000. Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode-infested plant roots using PCR. Mycological Research 104:435-439.

Hussey, R. S., and Barker, K. R. 1973. A comparison of methods of collecting inocula for *Meloidogyne* spp., including a new technique. Plant Disease Reporter 57:1025-1028.

Kerry, B. 1990. Fungi as biological control agents for plant parasitic nematodes. Pp. 153-170 *in* J. M. Whipps and R. D. Lumsden, eds. Biotechnology of fungi for improving plant growth. New York: British Mycological Society Symposia.

Kerry, B. R., and Hirsch, P. R. 2011. Ecology of *Pochonia chlamydosporia* in the rhizosphere at the population, whole organism and molecular scales. Pp. 171-182 *in* K. Davies and Y. Spiegel, eds. Biological control of plant-parasitic nematodes: building coherence between microbial ecology and molecular mechanisms. Progress in Biological Control 11. Berlin: Springer.

Kerry, B. R., Irving, F., and Hornsey, J. C. 1986. Variation between strains of the nematophagous fungus, *Verticillium chlamydosporium* Goddard. I. Factors affecting growth *in vitro*. Nematologica 32:461-473.

Kerry, B. R., Simon, A., and Rovira, A. D. 1984. Observations on the introduction of *Verticillium chlamydosporium* and other parasitic fungi into soil for control of the cereal cyst-nematode *Heterodera avenue*. Annals of Applied Biology 105:509-516.

Loffredo, A., Yang, J., Borneman, J., and Becker, J. O. 2010. Identification of a *Meloidogyne incognita*-suppressive soil and its potentially causal agent. Journal of Nematology 42:252 (Abstr.).

Manzanilla-López, R. H., Clark, I. M., Atkins, S. D., Hirsch, P. R., and Kerry, B. R. 2009. Rapid and reliable DNA extraction and PCR fingerprinting methods to discriminate multiple biotypes of the nematophagous fungus *Pochonia chlamydosporia* isolated from plant rhizospheres. Letters in Applied Microbiology 48:71-76.

Mauchline, T. H., Kerry, B. R., and Hirsch P. R. 2004. The biocontrol fungus *Pochonia chlamydosporia* shows nematode host preference at the infraspecific level. Mycological Research 108:161-169.

Moosavi, M. R., Zare, R., Zamanizadeh, H. R., and Fatemy, S. 2010. Pathogenicity of *Pochonia* species on eggs of *Meloidogyne javanica*. Journal of Invertebrate Pathology 104:125-133.

Morton, C. O., Mauchline, T. H., Kerry, B. R., and Hirsch, P. R. 2003. PCR-based DNA fingerprinting indicates host-related genetic variation in the nematophagous fungus *Pochonia chlamydosporia*. Mycological Research 107:198-205.

Müller, J. 1982. The influence of fungal parasites on the population dynamics of *Heterodera schachtii* on oil radish. Nematologica 28:161.

Omwega, C. O., Thomason, I. J., and Roberts, P. A. 1988. A nondestructive technique for screening bean germ plasm for resistance to *Meloidogyne incognita*. Plant Disease 72:970-972.

Schneider, R. 1958. Untersuchungen über Variabilität und Taxonomie von *Fusarium avenaceum* (Tr) Sacc. Phytopathologische Zeitschrift 32:95-126.

Segers, R., Butt, T. M., Kerry, B. R., Beckett, A., and Peberdy, J. F. 1994. The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins *in situ*. Microbiology 140:2715-2723.

Segers, R., Butt, T. M., Kerry, B. R., Beckett, A., and Peberdy, J. F. 1996. The role of the proteinase VCP1 produced by the nematophagous *Verticillium chlamydosporium* in the infection process of nematode eggs. Mycological Research 100:421-428.

Siddiqui, Z. A., and Mahmood, I. 1996. Biological control of plant parasitic nematodes by fungi: A review. Bioresource Technology 58:229-239.

Tibayrenc, M. 2009. Molecular epidemiology of microorganisms. Methods in Molecular Biology 551:13-25.

Usami, T., Fukaya, M., and Amemiya, Y. 2008. Electrophoretic karyotyping and mapping of pathotype-specific DNA sequences in Japanese isolates of *Verticillium dahliae*. Journal of General Plant Pathology 74:61-65.

Van Damme, V., Hoedekie, A., and Viaene, N. 2005. Long-term efficacy of *Pochonia chlamydosporia* for management of *Meloidogyne javanica* in glasshouse crops. Nematology 7:727-736.

Zeck, W. M. 1971. A rating scheme for field evaluation of root-knot nematode infestations. Pflanzenschutz-Nachrichten Bayer 24:141-144.

Summary

The research described in this dissertation examined several agricultural phenomena using traditional plant pathology methods as well as recently developed molecular microbial ecology methods. In the peach replant disease research, the experiments endeavored to identify microbes associated with the peach replant disease in a California soil. In the pathogen suppressive soil research, two nematode biological control systems were studied. First, a series of experiments investigated the population dynamics between *Dactylella oviparasitica* and *Heterodera schachtii*. Second, biocontrol efficacy tests against southern root knot nematode were performed for 3 strains of *Pochonia chlamydosporia* var. *chlamydosporia*.

In Chapter 1, the study was designed to identify bacteria, fungi and oomycetes associated with peach replant disease in a soil located at the UC Kearney Agricultural Center in California. Soil samples were subjected to treatments to create various levels of replant disease symptoms. Peach seedlings were grown in the treated soils in greenhouse trials. After 6 weeks, plant growth parameters were measured, and culture and culture-independent analyses were performed on DNA extracted from the plant roots to identify bacteria, fungi and oomycetes. A total of 9,320 bacterial operational taxonomic units (OTU) were identified. Among the 60 most abundant OTUs, 27 showed significant ($P < 0.05$) negative correlation with peach top weights while 10 were positively correlated. Most of these OTUs belonged to the bacterial phylum

Proteobacteria (96%), including the classes Gammaproteobacteria (44.4%), Betaproteobacteria (33.3%) and Alphaproteobacteria (22.2%), and the orders Pseudomonadales, Burkholderiales, Chromatiales, Rhodocyclales, and Sphingomonadales. The most abundant fungal taxa were *Trichoderma asperellum*, *Trichoderma virens, Fusarium oxysporum, Ceratocystis fimbriata* and *Fusarium solani*. The most abundenat oomycetes taxa were *Pythium vexans*, *Pythium violae* and an unidentified *Aplanochytrium* species. Sequence-selective quantitative PCR analyses identified negative and positive associations between *P. vexans* and *Trichoderma* sp. and peach top weights, respectively.

This work produced a list of microbes that are putatively causal or protective agents. The next step in this research will be to isolate these organisms and test them. For the organisms that showed negative correlations with plant growth, performing Koch's postulates experiments in greenhouse or field conditions would likely provide more information about the putatively causal organisms. On the other hand, adding organisms with positive correlations to soils exhibiting replant disease symptoms might lead to the discovery of possible plant growth promoting microbes and/or new biological agent(s) against peach replant disease.

In Chapter 2, a series of investigations were performed to examine the population dynamics of the sugarbeet cyst nematode, *Heterodera schachtii*, and the fungus *Dactylella oviparasitica*. After two nematode generations, the

population densities of *H. schachtii* were measured in relation to various initial population densities of both *D. oviparasitica* and *H. schachtii*. In general, higher initial levels of *D. oviparasitica* were associated with lower final levels of *H. schachtii*. Regression models showed that the initial densities of *D. oviparasitica* were only significant when predicting the final densities of *H. schachtii* J2 and eggs as well as fungal egg parasitism, while the initial densities of J2 were significant for all final *H. schachtii* measurements. We also showed that the densities of *H. schachtii*-associated *D. oviparasitica* fluctuate dramatically, with rRNA gene numbers going from zero in most field-soil-collected cysts to an average of 4.24×10^8 in cysts collected from root surfaces. Finally, phylogenetic analysis of rRNA genes suggested that *D. oviparasitica* belongs to a clade of nematophagous fungi with a large geographical distribution. We posit that the findings in the study will provide foundational data facilitating the development of more effective sugar beet planting decision models.

This work also led to the identification of a new phylotype of *D. oviparasitica*. Future work will involve obtaining pure cultures of this organism. Further characterization of its parasitic ability of nematodes would lead to a better understanding of this organism and enable assessment of its utility in biological control applications. In addition, a greater understanding of the causes of the dramatic fluctuations in the population densities of *D. oviparasitica* associated with cysts should lead to more effective pathogen management strategies.

In Chapter 3, three *Pochonia chlamydosporia* var. *chlamydosporia* strains were isolated from a *Meloidogyne incognita*-suppressive soil, and then genetically characterized with multiple *Pochonia*-selective typing methods based on analysis of ß-tubulin, rRNA internal transcribed spacer (ITS), rRNA small subunit (SSU), and enterobacterial repetitive intergenic consensus (ERIC) PCR. All strains exhibited different patterns with the ERIC analysis. Strains 1 and 4 were similar with PCR analysis of ß-tubulin and ITS. The strains' potential as biological control agents against root-knot nematodes were examined in greenhouse trials. All three *P. chlamydosporia* strains significantly reduced the numbers of nematode egg masses. Strain 4 reduced almost 50% of the eggs, and showed effects on the numbers of J2 and on nematode-caused root-galling. A newly developed SSU-based PCR analysis differentiated strain 4 from the others, and could therefore potentially be used as a screening tool for identifying other effective biocontrol strains of *P. chlamydosporia* var. *chlamydosporia*.

There are also other root knot nematodes that are considered economically important, such as *M. hapla* and *M. javanica*. Future research will involved performing pathogenicity tests of our *P. chlamydosporia* isolates against these other root knot nematodes. Since co-evolution between pathogen and host are commonly observed, the strains that were less effective against *M. incognita* may prove to be more useful for *M. hapla* and *M. javanica.* New molecular assays could also be developed that utilize mutiloci markers.

Such assay could lead to the development of more effective screening tools for identifying other successful biological control strains.

In conclusion, the research described in this dissertation focused on microbial ecological aspects of nematode suppression and replant disease. The findings from the peach replant studies provide insights into disease etiology and possible disease management strategies. Foundational data facilitating the development of more effective sugar beet planting decision models were obtained from the *D. oviparasitica* studies, and implementation of these results could lead to better nematode management strategies. *P. chlamydosporia* strain 4 was shown to be a potential biological control agent against southern root knot nematode, providing new pathogen control options. Overall, with the increased knowledge of microbial interactions in agriculture systems, disease control strategies that are effective, environmentally friendly and low cost, will hopefully be available in the near future.