APPLIED T-RFLP ANALYSES FOR THE IDENTIFICATION AND CHARACTERIZATION OF MICROBIAL POPULATIONS ASSOCIATED WITH DAMPING-OFF INCIDENCE IN A TRANSITIONAL ORGANIC CROPPING SYSTEM

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

María Soledad Benítez, M.S.

* * *

The Ohio State University
2008

Dissertation Committee: Approved by:
Dr. Brian McSpadden Gardener, Adviser
Dr. Terrence L. Graham
Dr. Warren Dick
Dr. Enrico Bonello

___________________________
Adviser

Plant Pathology Graduate Program
ABSTRACT

Within agricultural ecosystems the abundance and activities of diverse microbial populations affecting plant health can be significantly influenced by farm management practices. This work describes the effects of transitional cropping strategies on damping-off incidence and on soil and rhizosphere bacterial, fungal and oomycete communities. Specifically, individual microbial populations were associated with damping-off incidence and the suppression thereof, and two novel bacterial species were characterized for their ability to reduce seedling disease severity. Soils from an organic transition experiment exhibited different levels of damping-off suppression, with a perennial mixed hay transition consistently suppressing damping-off of tomato and soybean. Microbial community analyses were performed from the rhizosphere and surrounding soil of tomato and soybean seedlings grown in soils previously exposed to different transition strategies. The abundance of *Pseudomonas* and DAPG-producing *Pseudomonas* populations, did not clearly associate with disease levels. However, the analysis of bacterial, fungal and oomycete community profiles (generated from terminal restriction fragment length polymorphism of 16S and ITS genes, respectively) revealed individual members of the community (expressed as a terminal restriction fragment, TRF) that statistically associated with disease suppression or disease. Bacterial TRF more abundant in the mixed hay suppressive treatment were negatively correlated with disease incidence. *In*
silico analysis of bacterial database sequences indicated that species of known biocontrol agents produce a TRF size corresponding to those identified in this study. Fungal and oomycete TRF abundance and diversity tended to be lower in the mixed hay transition strategy. Fungal and oomycete isolates of the genus *Fusarium, Chaetomium* and *Pythium*, among others, generate TRF that match those associated with disease in the studied transition strategies. Sequence information obtained from the bacterial T-RFLP data was used to identify and isolate two novel bacterial species from the mixed hay soils. *Mitsuaria* and *Burkholderia* isolates were phylogenetically related to the TRF sequences associated with disease suppression, inhibited pathogen growth *in vitro*, and reduced seedling disease severity. This work establishes the power of T-RFLP based screening of microbial communities to identify and direct the recovery of functionally important microorganisms in soil.
To my parents
ACKNOWLEDGMENTS

I would like to acknowledge first my advisor, Brian McSpadden Gardener for his support and guidance during this process, and especially for believing in me and encouraging me hard enough so to convince me to move into the PhD program. In addition thanks for starting me into a project that resulted being not only very productive and full of learning experiences but more than anything very fun to work in.

I would like to thank the members of my student advisory committee, Terrance Graham, Warren Dick and Enrico Bonello, for all your helpful comments and support.

Special thanks to the Department of Plant Pathology, professors, staff and students, for allowing me to be part of this program and making my time at this department so enjoyable but at the same time intellectually challenging.

Thanks to all the people that in these years have shared the lab with me: Seth, Sonia, Jeff, Alejandra, Carrie, Kelly, Gloria, Dario, Jessica, Fulya, Guleray, Raghu, Laura, Amara, Clara, Jackie, Ethan, with a very special thank you for Rosa, Sunny, Dorith and Pal.

A very special thank you to all those with whom I shared very nice moments during my stay at Ohio State: Wirat, Angel, Jose, Santiago, Miguel, Alieta, Frances, Andrea, Carla, Julio, Audrey, Diego, Fulya, Sawsan, Xiulan, Kirk, Luciana, Juan Pablo,
Sandra, Rodrigo, Alex, Sanja, Hanbae, Vanessa, Maggie, Hehe, Zhifen, Joe, Jorunn, Jing, Vero, Kristhen, Jhonny, Freddy, Carmen, Loren, Lynn...

Of course thanks to my friend Gaby, who from California was a great support during these times, also thanks to Nessy, David and Pancho.

The greatest thanks of all to my family, for their patience and support. Thanks to my mom for teaching me so early about the scientific method. Also for coming so many times to visit me, being such a good company and a great friend of my friends, and thanks to my dad, my sister and my nieces for letting her come. Thanks for my dad for being my scientific and work ethic role model. Thanks to my sister’s unconditional support. Thanks for Ale for her patience and understanding. Thanks to my brother who I know is proud of me.

Finally thanks to all of you who will eventually be reading this thesis. Don’t wonder too much why I didn’t mention your names in it, it will have taken too much space. That includes my “witch” friends, my high school friends, my college friends, my cousins, aunts and grandma, and all other “unclassified” friends.
VITA

1978 Born, Quito, Ecuador

1996-2002 Licenciatura, Biological Sciences, Pontificia Universidad Católica del Ecuador, Quito

2002-2004 Auxiliary Professor and Research Associate, Department of Biological Sciences, Pontificia Universidad Católica del Ecuador, Quito

2007 MSc, The Ohio State University, obtained as a successful completion of the candidacy examination

2004 – present Graduate Research Associate, Department of Plant Pathology, The Ohio State University

PUBLICATIONS


**FIELDS OF STUDY**

Major Field: Plant Pathology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiv</td>
</tr>
</tbody>
</table>

Chapters:

1. Setting up the stage: Identification of a disease suppression differential
   - Introduction                                                                 | 1    |
   - Identification of a disease suppressive differential: Transitional organic cropping system effects on soybean and tomato seedling damping-off and vigor | 6    |
   - Transition strategy and compost amendment effects on soil chemistry      | 8    |
   - Transition strategy and compost amendment effects on soybean and tomato damping-off and seedling vigor | 10   |
   - Transition strategy effects on the abundance and incidence of DAPG-producing *Pseudomonas* colonizing the rhizosphere of tomato and soybean | 13   |
   - Summary                                                                  | 19   |
   - References                                                               | 21   |

2. Multiple statistical approaches of community fingerprint data reveal bacterial populations associated with general disease suppression arising from the application of different organic field management
   - Abstract                                                                  | 41   |
   - Introduction                                                             | 42   |
   - Materials and Methods                                                   | 46   |
   - Results                                                                  | 50   |
   - Discussion                                                               | 58   |
   - Acknowledgements                                                        | 64   |
   - References                                                               | 65   |
3. Association of multiple fungal and oomycete populations with damping-off disease incidence in soils previously managed under different organic transition strategies
   Abstract ........................................................................................................... 80
   Introduction ..................................................................................................... 81
   Materials and Methods .................................................................................. 85
   Results ............................................................................................................ 91
   Discussion ...................................................................................................... 99
   Acknowledgements ...................................................................................... 106
   References .................................................................................................... 106

4. Linking sequence to function in soil: sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression
   Abstract ........................................................................................................... 126
   Introduction ..................................................................................................... 127
   Materials and Methods .................................................................................. 129
   Results ............................................................................................................ 136
   Discussion ...................................................................................................... 142
   Acknowledgements ...................................................................................... 146
   References .................................................................................................... 146

5. Conclusions ................................................................................................... 167
   References .................................................................................................... 174

Bibliography ...................................................................................................... 178
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>pH, % N, and % C from soils exposed to four different transition strategies, with and without compost amendment, at three different sampling dates</td>
</tr>
<tr>
<td>1.2</td>
<td>Extractable nutrient status from soils exposed to four different transition strategies, with and without compost amendment, at three different sampling dates</td>
</tr>
<tr>
<td>1.3</td>
<td>Vigor of soybean seedlings grown in the greenhouse in soils previously exposed to different field management strategies</td>
</tr>
<tr>
<td>1.4</td>
<td>Vigor of soybean seedlings grown in an organic field after three years of transition under different management strategies</td>
</tr>
<tr>
<td>1.5</td>
<td>Vigor of tomato seedlings grown in an organic field after three years of transition under different management strategies</td>
</tr>
<tr>
<td>1.6</td>
<td>Abundance and incidence of rhizosphere colonizing <em>Pseudomonas</em> and <em>phlD</em>+ <em>Pseudomonas</em> in tomato and soybean seedlings, grown in the greenhouse and in the field, in soils previously subjected to different organic transition strategies</td>
</tr>
<tr>
<td>1.7</td>
<td>Spearman’s pair-wise correlations for the abundance of rhizosphere colonizing pseudomonads in tomato and soybean samples and damping-off incidence, for bioassays performed in the greenhouse and the field</td>
</tr>
<tr>
<td>1.8</td>
<td>Damping-off incidence groups, according to % damping-off severity observed in tomato and soybean grown in the greenhouse and the field, in soils previously cropped with one of the studied transition strategies</td>
</tr>
<tr>
<td>2.1</td>
<td>16S rDNA terminal restriction fragments with high factor loadings on the first three principal components for each experimental context</td>
</tr>
</tbody>
</table>
2.2 Relative abundance of a selected subset of 16S rDNA terminal restriction fragments showing significant differences between transition strategies, for each experimental context

2.3 Damping-off incidence levels observed in tomato and soybean grown in the greenhouse and the field, in soils previously cropped with different transition strategies

2.4 Comparison of the proportion of negative correlations between percent pre-emergence damping-off and the relative abundance of individual 16S rDNA terminal restriction fragments associated with transition strategies exhibiting different damping-off incidence levels across multiple experiments

2.5 Prevalence of negative correlations between percent pre-emergence damping-off and the relative abundance of individual 16S rDNA terminal restriction fragments associated with transition strategies exhibiting the lowest damping-off incidence

2.6 Bacterial genera, based on in silico analysis, predicted to generate a terminal restriction fragment corresponding to the size of the TRF associated with damping-off suppression

3.1 Fungal and oomycete ITS terminal restriction fragments with high factor loadings on the first three principal components for each experimental context

3.2 Average richness of fungal and oomycete ITS terminal restriction fragments obtained from samples originating of tomato and soybean grown in soils previously exposed to different transition strategies

3.3 Average diversity of fungal and oomycete ITS terminal restriction fragments obtained from samples originating of tomato and soybean grown in soils previously exposed to different transition strategies

3.4 Relative abundance of a selected subset of fungal and oomycete ITS terminal restriction fragments showing significant differences between transition strategies
3.5 Proportion of positive and negative correlations between percent damping-off and the relative abundance of individual fungal/oomycete ITS terminal restriction fragments associated with transition strategies exhibiting high disease incidence in tomato seedlings 116

3.6 Proportion of positive and negative correlations between percent damping-off and the relative abundance of individual fungal/oomycete ITS terminal restriction fragments associated with transition strategies exhibiting high disease incidence in soybean seedlings 117

3.7 Proportion of positive and negative correlations between total 16S T-RFLP fluorescence signal and total ITS T-RFLP fluorescence signal 118

3.8 Identity of fungal and/or oomycete isolates that generate a terminal restriction fragment corresponding in size to the TRF associated with disease incidence in tomato and soybean 119

4.1 Classification of 16S rDNA clones that match the size of targeted MspI-generated terminal restriction fragments 154

4.2 Classification of 16S rDNA clones, representing extended sequences of M139 and M141 TRF 155

4.3 Lesion severity in soybean seedlings treated with Mitsuaria isolates and challenged with Pythium aphanidermatum, Rhizoctonia solani and Phytophthora sojae 156

4.4 Lesion severity in tomato seedlings treated with Mitsuaria isolates and challenged with Pythium aphanidermatum and Rhizoctonia solani 157

4.5 Lesion severity in soybean seedlings treated with Burkholderia isolates and challenged with Rhizoctonia solani 158

4.6 Lesion severity in soybean seedlings treated with Burkholderia isolates and challenged with Rhizoctonia solani 159
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Damping-off of soybean seedlings grown in the greenhouse in soils previously subjected to different organic transition strategies</td>
<td>37</td>
</tr>
<tr>
<td>1.2</td>
<td>Damping-off of soybean seedlings grown in field plots previously subjected to different organic transition strategies</td>
<td>38</td>
</tr>
<tr>
<td>1.3</td>
<td>Damping-off of tomato seedlings grown in field plots previously subjected to different organic transition strategies</td>
<td>39</td>
</tr>
<tr>
<td>1.4</td>
<td>Step-wise approach used in this study for the identification of microbial populations associated with disease suppression and disease in an organic transition field experiment</td>
<td>40</td>
</tr>
<tr>
<td>2.1</td>
<td>Bacterial community profiles obtained from the rhizosphere of tomatoes grown in soils previously exposed to different transition strategies</td>
<td>76</td>
</tr>
<tr>
<td>2.2</td>
<td>Bacterial community profiles obtained from the rhizosphere of soybeans grown in soils previously exposed to different transition strategies</td>
<td>77</td>
</tr>
<tr>
<td>2.3</td>
<td>Bacterial community profiles obtained from soils were tomatoes grown in soils previously exposed to different transition strategies</td>
<td>78</td>
</tr>
<tr>
<td>2.4</td>
<td>Bacterial community profiles obtained from soils were soybeans grown in soils previously exposed to different transition strategies</td>
<td>79</td>
</tr>
<tr>
<td>3.1</td>
<td>Separation of ITS-based community profiles based on variation in damping-off incidence levels of soils where tomatoes were grown</td>
<td>120</td>
</tr>
<tr>
<td>3.2</td>
<td>Separation of ITS-based community profiles from the rhizosphere of tomato seedlings based on variation in damping-off incidence levels of soils where tomatoes were grown</td>
<td>121</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>Separation of ITS-based community profiles based on variation in damping-off incidence levels of soils where soybeans were grown</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>Separation of ITS-based community profiles from the rhizosphere of soybean seedlings based on variation in damping-off incidence levels of soils where tomatoes were grown</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>Mean total fluorescence signal of ITS terminal restriction fragments obtained from tomato samples grown in soils previously exposed to different transition strategies</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>Mean total fluorescence signal of ITS terminal restriction fragments obtained from soybean samples grown in soils previously exposed to different transition strategies</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>DNA-sequence alignment showing the position and variation of the first variable region of the 16S rRNA of representative species within the order Burkholderiales and clones generated in this study</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>Sequence alignments showing overlap region between TRF clones and extended TRF sequence clones</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>Classification of M139-associated isolates as <em>Mitsuaria</em> sp. based on 16S rDNA sequence analyses</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>Classification of <em>Mitsuaria</em> isolates identified in this study in relation to the other known members of the genus</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>Classification of M141-associated isolates as a novel <em>Burkholderia</em> sp. based on 16S rDNA sequence analyses</td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>Frequency of positive <em>in-vitro</em> inhibition activity of <em>Mitsuaria</em> and <em>Burkholderia</em> isolates identified in this study against multiple fungal and oomycete tomato and soybean pathogens</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Chitinolytic activity of <em>Mitsuaria</em> isolates</td>
<td></td>
</tr>
</tbody>
</table>
5.1 Step-wise approach followed in this dissertation for the understanding of the of microbial populations associated with disease suppression and disease in an organic transition field experiment
CHAPTER 1

SETTING UP THE STAGE: IDENTIFICATION OF A DISEASE SUPPRESSION DIFFERENTIAL

INTRODUCTION

Development of more sustainable agricultural management practices is becoming more important and recognized. Factors contributing to their wider implementation include high energy costs involved in conventional agriculture and environmental concerns associated with the use of synthetic chemicals and to soil and water management (Cook, 2006). Sustainable agriculture relies on farm management practices that promote agroecosystem resilience, minimize environmental impact and improve profitability (MacRae et al., 1990). Favorable soil characteristics in a sustainable agricultural context involve fertility and yield promotion, as well as the maintenance of plant health and the support of beneficial microbial populations that contribute to plant disease suppression (Cook, 2006; Janvier et al., 2007).

Understanding the roles of indigenous microbial populations in the control or suppression of plant diseases is necessary for the development and application of adequate field management strategies (Mazzola, 2004). Pathogen survival and growth is often limited due to biological parameters of soils, which can be affected by the farm
management of choice. Observations that different soil types and managements have an effect on plant performance and disease development date back to ancient agricultural-based settlements (Cook and Baker, 1983). The term suppressiveness was first used to describe reduction in disease development in 1931, the same year in which the first demonstration of microbial-based pathogen suppression in soil was presented (Cook and Baker, 1983). In 1963 Park discussed the ecology of soil-borne diseases, including the interactions among resident microbial populations in soil and pathogen populations. Baker and Cook (1974) defined disease suppressive soils as soils in which in spite of pathogen presence pathogens fail to establish or cause disease, or if disease develops it declines over time. Cook and Baker (1983) further reviewed characteristics of suppressive soils, as summarized below. Soils can naturally be suppressive or conducive for plant disease development. In a broad sense, two types of biologically-based disease suppression have been described: general suppression, which results as an effect of the microbial community as a whole, presumably through more than one mode of action; and specific suppression which relates to the activity of one or a few microbial populations, against plant pathogens, most likely through a direct interaction (e.g. antagonism or parasitism). However, soilborne disease suppression probably involves a combination of both.

Soil and rhizosphere microbial community structure and diversity can be affected by soil type, plant species and/or land management or use (Garbeva et al., 2004a). Agricultural management practices have been shown to significantly affect microbial populations in soils. For instance, studies on tillage (Feng et al., 2003), rotation (Larkin, 2003; Lupwayi et al., 1999), cover crops (Schutter et al., 2001; Schutter and Dick, 2002),
organic amendments and mulch (Parham et al., 2003; Perez-Piqueres et al., 2006; Rotenberg et al., 2007a; Tiquia et al., 2002), fertilizers (Murray et al., 2006), crop species (Berg et al., 2002) have shown changes in microbial communities as a whole, in response to management. Among the soil characteristics affected by management and commonly associated to changes in microbial communities are organic matter in soil, soil fraction, texture class, cation exchange capacity and pH (Blackwood and Paul, 2003; Rotenberg et al., 2007b; Schutter et al., 2001). Contrasting work, however, has also been presented by Hiddink et al. (2005) and Girvan et al. (2003), who observed slight or no differences in microbial communities in response to crops (plant type) and cropping systems (management), whereas most of the differential was associated with soil type (site). Cavigelli et al. (2005) also described effects of soil type on multiple microbial properties (including activity and community measures), however, specific soil and microbial properties were associated differently depending on how soil types were defined which again indicates the complexity of these associations.

Multiple farming practices have shown to promote disease suppression. These include the use of organic amendments, rotation sequences (Larkin and Honeycutt, 2006), mixed cropping systems (Seigies and Pritts, 2006; Vilich, 1993) and, in some instances, monoculture (Weller et al., 2002). Disease suppression promotion most likely occurs as a result of changes in the structure of microbial populations, particularly of those that act to suppress plant pathogens and promote plant growth. The mechanisms of suppression, as well as the context in which plant beneficial microbial populations are induced has been a major focus of study. For instance, Hoitink and Fahy (1986) present several examples of compost-induced disease suppression and its relationships with physical, chemical and
biological factors. Multiple microbial populations have been associated with the suppression of soilborne pathogens such as *Rhizoctonia* and *Pythium* damping-off by bark-based (Boehm et al., 1993; Kuter et al., 1983; Nelson and Hoitink, 1983; Tunlid et al., 1989) and other types of compost. However, the structure and activity of compost-associated disease suppressive communities is dependent on the type of organic matter used during composting, as well as the decomposition level (Boehm et al., 1993; Hoitink and Boehm, 1999). Recent research continues to explore the use of compost from different sources, as well as other organic amendments, for the use of suppressiveness in multiple pathosystems (Bulluck and Ristaino, 2002; Scheuerell et al., 2005; Termorshuizen et al., 2006; Rotenberg et al., 2007a). Although results tend to be variable there is a trend towards suppressiveness or no effect on pathogen, rather than increased disease in response to amendment (Termorshuizen et al., 2006).

A number of studies have described farm management effects on populations of specific beneficial microbial groups. For instance, wheat monoculture is known to result in an increase in the abundance of antibiotic (2,4-diacetylphloroglucinol, DAPG) producing *Pseudomonas*, concomitant with the reduction of take-all disease severity (Weller et al., 2002). More recently, DAPG-producing *Pseudomonas* populations have shown to respond in a predictable manner to rotation sequence, tillage, compost amendments and seed treatments (Rotenberg et al., 2007b). Similarly, the diversity of *Pseudomonas*, *Burkholderia* and *Bacillus* species, and subset populations with inhibitory capacity against *Rhizoctonia solani*, differed between soils exposed to different land management practices in the Netherlands (Garbeva et al., 2004b; Salles et al., 2004; Garbeva et al., 2006; Salles et al., 2006a; Salles et al., 2006b). In the latter studies, the
grassland system harbored the greatest proportion of beneficial populations, than the arable land treatments.

Microorganisms associated with disease suppression could be used for the development of disease management strategies, either directly through the application of microorganisms or their products, or indirectly through cropping systems that favor their populations (Mazzola, 2004; Borneman and Becker, 2007). Borneman et al (2007) described a population-based approach for the identification of microorganisms involved in a specific function in situ. This is a multi-stepped approach that involves first the correlation between a known function and members of the microbial community (i.e. microbial community fingerprint profile). This is achieved through comparative microbial community analyses of multiple levels of a functional soil parameter (e.g. disease suppression differential). The following step involves a quantitative validation of the correlations, which is generally achieved through a method targeted to the population of interest (e.g. quantitative real-time PCR). The final step involves the isolation of the microorganisms of interest and the confirmation of function. Through the use of this experimental design, the fungus *Dactylella oviparasitica* was identified as a cyst-resident antagonist of the beet cyst nematode in California (Olatinwo et al. 2006). This approach also provides a baseline that could be used for determining associations between microbial parameters with soil physico-chemical characteristics and soil management effects. As described in Janvier et al. (2007), it is necessary to move ahead from descriptive observations of plant disease suppressiveness towards the quantitative association between the multiple factors contributing to this function. In addition, the use of microbial community profiling methodologies, as a screening strategy, can provide a
broader scope of microbial populations analyzed (Weller et al., 2002). Hence contributing not only to the discovery of novel microorganisms associated with plant disease suppression, but also providing information on potential biological control agents.

IDENTIFICATION OF A DISEASE SUPPRESSIVE DIFFERENTIAL:
TRANSITIONAL ORGANIC CROPPING SYSTEM EFFECTS ON SOYBEAN AND TOMATO SEEDLING DAMPING-OFF AND VIGOR

Environmental, health and economic concerns have driven rapid growth in organic farming systems around the world. The underlying philosophy of organic farming refers to the “farm as an organism, in which all the component parts interact to create a coherent whole” (Stockdale et al., 2001, pg. 264). This involves the restructuring of the whole farm system to maintain organic matter and soil and nutrient cycling to achieve production of high quality food and fiber. In addition it aims at maintaining the quality of life for the farmer and workers and minimize environmental impact (Stockdale et al., 2001). The National Standards on Organic Agriculture established the required standards for organic production and certification, which includes the prohibition of application of certain substances in land destined for organic farming during at least three years. During these three years of transitions, farmers can choose to manage their soils differently, but often reduction of yield is observed due to pathogen and pest pressure and a reduction of fertilizer input during this period (Liebhardt et al., 1989; Tu et al., 2006). Management strategies during this period not only have an effect in the rate of nutrient and soil structure build up, but also in microbial biomass and activity in soil (Tu et al., 2006).
An organic transition field experiment was established at the Ohio Agricultural Research and Development Center (OARDC) in 2003. This experiment aimed to address economic, environmental and biological impacts of management strategies during the three-year transition period from conventional to organic farming (Paths of Transition: Strategies for Peri-Urban Organic Farmers; USDA CSREES Integrated Res., Ext, and Ed., Organic Transitions Grants Program). The experiment was set up as a split-plot design, with four main transition strategies: tilled fallow (TF), perennial mixture of hay species (H), open field vegetable production (FV) and intensive vegetable production under high tunnels (HTV). These transition strategies represent a range from low-intensity (e.g. TF) to high-intensity (e.g. HTV) management. Split-plots within the main transition strategies were arranged to address the effects of composted dairy manure amendments (18.6 t ha\(^{-1}\) application rate). In 2006 the field plot was planted all in tomato and was certified as organic by The Ohio Ecological Food & Farm Association (OEFFA) according to the National Standards on Organic Agriculture.

Within this context, tomato and soybean damping-off incidence and seedling vigor were studied. We hypothesized that transition strategies contributing to greater organic matter build-up, as well as maintaining greater plant diversity over time (i.e. mixed hay system) will result in greater plant vigor and lowest damping-off incidence of subsequent crops. Furthermore we hypothesized that transition strategies with these characteristics will favor greater microbial diversity and activity resulting in the development of general soilborne disease suppression (expressed as lowest damping-off incidence). Results regarding transition strategy effects on soil chemistry, seedling vigor and damping-off incidence, have been in most part published in Baysal et al (2008;
Benitez, M-S. as a second author). These data are discussed in the following sections, within the context of identifying soils differing in disease suppression and the identification of microbial components associated to this suppression.

TRANSITION STRATEGY AND COMPOST AMENDMENT EFFECTS ON SOIL CHEMISTRY

Previous studies have described the effects of organic transition strategies in crop yields, soil properties and fertility, and microbial biomass and activity (Delate and Cambardella, 2004). Since organic farming properties involve, among others, the build up and maintenance of organic matter in soil (Stockdale et al., 2001), transition strategies positively contributing to this build-up could represent a start-up for a newly established organic farm. In addition, organic matter and nutrient content will influence the structure and activity of microbial communities in the soil (Weller et al., 2002). To determine the effects of transition strategy on soil chemistry, soil analyses were performed from soil samples collected from the Peri-Urban field experiment at three different time points: Spring and Fall of 2005 and Summer of 2006. In addition soils collected in the Spring and Fall of 2005 were utilized for tomato and soybean damping-off suppression bioassays performed in the greenhouse (see below).

In the Spring and Fall of 2005 four soil samples were taken from each sub-plot, with a 900 cm² x 10 cm deep soil cover, mixed and placed in a plastic bucket. The soils were stored in the greenhouse for up to four months prior to use. In the Summer of 2006 two soil samples were taken from each subplot and analyzed independently. Soil analyzes were performed at the Service Testing and Research (STAR) laboratory (OARDC,
Wooster, OH). Soil manipulation and methods used for analysis are described in Baysal et al. (2008). Soil data were analyzed as a split-plot design, with transition strategies as the main treatment and compost amendment as the secondary treatment. Pair-wise comparisons were performed using Tukey-Kramer HSD within amended or un-amended sub-plots (Tables 1.1 and 1.2). Thorough analysis and discussion of Fall of 2005 data is presented in Baysal et al. (2008).

Transition strategy effects (Tables 1.1 and 1.2) were observed for all the measured variables, except for Fe and Mn, and patterns of effects were consistent among the three sampling-dates. The highest pH was observed within the high tunnels, and the mixed hay resulted in the greatest %N and %C (Table 1.1). In terms of extractable nutrients, soils from the high tunnels had a tendency of higher values overall (Table 1.2). Compost x transition strategy interactions were observed, in which differences in nutrient content were evident only when comparing within amended subplots (e.g. P and K highest in the high tunnels in Fall 05; Table 1.2). Comparisons among amended and un-amended counterparts revealed also the overall effect of compost in increasing nutrient content (except for Fe and Mn). The treatment x time interaction was assessed following the repeated measures procedure, using the general lineal model. This comparison indicated a significant interaction for all of the soil parameters studied (Tables 1.1 and 1.2), except pH, Ca and Mn. Fall 05 data had the highest means overall and showed the greatest differential (more significant observations compared to SP-05 and SU-06). Overall the greatest effects in soil chemistry were observed in response to compost application. For some of the measured parameters, however, transition strategy effects were also observed. It is interesting to note the contribution of the mixed hay cropping system to
%N and %C regardless of compost application (Table 1.1). In addition, the soils from the high tunnels showed the greatest differential in pH and nutrient content when compared to the other transition strategies.

TRANSITION STRATEGY AND COMPOST AMENDMENT EFFECTS ON SOYBEAN AND TOMATO SEEDLING VIGOR AND DAMPING-OFF

Management effects on tomato and soybean damping-off and seedling vigor were assessed in multiple assays in the greenhouse and the field. For the greenhouse bioassays, soils were collected from the field in Spring (experiments 1 and 2) and Fall 2005 (experiment 3) and field bioassays were performed in the Summer of 2006. In the greenhouse 20 tomato (cv. Tiny Tim) and six soybean (cv. Sayamusume) seeds per-pot were planted in soils previously exposed to different transition strategies, with or without added disease pressure (i.e. soilborne pathogen inocula). Damping-off incidence and plant vigor measurements were recorded at approximately 21 days for tomato (four true leaves) and 17 days for soybean (vegetative 1 stage for soybean, V1). For the field experiments, 24 tomato and 16 soybean seeds were planted in two separate 45 x 45 cm areas per subplot, and seedlings were sampled at equivalent stages. Seedling vigor and damping-off data from the greenhouse bioassays were analyzed as a randomized complete block design with soil transition treatments as the main factors. Field bioassays were analyzed as a split plot design with the primary treatments being transition strategy and the secondary treatment compost amendment. More detailed description of the methodology is found in Baysal et al. (2008).
In the greenhouse, soybean seedling vigor differed in response to transition strategy and compost amendment (Table 1.3), with the differences being more evident for experiment 2 with added inocula. For the un-amended plots significant effects were observed only for experiment 2, with the mixed hay, followed by the high tunnels, resulting in the greatest fresh weight. Similarly, more plants grown in the mixed hay reached V1 stage, when compared with the other treatments. Compost amendment resulted in overall increase of plant vigor, compared to the un-amended counterparts, except for the field vegetable system. Within amended strategies, the field vegetables resulted in significantly lower shoot weight, compared to the other three strategies, whereas the mixed hay had significantly higher shoot weight and number of plants that reached V1 in experiments 2 and 3 (with pathogen inocula). Consistent with our observations for soybean, compost amendment resulted in overall increase of tomato vigor, and between transition strategies the high tunnel and the mixed hay treatments showed a tendency of the greatest tomato vigor. Tomato seedling vigor data are presented in more detail in Baysal et al. (2008).

In the greenhouse, effects of transition strategy and compost amendment in damping-off incidence were observed. For soybean, the mixed hay transition strategy resulted in the lowest damping-off incidence in all three experiments (Figure 1.1). Significant reduction, however, was observed within the amended and un-amended strategies in Experiment 2 and the amended strategies in Experiment 3 (Figure 1.1). Baysal et al. (2008) described a similar trend observed on the tomato experiments, with the mixed hay soils showing lowest damping-off incidence yet significance was observed only in Experiment 3.
Transition strategy effects on tomato and soybean seedling vigor and damping-off incidence were observed in the field and these results were consistent with the greenhouse observations (Tables 1.4 and 1.5). Soybean fresh weight was significantly higher in the mixed hay compared to the field vegetables, within the amended sub-plots. Similarly the number of plants that reached V1 stage was significantly higher in the amended mixed hay than the other amended strategies. While non-significant, the same trends were observed for the un-amended transition strategies, and in Experiment 2 for fresh weight only. Tomato seedling vigor did not show significant responses to amended or un-amended transitions strategies (Table 1.5). Transition strategy significantly affected damping-off incidence of both crops, with the amended mixed hay resulting in the lowest damping-off incidence in soy (Figure 1.2) and the un-amended mixed hay in tomato (Figure 1.3). In addition, lowest damping-off in the mixed hay soils (regardless of amendment) was observed two out of three times for the non-significant scenarios. Data were modified for presentation purposes from Baysal et al. (2008).

Overall, the mixed hay transition strategy, regardless of compost amendment, resulted in the lowest damping-off incidence across crops and experiments. Significant effects were observed in five out of the eight experiments, with other two experiments following the same, but non-significant, pattern. In addition, soybean seedlings grown in mixed hay soils had greatest plant vigor (measured as fresh weight and the number of plants that reached V1 stage), especially under conditions of high disease pressure (greenhouse experiments 2 and 3 and field experiments). These data indicates that the mixed hay transition strategy can enhance the suppression of damping-off of subsequent crops and provide nutrients that promote soybean vigor.
TRANSITION STRATEGY EFFECTS ON THE ABUNDANCE AND INCIDENCE OF
DAPG-PRODUCING *PSEUDOMONAS* COLONIZING THE RHIZOSPHERE OF
TOMATO AND SOYBEAN

The ability of multiple *Pseudomonas* species to suppress plant diseases and
promote plant growth is well recognized (Haas and Defago, 2005; McSpadden Gardener,
2007; Weller, 2007). The mechanisms through which *Pseudomonas* species induce
disease suppression and plant growth promotion include the production of antibiotics,
siderophores and/or mimics of plant growth regulators, as well as the induction of
systemic resistance in plants (Haas and Defago, 2005). Antibiotic producing
*Pseudomonas* strains have been widely studied since high abundance was reported in
soils suppressive to wheat and tobacco root diseases, and seed treatments resulted in
disease suppression (Cook and Rovira, 1976; Weller and Cook, 1983; Stutz et al., 1986;
lipopeptides are the most common antibiotics produced by disease suppressive
*Pseudomonas* populations (Haas and Defago, 2005). DAPG-producing *Pseudomonas*
strains are known for their ability to colonize a variety of crop species including tomato
and soybean (Sharifi-Tehrani et al., 1998; McSpadden Gardener et al., 2005), and to
inhibit growth of a variety of soilborne fungal, oomycete, and nematode pathogens
(McSpadden Gardener et al., 2005; Siddiqui et al., 2005; Siddiqui and Shaukat, 2003;
Weller et al., 2002). The induction of host resistance by a DAPG-producing strain was
reported for the *Arabidopsis thaliana-Peronospora parasitica* pathosystem (Iavicoli et
al., 2003). In addition, seed treatments with these bacteria increase stand and yield of
various field crops, including corn, soybean, and wheat (Cook et al., 2002; McSpadden
Gardener et al., 2005; McSpadden Gardener, 2007). Rotenberg et al. (2007b) described management effects (tillage, rotation, seed treatments and amendments) on DAPG-producing *Pseudomonas* population abundance. In addition, relationships between soil characteristics (e.g. pH), crop vigor and DAPG-producing *Pseudomonas* populations were observed, with variations related to context. The DAPG-producing *Pseudomonas* populations associate with disease suppression in multiple systems and their abundance in the rhizosphere responds to management. Therefore, we hypothesize that DAPG-producing *Pseudomonas* are more abundant in the rhizosphere of tomato and soybeans grown in soils from the mixed hay cropping system (i.e. transitions strategy that favor plant vigor and reduce damping-off incidence, see above and Baysal et al. 2008).

To determine the abundance and incidence of total and DAPG-producing *Pseudomonas* the rhizosphere of soybean and tomato plants, grown in soils previously exposed to different transition strategies, were sampled in multiple experiments. For soybean, rhizosphere samples were collected only for greenhouse experiment two and for field experiment one. For tomato, rhizosphere colonizing *Pseudomonas* were sampled in all greenhouse and field experiments. The rhizosphere of one plant (roots plus remaining soil after shaking) was sampled individually, for n=6 plants per treatment in the greenhouse and n=8 plants per treatment in the field, when available. Enumeration of total *Pseudomonas* and DAPG-producing *Pseudomonas* was done according to McSpadden Gardener et al. (2001). Briefly, bacteria were dislodged from roots and the rhizosphere wash was serially diluted in 1/3 Kings Medium B. Culture plates were incubated for 48 hours at room temperature, and bacterial growth was assayed spectrophotometrically. Cultures were frozen prior to use as template for whole cell PCR
for the amplification of the *phlD* gene. Amplification was performed using B2BF and BPR4 primers according to McSpadden Gardener et al. (2001). The terminal dilution of bacterial growth and the terminal dilution at which positive amplification of the *phlD* gene was observed were used to estimate colony-forming units of bacteria per gram of rhizosphere sample. Abundance data were analyzed following the non-parametric Kruskall-Wallis test to determine transition strategy and/or compost effects on the colonization of tomato and soybean roots by *Pseudomonas* and DAPG-producing *Pseudomonas*. Individual pair-wise comparisons were performed using the Wilcoxon 2-sample test. For this and further microbial analysis, only the open-field transition strategies were considered to avoid confounding with expected differences with the high tunnel system, based on greatest environmental variation (e.g. see soil data above, and differences in field and high tunnel temperatures shown in Briar, 2007). In addition, field plot logistics did not permit for the suppressiveness assays to be run inside the high tunnels in summer 2006.

Compost amendment had an effect on the abundance of rhizosphere colonizing *Pseudomonas* (Table 1.6). For all of the experimental contexts, except the soybean field assay, compost amendment resulted in an increase in total *Pseudomonas* populations (*P*<0.02). Significant effects of compost within transition strategies, was observed on tomatoes grown in the field vegetables and tilled fallow soils in the greenhouse, tomatoes grown in the field vegetables soils in the field, and soybeans grown in the mixed hay soils in the greenhouse. Though Table 1.6 shows only the results of experiment 2 for tomato, similar trends were observed in the other experiments. Contrary to these observations Rotenberg et al. (2007b) did not observe compost effects in total *Pseudomonas*
populations colonizing the rhizosphere of sweet corn. Compost effects on the populations of rhizosphere colonizing DAPG-producing *Pseudomonas* were not detectable across most experiments (Table 1.6). Significant effects of compost on DAPG-producing populations were observed only in the tomato field assay, where the abundance of these populations was lower in the un-amended plots of the field vegetable transition. Rotenberg et al. (2007b), however, described a more consistent increase in DAPG-producing *Pseudomonas* populations in the rhizosphere of sweet corn in response to compost. In that work, a greater rate of compost (12 t ha\(^{-1}\) vs 6 t ha\(^{-1}\)) resulted in a greater increase in population abundance. Therefore at a rate of 18 t ha\(^{-1}\) a significant increase in population abundance was expected, but not observed, in this study.

No consistent effects of transition strategy on the abundance and incidence of *Pseudomonas* and of DAPG-producing *Pseudomonas* populations were observed across experiments. In addition, in only one instance was there a significant response, on abundance of DAPG-producing *Pseudomonas* populations, to transition strategy; where the un-amended field vegetable strategy, resulted in greatest colonization of tomato rhizosphere in the field experiment. Though non-significant, within the amended strategies the same pattern is observed for tomatoes grown in the field (with significance observed within pooled amended and un-amended data, \(P<0.05\)). Rotenberg et al. (2007b) described field management effects on DAPG-producing *Pseudomonas* population. In that work significant differences in abundance in response to management within individual experiments were not observed. However, in Rotenberg et al. (2007b) patterns of abundance were reproducible across multiple experiments and years. Compost effects, as well as crop and site (greenhouse or field) seem to be more evident than the
effects of transition strategy on the colonization of tomato and soybean roots by DAPG-producing *Pseudomonas* in this system. Overall, incidence of colonization of DAPG-producing *Pseudomonas*, as well as the proportion of the total pseudomonads population comprised of DAPG-producers was greater in the greenhouse bioassays compared to the field bioassays, and greatest incidence of DAPG-producing *Pseudomonas* colonization was observed in tomato roots, compared to soybeans (Table 1.6). Previous work has shown similar variation in abundance and incidence of DAPG-producing *Pseudomonas* in relation to host, both at the crop and cultivar level (Mazzola et al., 2004; Bergsma-Vlami et al., 2005; McSpadden Gardener et al., 2005). For instance, the incidence of colonization of soybean rhizosphere by DAPG-producing *Pseudomonas* is lower than in corn, although when colonized, soybean rhizosphere harbors greater DAPG-producing *Pseudomonas* abundance (McSpadden Gardener et al., 2005).

Pair-wise correlation analyses were performed to further explore the contributions of total *Pseudomonas* and DAPG-producing *Pseudomonas* population to disease suppression in this system. The relationship between these bacterial populations and damping-off incidence was analyzed using the Spearman correlation coefficient ($\rho$) which was calculated on a per pot or field site basis (Table 1.7). Since a high-incidence of damping-off was observed, especially for soybean bioassays, and to ensure that correlations were performed with $n \geq 5$, data were arranged into three disease incidence levels: high, intermediate and low damping-off according to the results described above (Table 1.8, and modified from Benítez et al., 2007). Strong and significant correlations were observed only under high disease pressure conditions (Table 1.7), indicating either a greater influence of these populations on disease suppression in more conducive
situations or an overall bacterial population response to pathogen invasion. Increases in bacterial population abundance, including *Pseudomonas*, in response to pathogen infection have been described previously for take-all disease in wheat (McSpadden Gardener and Weller, 2001). A positive correlation was observed between the abundance of total *Pseudomonas* population and damping-off incidence for the greenhouse bioassays of tomato and soybean. In contrast, in the field conditions these correlations were negative for both crops. For DAPG-producing *Pseudomonas* a strong negative correlation with damping-off incidence was observed only in the tomato greenhouse bioassay; whereas, on the other contexts the correlations tend to be positive, but not as strong or significant. Negative correlations between crop health and DAPG-producing *Pseudomonas* abundance have been described previously for soybean rhizosphere populations, probably as a result of faster colonization after pathogen infection (Rotenberg et al., 2007b). If the correlation analysis is performed considering the proportion of the *Pseudomonas* populations comprising DAPG-producing *Pseudomonas*, some consistency in the results are observed among experimental contexts. The correlations between the proportion of the population harboring the *phlD*-gene and damping-off incidence under high disease pressure is negative for the greenhouse bioassays and positive for the field bioassays, for both crops. In conditions where DAPG-producing *Pseudomonas* populations represent a greater proportion of the population (i.e. greenhouse) they exhibit a negative correlation with disease (Table 1.7).

The analysis of *Pseudomonas* and DAPG-producing *Pseudomonas* populations in the study system resulted in some observations consistent with previous studies; however no clear association was observed between these bacterial populations and transition
strategy and/or damping-off suppression. Though these populations might contribute to a certain extent to disease suppression, especially in conditions of high disease pressure, those contributions were not detectable through our analysis. It seems likely that crop effects (tomato vs. soybean) and site of experiment (field vs. greenhouse) mask the transition strategy effects in the studied system, indicating once again the context-dependent contributions of microbial populations to plant health.

SUMMARY

The first step for studying the associations between microorganisms and function involves the identification of a measurable difference in phenotype in situ (Borneman et al., 2007), which in this case involves the identification of a disease suppressive system. From the work described in this chapter, a transition strategy (i.e. mixed hay cropping system), resulted in consistent damping-off suppression of tomato and soybean seedlings, as well as greatest soybean seedling vigor. Soil chemical analysis showed an increase in %C and %N in response to the mixed hay transition strategy; though these differences were marginal compared to the effects of compost amendment (Table 1.1). Compost amendment, however, did not consistently result in damping-off suppression in this system (Figures 1.1-1.3). Even though the added organic matter could play a role in damping-off suppression, there must be qualitative differences between the mixed hay and the compost-derived organic matter that contribute to suppression. The type and quality of organic matter has been shown to influence microbial-based compost-induced suppressiveness to soilborne diseases (Hoitink and Boehm, 1999). In addition, characteristics associated with the mixed hay system, such as length of cropping (i.e.
perennial) and diversity (i.e. mixture of eight species), as well as influence on soil aggregate size and structure, which will in turn affect water holding capacity and temperature (parameters not measured in this work) could have contributed to the observed suppressiveness. Soilborne diseases such as those caused by *Pythium* spp. are known to be dependent on soil moisture, temperature and pH, in addition to nutrients such as carbon (Martin and Loper, 1999). Similarly, the severity of *Fusarium* wilt is higher in sandy soils than soils with greater clay content (Cook and Baker, 1983). Therefore, the suppression observed may have resulted, in part, from changes in the physical characteristics of the soil which were not measured in this work.

The putative contributions of a subset of the microbial community (i.e. certain *Pseudomonas* spp.) known to exhibit pathogen suppressive and plant growth promoting activities were presented in this chapter. No consistent associations with suppressiveness were observed; however, based on the data available it is not possible to rule out their contribution to disease suppression. It is possible that the effects of multiple bacterial populations present in the mixed hay soils are additive, hence resulting in a more general mechanism of suppression, in contrast to specific suppressive systems such as in the take all-decline in wheat. In take-all decline, decrease in disease severity and pathogen populations correlate with an increase in populations of antibiotic producing *Pseudomonas* after several cropping cycles (Weller et al. 2002).

The overall objective of this thesis is the identification and description of some of the microbial components involved in disease and disease suppression in a transitional organic field experiment. Figure 1.4 outlines the experimental approach followed in this work, which was designed based on the guidelines described by Borneman et al. (2007).
Step 1, the identification of a disease suppressive differential was presented in this chapter, as a prelude of the following chapters which will focus on analyses of microbial communities associated to this system. Chapter 1 described also the initial assessment of microbial communities, focused on populations of total and DAPG-producing *Pseudomonas*. Chapter 2 and 3 further describe the use of microbial community profiles to determine the association of bacterial (Chapter 2), fungal and oomycete populations (Chapter 3) with the differences in damping-off incidence exhibited by the different transition strategies (Step 2, Figure 1.4). Chapter 4 describes the identification, isolation and confirmation of function (i.e. disease suppression) of bacterial populations previously associated with disease suppression in Chapter 2 (Steps 3 and 4, Figure 1.4). Chapter 5 is presented as an epilogue, and includes concluding remarks for the overall thesis and future research directions to build upon this work.

REFERENCES


Soils were sampled from field plots undergoing the transition from conventional to organic farming and subject to tilled fallowing (TF), mixed hay cropping system (H), field vegetable cropping (FV) and high tunnel vegetable cropping (HTV). Each transition strategy included compost amended and un-amended split plots.

Soils sampled in SP-05 and F-05 were used for greenhouse damping-off suppressiveness bioassay, whereas in SU-06, suppressiveness assays were performed in the field.

Values are the means of: SP-05, 2 replications; F-05, 4 replications; SU-06, 8 replications.

Compost within transition strategy interaction as assessed using a split-plot design within the general linear model, \( P \) values shown.

Means followed by a different letter are significantly different at \( P < 0.05 \) (Tukey-Kramer HSD). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only. For SP-05 no statistics presented due to low replication.

Time within treatment interaction was assessed using a repeated measures design within the general linear model, \( P \) values shown.

Table 1.1. pH, % N, and % C from soils exposed to four different transition strategies, with and without compost amendment, at three different sampling dates

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Compost</th>
<th>Transition strategy</th>
<th>pH</th>
<th>% N</th>
<th>% C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-05</td>
<td>Un-amended</td>
<td>TF</td>
<td>6.1</td>
<td>0.11</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6.4</td>
<td>0.13</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>6.5</td>
<td>0.12</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT</td>
<td>7.1</td>
<td>0.12</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Amended</td>
<td>TF</td>
<td>6.6</td>
<td>0.16</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6.7</td>
<td>0.23</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>6.9</td>
<td>0.19</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HT</td>
<td>7.3</td>
<td>0.29</td>
<td>3.3</td>
</tr>
</tbody>
</table>

| F-05          | Un-amended  | TF                  | 6.3    | 0.10| B   |
|               |             | H                   | 6.5    | 0.14| A   |
|               |             | FV                  | 6.8    | 0.10| B   |
|               |             | HT                  | 7.4    | 0.11| B   |
|               | Amended     | TF                  | 6.8    | 0.2 | b   |
|               |             | H                   | 6.8    | 0.4 | a   |
|               |             | FV                  | 7.1    | 0.2 | b   |
|               |             | HT                  | 7.5    | 0.4 | a   |

| SU-06         | Un-amended  | TF                  | 6.4    | 0.12| B   |
|               |             | H                   | 6.4    | 0.14| A   |
|               |             | FV                  | 6.9    | 0.13| B   |
|               | Amended     | TF                  | 6.8    | 0.17| 1.8 |
|               |             | H                   | 6.8    | 0.19| 2.1 |
|               |             | FV                  | 7.3    | 0.18| 1.8 |

| Trans x compost | <0.001 | <0.001 | <0.001 |
| Time x treatment | > 0.2  | <0.001 | <0.001 |
Soils were sampled from field plots undergoing the transition from conventional to organic farming and subject to tilled fallowing (TF), mixed hay cropping system (H), field vegetable cropping (FV) and high tunnel vegetable cropping (HTV). Each transition strategy included compost amended and un-amended split plots.

Soils sampled in SP-05 and F-05 were used for greenhouse damping-off suppressiveness bioassay, whereas in SU-06, suppressiveness assays were performed in the field.

Values are the means of: SP-05, 2 replications; F-05, 4 replications; SU-06, 8 replications.

Compost within transition strategy interaction as assessed using a split-plot design within the general linear model, P values shown.

Means followed by a different letter are significantly different at P < 0.05 (Tukey-Kramer HSD). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only. For SP-05 no statistics presented due to low replication.

Time within treatment interaction was assessed using a repeated measures design within the general linear model. P values shown.

Table 1.2. Extractable nutrient status from soils exposed to four different transition strategies, with and without compost amendment, at three different sampling dates

<table>
<thead>
<tr>
<th>Date</th>
<th>Compost</th>
<th>Transition strategy</th>
<th>P (μg g⁻¹)</th>
<th>K (μg g⁻¹)</th>
<th>Ca (μg g⁻¹)</th>
<th>Mg (μg g⁻¹)</th>
<th>Fe (μg g⁻¹)</th>
<th>Mn (μg g⁻¹)</th>
<th>Zn (μg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP05</td>
<td>Un-amended</td>
<td>TF</td>
<td>42²</td>
<td>88</td>
<td>998</td>
<td>191</td>
<td>200</td>
<td>185</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>38</td>
<td>81</td>
<td>1243</td>
<td>188</td>
<td>195</td>
<td>208</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>40</td>
<td>93</td>
<td>1175</td>
<td>220</td>
<td>193</td>
<td>215</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT</td>
<td>47</td>
<td>84</td>
<td>1539</td>
<td>290</td>
<td>197</td>
<td>239</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Amended</td>
<td>TF</td>
<td>91</td>
<td>219</td>
<td>1374</td>
<td>249</td>
<td>206</td>
<td>196</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>127</td>
<td>327</td>
<td>1850</td>
<td>332</td>
<td>199</td>
<td>191</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>121</td>
<td>282</td>
<td>1575</td>
<td>308</td>
<td>200</td>
<td>202</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT</td>
<td>222</td>
<td>1707</td>
<td>2328</td>
<td>589</td>
<td>181</td>
<td>198</td>
<td>14.9</td>
<td></td>
</tr>
<tr>
<td>Trans x compost⁴</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.68</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>F05</td>
<td>Un-amended</td>
<td>TF</td>
<td>40</td>
<td>108</td>
<td>947</td>
<td>221</td>
<td>158</td>
<td>152</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>34</td>
<td>103</td>
<td>1112</td>
<td>226</td>
<td>160</td>
<td>158</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>37</td>
<td>101</td>
<td>1119</td>
<td>250</td>
<td>153</td>
<td>172</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT</td>
<td>48</td>
<td>87</td>
<td>1523</td>
<td>338</td>
<td>165</td>
<td>192</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Amended</td>
<td>TF</td>
<td>118 b²</td>
<td>638 b</td>
<td>1493 c</td>
<td>370 c</td>
<td>172</td>
<td>155</td>
<td>8.0 c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>143 b</td>
<td>859 ab</td>
<td>2093 ab</td>
<td>521 b</td>
<td>175</td>
<td>149</td>
<td>15.1 a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>140 b</td>
<td>741 b</td>
<td>1633 bc</td>
<td>432 bc</td>
<td>168</td>
<td>169</td>
<td>9.2 bc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT</td>
<td>255 a</td>
<td>1265 a</td>
<td>2401 a</td>
<td>700 a</td>
<td>167</td>
<td>158</td>
<td>13.9 ab</td>
<td></td>
</tr>
<tr>
<td>Trans x compost⁴</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.34</td>
<td>0.79</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>SU06</td>
<td>Un-amended</td>
<td>TF</td>
<td>47 A</td>
<td>214</td>
<td>976</td>
<td>213 B</td>
<td>179</td>
<td>179</td>
<td>43.4</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>32 B</td>
<td>164</td>
<td>1047</td>
<td>217 AB</td>
<td>184</td>
<td>164</td>
<td>43.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>37 B</td>
<td>186</td>
<td>1106</td>
<td>254 A</td>
<td>188</td>
<td>184</td>
<td>51.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT</td>
<td>76 A</td>
<td>281</td>
<td>1705</td>
<td>276 ab</td>
<td>203</td>
<td>170</td>
<td>51.2</td>
<td></td>
</tr>
<tr>
<td>Amended</td>
<td>TF</td>
<td>75 A</td>
<td>352</td>
<td>1439</td>
<td>268 b</td>
<td>197</td>
<td>189</td>
<td>45.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>76 A</td>
<td>281</td>
<td>1705</td>
<td>276 ab</td>
<td>203</td>
<td>170</td>
<td>51.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>91 A</td>
<td>421</td>
<td>1470</td>
<td>337 a</td>
<td>207</td>
<td>222</td>
<td>45.9</td>
<td></td>
</tr>
<tr>
<td>Trans x compost⁴</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.007</td>
<td>&lt;0.001</td>
<td>0.95</td>
<td>0.44</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Time x treatment⁴ |  |  | <0.001 | <0.001 | > 0.2 | <0.001 | <0.001 | > 0.2 | <0.001 |

Table 1.2. Extractable nutrient status from soils exposed to four different transition strategies, with and without compost amendment, at three different sampling dates.
Soils were sampled from field plots undergoing the transition from conventional to organic farming and subject to tilled fallowing (TF), mixed hay cropping system (H), field vegetable cropping (FV) and high tunnel vegetable cropping (HTV). Each transition strategy included compost amended and un-amended split plots.

Experiment 1, non-inoculated; Experiment 2 and 3 inoculated with *P. sojae* and *P. ultimum*. Soil was inoculated with 10 agar plugs per pathogen, as described in Baysal et al. (2008).

Values are the means of 12 replications (3 blocks/4 replicates per block).

Means followed by a different letter are significantly different at $P < 0.05$ (Tukey-Kramer HSD). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only.

Table 1.3. Vigor of soybean seedlings grown in the greenhouse in soils previously exposed to different field management strategies

<table>
<thead>
<tr>
<th>Compost strategy</th>
<th>Experiment 1 $^b$</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amended</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>2.40 $^{a,b}$ A $^d$</td>
<td>10.0 A A</td>
<td>0.3 B a 0.2 b 0.25 ab 0.2 b</td>
</tr>
<tr>
<td>H</td>
<td>2.38 $^{a,b}$ ab</td>
<td>0.76 B a 0.3 b</td>
<td>0.25 ab 0.2 b</td>
</tr>
<tr>
<td>FV</td>
<td>2.22 $^b$ ab</td>
<td>0.67 b a 0.2 b</td>
<td>0.00 b 0.0 b</td>
</tr>
<tr>
<td>HTV</td>
<td>2.55 $^b$ a</td>
<td>1.11 b a 0.4 b</td>
<td>0.17 b 0.1 b</td>
</tr>
<tr>
<td>Un-amended</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>2.20 $^a$ A $^d$</td>
<td>0.47 C 0.2 B 0.05 A 0.0 A</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>2.25 A 1.8 A 2.8 A</td>
<td>0.25 A 0.2 A</td>
<td></td>
</tr>
<tr>
<td>FV</td>
<td>2.32 A 1.3 A 1.3 A</td>
<td>0.25 A 0.2 A</td>
<td></td>
</tr>
<tr>
<td>HTV</td>
<td>2.05 A 1.5 A 1.5 A</td>
<td>0.00 A 0.0 A</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Soils were sampled from field plots undergoing the transition from conventional to organic farming and subject to tilled fallowing (TF), mixed hay cropping system (H), field vegetable cropping (FV) and high tunnel vegetable cropping (HTV). Each transition strategy included compost amended and un-amended split plots.

$^b$ Experiment 1, non-inoculated; Experiment 2 and 3 inoculated with *P. sojae* and *P. ultimum*. Soil was inoculated with 10 agar plugs per pathogen, as described in Baysal et al. (2008).

$^c$ Values are the means of 12 replications (3 blocks/4 replicates per block).

$^d$ Means followed by a different letter are significantly different at $P < 0.05$ (Tukey-Kramer HSD). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only.
Field plots considered were subject to tilled fallowing (TF), mixed hay cropping system (H) and field vegetable cropping (FV). Each transition strategy included compost amended and un-amended split plots. Field bioassays were not conducted within the high tunnel vegetable cropping.

Values are the means of 8 replications (4 blocks per 2 replicates per block).

Means followed by a different letter are significantly different at $P < 0.05$ (Tukey-Kramer HSD). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only.

Table 1.4. Vigor of soybean seedlings grown in an organic field after three years of transition under different management strategies

<table>
<thead>
<tr>
<th>Compost</th>
<th>Transition strategy</th>
<th>Experiment 1</th>
<th></th>
<th></th>
<th>Experiment 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fresh weight per plant (g)</td>
<td>Mean No. V plants</td>
<td></td>
<td>Fresh weight per plant (g)</td>
<td>Mean No. V1 plants</td>
<td></td>
</tr>
<tr>
<td>Un-amended</td>
<td>TF</td>
<td>2.0$^b$</td>
<td>A</td>
<td>0.6</td>
<td>A</td>
<td>0.5</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>3.0</td>
<td>A</td>
<td>1.3</td>
<td>A</td>
<td>0.8</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>0.9</td>
<td>A</td>
<td>0.4</td>
<td>A</td>
<td>0.6</td>
<td>A</td>
</tr>
<tr>
<td>Amended</td>
<td>TF</td>
<td>2.3</td>
<td>ab$^c$</td>
<td>0.1</td>
<td>b</td>
<td>0.8</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>4.5</td>
<td>a</td>
<td>2.5</td>
<td>a</td>
<td>1.4</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>0.5</td>
<td>b</td>
<td>0.0</td>
<td>b</td>
<td>0.4</td>
<td>a</td>
</tr>
</tbody>
</table>

$^a$Field plots considered were subject to tilled fallowing (TF), mixed hay cropping system (H) and field vegetable cropping (FV). Each transition strategy included compost amended and un-amended split plots. Field bioassays were not conducted within the high tunnel vegetable cropping.

$^b$Values are the means of 8 replications (4 blocks per 2 replicates per block).

$^c$Means followed by a different letter are significantly different at $P < 0.05$ (Tukey-Kramer HSD). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only.
<table>
<thead>
<tr>
<th>Compost Strategy</th>
<th>Transition Strategy</th>
<th>Plant Height per plant (cm)</th>
<th>Fresh weight per plant (g)</th>
<th>Leaf area index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-amended</td>
<td>TF</td>
<td>5.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.89</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>4.2</td>
<td>1.01</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>4.3</td>
<td>1.15</td>
<td>2.8</td>
</tr>
<tr>
<td>Amended</td>
<td>TF</td>
<td>5.7</td>
<td>2.51</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>5.6</td>
<td>2.23</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>4.7</td>
<td>2.15</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Field plots were subject to tilled fallowing (TF), mixed hay cropping system (H) and field vegetable cropping (FV). Each transition strategy included compost amended and un-amended split plots. Field bioassays were not conducted within the high tunnel vegetable cropping.<br>
<sup>b</sup> Leaf area index was assessed based on a four-level rating system, where 0 = growth through the second true leaf visible, 1 = first compound leaf emerging, 2 = first compound leaf fully expanded, and 3 = the layer of compound leaves ≥ 100 cm².<br>
<sup>c</sup> Values are the means of 8 replications (4 blocks and 2 replicates per block).

Table 1.5. Vigor of tomato seedlings grown in an organic field after three years of transition under different management strategies.
Pseudomonas strains PCR positive in the phlD gene, component of the biosynthetic pathway of the antibiotic 2,4-diacetylphloroglucinol (DAPG)

Plots considered were subject to tilled fallowing (TF), mixed hay cropping system (H) and field vegetable cropping (FV). Each transition strategy included compost amended and un-amended split plots. Pseudomonas counts were not conducted for the high tunnel vegetable cropping

Mean population size on plants, expressed as CFU g⁻¹ of root

Proportion of root samples harboring phlD⁺ Pseudomonas at the indicated abundance levels

Percentage of Pseudomonas populations harboring the the phlD gene

For tomato greenhouse bioassays, data presented correspond to Experiment 2. These results are representative for the other two (Experiments 1 and 3) experiments.

Means followed by a different letter are significantly different at $P < 0.1$ (Wilcoxon 2-sample test). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only.

Table 1.6. Abundance and incidence of rhizosphere colonizing Pseudomonas and phlD⁺ Pseudomonas in tomato and soybean seedlings, grown in the greenhouse (GH) and in the field, in soils previously subjected to different organic transition strategies

<table>
<thead>
<tr>
<th>Crop</th>
<th>Site</th>
<th>Compost</th>
<th>Transition strategy</th>
<th>$P_s^a$ (log g⁻¹)</th>
<th>Abundance$^b$ phlD⁺ (log g⁻¹)</th>
<th>% phlD⁺</th>
<th>Incidence$^e$</th>
<th>&gt;log 3.4</th>
<th>&gt;log 4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>GH</td>
<td>Un-amended</td>
<td>TF</td>
<td>6.5</td>
<td>5.1</td>
<td>28.3</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H</td>
<td>6.3</td>
<td>5.3</td>
<td>28.1</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FV</td>
<td>6.2</td>
<td>5.2</td>
<td>51.5</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amended</td>
<td>TF</td>
<td>7.2</td>
<td>5.9</td>
<td>12.7</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H</td>
<td>7.2</td>
<td>5.9</td>
<td>51.7</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FV</td>
<td>7.4</td>
<td>5.5</td>
<td>8.9</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>Un-amended</td>
<td>TF</td>
<td>5.8</td>
<td>3.9</td>
<td>B$^h$</td>
<td>18.9</td>
<td>29</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>5.9</td>
<td>3.8</td>
<td>B</td>
<td>1.6</td>
<td>25</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>5.7</td>
<td>4.2</td>
<td>A</td>
<td>7.3</td>
<td>63</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amended</td>
<td>TF</td>
<td>6.1</td>
<td>3.7</td>
<td>4.0</td>
<td>13</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6.3</td>
<td>3.8</td>
<td>0.7</td>
<td>25</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>6.8</td>
<td>4.0</td>
<td>0.6</td>
<td>29</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>Un-amended</td>
<td>TF</td>
<td>6.3</td>
<td>4.4</td>
<td>27.1</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>6.3</td>
<td>5.2</td>
<td>13.6</td>
<td>88</td>
<td>88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>6.6</td>
<td>5.0</td>
<td>8.0</td>
<td>80</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amended</td>
<td>TF</td>
<td>7.1</td>
<td>3.9</td>
<td>0.2</td>
<td>25</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>7.1</td>
<td>5.2</td>
<td>11.4</td>
<td>75</td>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>7.1</td>
<td>5.8</td>
<td>6.2</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>Un-amended</td>
<td>TF</td>
<td>5.3</td>
<td>3.5</td>
<td>2.5</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>5.6</td>
<td>3.5</td>
<td>1.6</td>
<td>14</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>5.6</td>
<td>3.3</td>
<td>2.2</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amended</td>
<td>TF</td>
<td>5.2</td>
<td>3.4</td>
<td>6.7</td>
<td>20</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>5.9</td>
<td>3.8</td>
<td>12.9</td>
<td>25</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FV</td>
<td>4.6</td>
<td>3.3</td>
<td>14.7</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Pseudomonas strains PCR positive in the phlD gene, component of the biosynthetic pathway of the antibiotic 2,4-diacetylphloroglucinol (DAPG)

$^b$Plots considered were subject to tilled fallowing (TF), mixed hay cropping system (H) and field vegetable cropping (FV). Each transition strategy included compost amended and un-amended split plots.

$^c$Pseudomonas counts were not conducted for the high tunnel vegetable cropping

$^d$Mean population size on plants, expressed as CFU g⁻¹ of root

$^e$Proportion of root samples harboring phlD⁺ Pseudomonas at the indicated abundance levels

$^f$Percentage of Pseudomonas populations harboring the the phlD gene

$^g$For tomato greenhouse bioassays, data presented correspond to Experiment 2. These results are representative for the other two (Experiments 1 and 3) experiments.

$^h$Means followed by a different letter are significantly different at $P < 0.1$ (Wilcoxon 2-sample test). Pairwise comparisons presented within amended (lower case) or un-amended (upper case) plots only.
<table>
<thead>
<tr>
<th>Crop</th>
<th>Site</th>
<th>Pseudomonas(^c)</th>
<th>(\text{phlD}^+)(^e)</th>
<th>% phlD(^f)</th>
<th>Damping off incidence(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>Tomato</td>
<td>GH</td>
<td>0.81(^d)</td>
<td>-0.81</td>
<td>-0.85</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Field</td>
<td>-0.37</td>
<td>0.25</td>
<td>0.34</td>
<td>-0.12</td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>0.67</td>
<td>0.11</td>
<td>0.52</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Field</td>
<td>-0.52</td>
<td>-0.20</td>
<td>-0.61</td>
<td>-0.10</td>
</tr>
</tbody>
</table>

\(a\) Abundance expressed as the mean population size on plants, expressed as CFU g\(^{-1}\) of root

\(b\) To calculate the \(\rho\) correlation coefficient, transition strategies were grouped into three levels of disease, based on \% pre-emergence damping-off

\(c\) Total Pseudomonas populations as determined by growth up to the terminal dilution on 1/3 KB

\(d\) Spearman correlation coefficient (\(n \geq 5\)). Values in bold indicate significant correlation at \(P<0.1\).

\(e\) Pseudomonas strains PCR positive to the \(\text{phlD}\) gene, component of the biosynthetic pathway of the antibiotic 2,4-diacyethylphloroglucinol (DAPG)

\(f\) Percentage of Pseudomonas populations harboring the \(\text{phlD}\) gene

Table 1.7. Spearman’s \(\rho\) for pair-wise correlations between the abundance of rhizosphere colonizing pseudomonads in tomato and soybean samples, and damping-off incidence, for bioassays performed in the greenhouse (GH) and the field
Crop  | Experiment | Transition Strategies exhibiting\(^a\)  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low damping-off incidence</td>
<td>Intermediate damping-off incidence</td>
<td>High damping-off incidence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td>GH</td>
<td>H+C(^b), TF+C</td>
<td>H, FV, FV+C</td>
<td>TF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Field</td>
<td>H+C, H</td>
<td>TF, TF+C</td>
<td>FV, FV+C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>H+C, H</td>
<td>TF+C, FV</td>
<td>TF, FV+C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Field</td>
<td>H+C</td>
<td>TF, H</td>
<td>TF+C, FV, FV+C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) N=12 pots in the greenhouse or n=8 field sites per treatment were analyzed for differences in damping-off under pathogen pressure. For the tomato, low is defined as 50-60% damping off; intermediate as 60-70%; and high, as 70-80%. For soybean in the greenhouse, low corresponds to 40-60% damping-off; intermediate 60-80%; and high > 80%. For soybean grown in the field, low 70-80%; intermediate, 80-90%; and high, >90%. See figures 1.2-1.4, text above and Baysal et al. (2008).

\(^b\) Abbreviations: TF, tilled fallow; H, mixed hay; FV, field vegetable; +C, compost amended treatment. Modified from Benítez et al., 2007.

Table 1.8. Damping-off incidence groups, according to % damping-off severity observed in tomato and soybean grown in the greenhouse (GH) and the field, in soils previously cropped with different transition strategies.
Figure 1.1. Damping-off incidence of soybean seedlings grown in the greenhouse in soils previously subjected to different organic transition strategies. Damping-off incidence is expressed as the percentage of non-germinated seedlings. TF, tilled fallowing; H, mixed hay cropping system; FV, field vegetables cropping; HTV, high tunnel vegetables; C, compost amended sub-plots; NC, un-amended plots. Bars labeled with a different letter are significantly different at $P < 0.05$ (Wilcoxon 2-sample test). Pair-wise comparisons presented within amended (lower case) or un-amended (upper case) plots only. Compost within transition effects ($P<0.05$) were observed only for Experiments 2 and 3.
Figure 1.2. Damping-off incidence of soybean seedlings grown in field plots previously subjected to different organic transition strategies. Damping-off incidence is expressed as the percentage of non-germinated seedlings. TF, tilled fallow; H, mixed hay cropping system; FV, field vegetables cropping; C, compost amended sub-plots; NC, un-amended plots. Bars labeled with a different letter are significantly different at $P < 0.05$ (Wilcoxon 2-sample test). Pair-wise comparisons presented within amended (lower case) or un-amended (upper case) plots only. Compost within transition effects ($P<0.05$) were observed only for Experiment 2.
Figure 1.3. Damping-off incidence of tomato seedlings grown in field plots previously subjected to different organic transition strategies. Damping-off incidence is expressed as the percentage of non-germinated seedlings. TF, tilled fallowing; H, mixed hay cropping system; FV, field vegetables cropping; C, compost amended sub-plots; NC, un-amended plots. Bars labeled with a different letter are significantly different at $P < 0.05$ (Wilcoxon 2-sample test). Pair-wise comparisons presented within amended (lower case) or un-amended (upper case) plots only. No compost within transition effects were observed ($P>0.5$).
Figure 1.4. Step-wise approach used in this study for the identification of microbial populations associated with disease suppression and disease in an organic transition field experiment.
CHAPTER 2

MULTIPLE STATISTICAL APPROACHES TO COMMUNITY FINGERPRINTING
REVEAL BACTERIAL POPULATIONS ASSOCIATED WITH GENERAL DISEASE
SUPPRESSION ARISING FROM THE APPLICATION OF DIFFERENT ORGANIC
FIELD MANAGEMENT STRATEGIES

ABSTRACT

Multiple statistical analyses of terminal restriction fragment length polymorphism (T-RFLP) data were used to screen and identify bacterial populations involved in general disease suppression in an organically managed soil. Prior to sampling, three different management strategies (i.e. mixed hay, tilled fallowing and open-field vegetables production) were used during the transition from conventional to organic farming, with and without compost amendment. The mixed hay transition strategy consistently led to the lowest damping-off disease incidence on two different crops in separate greenhouse and field experiments. Bacterial population structure in bulk soil and the rhizosphere of both crops was characterized using T-RFLP analyses of amplified 16S rDNA sequences. First, principal component analysis (PCA) revealed changes in the relative abundance of bacterial terminal restriction fragments (TRF) in response to transition strategy and/or compost amendment in eight different experimental contexts. In each context, a different
subset of TRF substantially contributed to the variation along the first two principal components. However, terminal restriction fragment M148 contributed significantly to the observed variation in 6 out of the 8 experiments, and moderately in the remaining 2 experiments. As a second approach, non-parametric analyses of variance revealed that the relative abundance of TRF differed among treatments. While the responsive subsets identified varied somewhat by experimental context, M137, M139 and M141 were more abundant in samples from the mixed hay transition strategy in multiple experimental contexts. Subsequent correlation analyses revealed that TRF associated with disease suppressive treatments (i.e. mixed hay with and without compost) were frequently negatively correlated with damping-off disease incidence. As a group, these TRF were disproportionately associated with lower disease levels further indicating their role in disease suppression. *In silico* analysis of the bacterial 16S rDNA sequence database revealed that the TRF identified in this study (e.g. M137, M139, M141, and M148) might correspond to well-characterized genera of bacterial biological control agents.

**INTRODUCTION**

Agricultural management practices impact soil and rhizosphere microbial diversity and community structure. The interactions between crop species, management strategy, and soil type, affect soil microbial communities (Garbeva et al., 2004a). For example, Berg et al. (2002) observed differences in bacterial communities in the rhizosphere of different crop species: potato, oilseed rape, and strawberry. In contrast, Hiddink et al. (2005), reported no significant differences in soil microbial communities between single and mixed crop cropping systems; and Girvan et al. (2003) described
greater differences in communities associated with soil types when compared to crop species. Tillage (Feng et al., 2003), rotation (Lupwayi et al., 1998; Larkin, 2003), use of mulches (Tiquia et al., 2002), cover crops (Schutter et al., 2001; Schutter and Dick, 2002), and amendments (Parham et al., 2003; Pérez-Piqueres et al., 2006) are also known to influence the structure and activity of microbial communities. The effects of different farming practices in the abundance of rhizosphere colonizing biocontrol *Pseudomonas*, specifically those producing the antibiotic 2,4-diacyetylphloroglucinol (DAPG), were described by Rotenberg et al. (2007). The abundance of rhizosphere-colonizing DAPG-producing *Pseudomonas*, which have previously been implicated in soilborne disease suppression (Weller et al., 2002; McSpadden Gardener, 2007), were positively correlated with stand and yield of corn and predictably responded to rotation sequence, tillage, compost amendments and seed treatments.

Soils differ in their ability to suppress plant disease development. Disease-suppressive soils are soils in which pathogens fail to establish or to produce disease (Baker and Cook, 1974). Pathogen survival and growth is often limited due to a variety of biological parameters of soil. Two types of biologically-based disease suppression have been described. General suppression, which occurs as an overall effect of the microbial community, principally through competition for resources, differs from specific suppression, which relates to a specific mode of action against pathogen populations. However, it seems likely that both general and specific suppressive activities occur to varying degrees in most soils. It is well established that particular farm management practices can be used to promote disease suppression. For example, the use of compost for the control of soilborne pathogens and the characteristics of these organic
amendments contributing to suppressiveness has been widely studied (Hoitink and Fahy, 1986; Hoitink and Boehm, 1999). Similarly, Larkin and Honeycutt (2006) demonstrated the importance of rotation sequence on the buildup of soil microbial communities suppressive to *Rhizoctonia* diseases of potato; and Berg et al. (2002) described the importance of host species for bacterial antagonists to *Verticillium dahliae*.

Microorganisms associated with disease suppressiveness may represent useful biological control agents. After soils with various disease suppressive levels have been recognized, a fingerprint of the microbial community can provide information about candidate microorganisms involved in this function (Borneman et al., 2004). Over ten years ago, Tunlid et al. (1989) and Boehm et al. (1993) used multivariate analyses of fatty acid profiles to demonstrate that multiple bacterial populations contributed to general suppression of *Rhizoctonia* and *Pythium* damping-off of cucumber. Specific microbial populations have also been implicated in disease suppression. For instance, populations of DAPG-producing *Pseudomonas* are more abundant in long-term wheat monoculture systems exhibiting suppression to take all disease of wheat (Weller et al., 2002). Similarly, the fungus *Dactylella oviparasitica*, identified through rRNA gene analysis, is involved in the suppression of the beet-cyst nematode by specific California soils (Olatinwo et al., 2006).

Culture-independent studies of microbial communities rely on the analysis of conserved DNA sequences. Terminal restriction fragment length polymorphism (T-RFLP) is a PCR-based technique which can be used to create community profiles based on differences in restriction fragment length of a specific DNA region (Marsh, 1999). T-RFLP has been used to study differences in community structure and diversity in
different systems, at the domain level (based on ribosomal DNA sequences) and for specific functional groups, such as denitrifiers (Braker et al., 2001).

This study was part of a multi-disciplinary research project aimed to evaluate economic, environmental and biological impacts of management strategies to be used during the transition from conventional to organic farming. In the United States, a three-year period without synthetic chemical inputs is required to obtain official organic certification. During this transition period growers typically experience yield reductions due to insect, weed and disease pressure, and do not always provide adequate fertility (Liebhardt et al., 1989; Tu et al., 2006). Growers may approach this prescribed transition period in several ways; and, their choices are driven by economic as well as agronomic concerns (McRae et al., 1990; Tu et al., 2006).

In this work, T-RFLP analyses were used to study differences in bacterial community structure in soil and rhizosphere samples taken from plants grown in soils previously experiencing different organic transition strategies. Plants grown in soils from different treatments varied in health and vigor, in both greenhouse and field bioassays (Baysal et al., 2008). Based on these results, we hypothesized that the different management treatments modified microbial community structure so as to alter the level of suppression to soilborne diseases, such as pre-emergence damping-off. Soil and rhizosphere microbial communities are known to be complex, and information obtained from molecular fingerprinting analyses could be limited. Hence, we used a variety of statistical approaches to mine community fingerprint data to identify associations between different microbial populations and soilborne disease suppression. The

45
approach consisted of a combination of multivariate and non-parametric univariate procedures that provide different perspectives on the extensive data sets generated by microbial community fingerprinting techniques.

MATERIALS AND METHODS

Field site description. An organic transition field experiment was established at the Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH. Soil at this site is a moderately well drained Wooster silt loam with approximately 2.2% organic matter, 40 mg kg\(^{-1}\) P, and 95 mg kg\(^{-1}\) K. The field was previously under a conventional corn and soybean rotation, and transition to organic farming started in 2003, and three transitional management strategies were studied. The three transition strategies were tilled fallow (TF), mixed hay species (H), and low intensity open field vegetables production (FV). Split-plots within the main transition strategy plots were arranged to address fertility management during this period. Half of each main plot received an annual addition of 18.6 t ha\(^{-1}\) dry weight composted dairy manure. The main plots were replicated four times in a randomized split plot design. Main field plots were 18.3 m x 17.1 m long. After the three year transition period (2006) the field was certified as organic, and the whole field was planted to tomato.

Greenhouse and field bioassays. Soils from the field site undergoing the transition from conventional to organic farming were sampled in spring 2005. Disease suppressiveness assays were performed in the greenhouse using collected soils. Tomato cv. Tiny Tim (Stokes Seed Ltd.) and soybean cv. Sayamusume (Territorial Seed Company) were grown in 10 x 10 x 9 cm pots filled with field soil inoculated with 10
agar plugs of oomycete pathogens. Agar plugs were obtained with #3 cork borers (6 mm diameter). Isolates used for tomato inoculations were provided by Sally Miller (OARDC), and included *Pythium aphanidermatum* isolate 349 and *P. ultimum*. For soybean bioassays soils were inoculated with *Phytophthora sojae* race 25 and *P. ultimum*, provided by Anne Dorrance (OARDC). Twenty tomato and 6 soybean seeds were planted per pot, with three greenhouse replicates from each field subplot. Watering was done to the top of the pot through a siphon delivery system, at a rate of 65 ml day$^{-1}$ pot$^{-1}$. The experiments were conducted in a 14/10 hr photoperiod and with a day/night temperature of 20/15°C and 55% relative humidity. Seedlings were sampled after 21 and 17 days in the greenhouse, or the equivalent of four true leaves in tomato, and vegetative stage 1 (V1) in soybean, respectively. Field bioassays were performed in the summer of 2006. Twenty four tomato seeds and 16 soybean seeds were planted in two 45 x 45 cm areas per subplot within the field. The rest of the field was planted with two tomato cultivars (Mountain Spring and Florida 47), on plastic covered raised beds. Seedlings were sampled at the equivalent stages (V1 and four true leaves) from the greenhouse bioassays.

**Seedling sampling.** Plant vigor and disease data were recorded for the greenhouse and field experiments. Stand counts per pot or field site were used as a measure of damping-off. Percent damping off was calculated as the proportion of plants that did not germinate in each pot per field site times 100. Plant vigor measurements included shoot fresh weight, plant height and a leaf area index measurement for tomato. For soybean, shoot fresh weight and developmental stage (expressed as the number of seedlings that reached V1 stage) were recorded and are reported elsewhere (Baysal et al., 2008). The roots of one plant per pot per field site and its adhering soil, after shaking
(rhizosphere), were sampled for DNA extraction. After plant removal and rhizosphere sampling, soil was mixed and homogenized by hand; and any remaining visible plant root material was removed. Samples of this root-free soil were obtained from each pot and field site. Rhizosphere and soil samples were stored at -20°C prior to DNA extraction.

**Bacterial community profiling.** Differences in bacterial community structure associated to transition strategy were determined through terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified bacterial 16S rDNA sequences. The Ultra-Clean soil DNA extraction kit (MoBio) was used for DNA extraction starting from 0.5 g of soil and 0.3 g of rhizosphere samples. 1:25 and 1:10 dilutions of soil and rhizosphere DNA respectively were used for PCR amplification of the 16S gene with the 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' ACG GCT ACC TTG TTA CGA CTT 3') primers, based on those described by Weisburg et al. (1991; fD1 and rP2). The 8F primer was labeled with the fluorescent WellRED dye D4 (Sigma, Proligo) for further visualization of the terminal restriction fragments (TRF). Amplification was carried out in 25 µl reactions containing 2.5 µl 10x Mg-free buffer (Promega Corp.), 2 or 1.5 µl (for soil and rhizosphere samples, respectively) 25 mM MgCl₂, 2.5 µl 2 mM deoxynucleoside triphosphates, 15 or 15.5 µl sterile water (Sigma, Molecular Biology Reagent), 0.25 µl of each primer (100 pmol µl⁻¹), 0.1 µl RNase A (10 mg ml⁻¹) (Novagen), 0.33 µl of *Taq* DNA polymerase (5 U µl⁻¹) (Promega), and 2.5 µl template. Amplification was performed with a PTC-200 Thermocycler (MJ Research Inc.). The cycling program consisted of a 5 min initial denaturation step at 95°C followed by 30 cycles of 94°C for 60 s, 54°C for 45 s, and 70°C for 60 s; and an 8 min final extension.
step at 70°C. Amplification products were separated on 1.5% agarose gels in 50% Tris-borate-EDTA buffer and visualized by ethidium bromide staining (1 mg l⁻¹).

Restriction digestion of PCR products were performed in 10 µl reactions containing 0.3 µl MspI enzyme (10 U µl⁻¹) (Promega), 0.5 µl Buffer B and 3.5 µl PCR product. Samples were incubated for 3 h at 37°C followed by 20 min at 65°C for enzyme inactivation. 3.5 µl of the digestion reaction were diluted into 7 µl of water and sent to the Molecular and Cellular Imaging Center of the OARDC, Wooster, OH. There, 0.1 µl of sample were mixed with 0.5 µl 600 bp size standard (CEQ DNA size standard kit 600) and 40 µl formamide (loading solution). Terminal fragments were loaded and separated on the CEQ 8800 Genetic Analysis System (Beckman Coulter) and individual profiles were analyzed with the CEQ fragment analysis software (CEQ 8000 Genetic Analysis System). Matrices containing incidence as well as peak height data of individual TRF were generated for all samples. The profiles obtained from each bioassay were analyzed separately with a total of eight experimental contexts (2 crops in 2 sites, and soil and rhizosphere samples for each one of those). The following criteria were used to define TRF in these assays: fragment size ≥ 100 bp, fluorescence intensity ≥ 200 fluorescence units (peak height). Binning was defined as ± 1 nt for TRF ≤ 300 bp, and ± 2 nt for TRF ≥ 300 bp. The relative abundance of each TRF was calculated as the proportion of the fluorescence (peak height) of a specific TRF in a sample, from the total fluorescence of that sample.

Statistical analyses. Ordination was performed through principal components analysis (PCA) on covariance matrices to determine variation in bacterial community structure due to treatment. This analysis was done on the relative abundance data of the
TRF, with each TRF considered as a different variable. Data from each experimental context (total of eight experimental contexts: 2 crops in 2 sites, and soil and rhizosphere samples for each) was analyzed separately using the JMP IN (v4.0.4. SAS Inc.) statistical software package. Ordination plots were created with SigmaPlot (v10.0 Systat Software Inc.) from the mean principal component scores for each treatment. Factor loadings were calculated for each TRF to determine the relative influence of each TRF on the variation among treatments along each principal component. A subset of TRF was selected for further analysis from the total TRF obtained in each experiment, based on their reproducibility within a treatment. These “selected TRF” were observed in ≥ 60% of the samples of at least one treatment. For comparison and correlation analyses, non-parametric procedures were used because the abundance of TRF is not linearly related to template abundance. The rank-based Kruskall-Wallis test was used to determine treatment differences in relation to the relative abundance of the selected TRF. Analyses were run on MINITAB (v14.2, Minitab, Inc., State College, PA) and pair-wise treatment comparisons were performed based on the Bonferroni-Dunn method. Quantitative associations between percent damping-off and the relative abundance of selected TRF (>60% criteria) were determined. The Spearman correlation coefficients were calculated from ranked data on a per pot or per field site basis, using MINITAB.

RESULTS

Principal component analysis on data from T-RFLP profiles. Principal component analysis (PCA) was performed to determine the overall effect of transition strategies on the observed population of 16S rDNA terminal restriction fragments (TRF).
For this analysis each TRF was considered as a different variable. In addition, each crop rhizosphere and soil sample set, and each individual bioassay were analyzed separately because data from each were generated independently (i.e. in different PCR and capillary electrophoresis runs) and comparisons between soil and rhizosphere samples were not a focus of this study. Ordination plots were generated from the mean principal component scores for each treatment and factor loadings were used to interpret the observed treatment separations. Treatment separation was evaluated based on non-overlapping error bars in each principal component axis. The observed variation explained by the first two principal components ranged between 58-86% among all bioassays and soil and rhizosphere samples. The third principal component contributed only 5-13% of the observed variation.

Transition strategy appeared to influence rhizosphere T-RFLP profiles. For tomato, separation between the three un-amended transition strategies was observed in the greenhouse and the field (Figure 2.1). In the greenhouse, the mixed hay (H) separated from the tilled fallow (TF) along the first PC; whereas, the three transition strategies separated on the second PC. In the field, the mixed hay (H) and field vegetables (FV) differed along the first PC and variation between the three strategies was more evident in the second PC. Similar to the tomato, soybean rhizosphere T-RFLP profiles were influenced by transition strategy (Figure 2.2). In the greenhouse, the mixed hay (H) and field vegetables (FV) differed from each other especially along the second PC. All three transition strategies were distinguished in the field, with greater variation along the first PC, but separation between the mixed hay (H) and field vegetables (FV) was also observed along the second PC.
Responses to compost amendment were also observed on the T-RFLP profiles of the rhizosphere of both crops (Figure 2.1 and 2.2). In the greenhouse experiments, the un-amended mixed hay (H) and tilled fallow (TF) differed from their amended (+C) equivalent in tomato and soybean. In the field tomato, effects from compost amendment were observed only for the mixed hay (H). In contrast, for the field soybean compost amended subplots of the tilled fallow (TF) and field vegetables (FV) differed from the un-amended subplots. In addition, compost amendment resulted in less separation of the three transition strategies when considering profiles of the rhizosphere of tomato grown in the greenhouse and the field, and soybean grown in the field.

Principal component analyses of T-RFLP profiles from the soil fraction of both crops also show differences due to transition strategy and in response to compost amendment (Figure 2.3 and 2.4). For tomato, the mixed hay (H) separate from the field vegetables (FV) in the greenhouse and the field; and for soybean, differences were observed between the three transition strategies. Effects from compost amendment (+C) were observed in all three transition strategies for tomato greenhouse and field soils, and for the tilled fallow and field vegetable treatments for the soybean field soils.

From all of the TRF obtained in each individual analysis (N = 29-83 for each), only a small subset considerably contributed to the variation observed in the PCA. Terminal restriction fragments with a factor loading of $|x| \geq 0.70$ for each of the first three principal components are summarized in Table 2.1. The first three principal components explained between 64 - 92% of the variation among the 8 different experimental contexts. TRF M148 greatly contributed to the variation in PC 1 in four out of the eight scenarios (tomato greenhouse soil and rhizosphere, tomato field soil and soybean greenhouse soil);
and in PC 2 in two scenarios (rhizosphere from tomato grown in the field and field soil from soybean). The only situations in which M148 did not have a factor loading of $|x| \geq 0.70$ were in the analyses of the TRF obtained from the rhizosphere of soybean (in the greenhouse and the field). In the latter experiments TRF M148 was still present in high abundance, and the factor loadings were high (i.e. $0.5 < |x| < 0.7$), but did not meet our selection criteria. In addition, the direction of the contribution of M148 was the same in seven out of the eight experimental contexts, with exception of the samples from the rhizosphere of field tomato. Other TRF that largely contributed to the variation to the first three principal components in more than one scenario were M151, M501, M488-490 and M493-494. These analyses indicate that small subsets of TRF were disproportionately responsible for separation of treatments by PCA, but that the composition of those subsets varied somewhat by experimental context.

**Treatment differences in the relative abundance of individual terminal restriction fragments.** As a second approach to identifying important differences in bacterial community structure, the non-parametric Kruskall-Wallis test was used to determine if the relative abundance of individual TRF was influenced by treatment. Because of the high degree of variability between samples, the data set of each experimental context was sifted to select TRF occurring with a useful degree of replication. Thus, terminal restriction fragments present in $\geq 60\%$ of the samples of at least one treatment were selected for further analysis. The number of TRF selected varied between bioassays, and overall the selected TRF represent the majority of the fluorescence of each bioassay. The proportion of fluorescence per sample was used to estimate differences in the relative abundance of each TRF. The analysis was performed
separately for each crop and experiment. The mean relative abundance of selected TRF that show differences between treatments \((P < 0.1)\) are reported in Table 2.2.

This approach revealed that different TRF were generated more abundantly in reactions representing different transition strategies, and the proportion of selected TRF showing significant differences between treatments varied among experiments. For the soils from tomato grown in the greenhouse, six TRF were selected from 35, and from these, three were more abundant, in terms of proportion of fluorescence, in the soils previously cropped with mixed hay (TRF M137, M139 and M141). In the soils from tomato grown in the field, 16 out of 63 possible TRF were selected; from which TRF M180 was significantly higher in the mixed hay plus compost treatment. Nineteen from 76 TRF were selected for the soils from soybean grown in the greenhouse. Twelve of these fragments were significantly higher for a treatment or set of treatments, with M137, M141 and M148 being highest in the mixed hay and mixed hay plus compost. Terminal fragments M127, M204, M490, and M494 were significantly higher in the soils from the field vegetables and tilled fallow treatments, and M162, M282 and M382 for the tilled fallow only. In the soybean field soils, 23 out of 83 TRF were selected and only three showed significant difference, M106 with the highest abundance in soils previously cropped as tilled fallow and field vegetables; and M123 and M136 in amended soils from tilled fallow and field vegetables.

Likewise, a small subset of all the TRF obtained from rhizosphere samples were shown to be more abundant in reactions representing different transition strategies. For tomato, the total number of TRF obtained in the rhizosphere was greater than in the soil, with 48 total fragments observed in the greenhouse, from which only four were selected.
None showed significant differences between treatments. In the field, 27 out of 68 TRF were selected, from which only two showed differences in terms of abundance. Terminal restriction fragment M141 was significantly more abundant in the mixed hay plus compost and tilled fallow plus compost transitions strategies; and TRF M182 was significantly higher in the mixed hay plus compost and the field vegetables regardless compost amendment. For the soybean more TRF were obtained from the soil samples than from the rhizosphere. From the greenhouse samples, nine out of 41 TRF were selected, from which only three were significantly higher in the field vegetables (M122), the tilled fallow (M403), and the amended mixed hay (M489), respectively. Also nine TRF were selected from the field samples (out of 29), three of which showed any differences. Terminal restriction fragment M139 was more abundant when plants were grown in the amended soils from the field vegetable and tilled fallow; and M159 and M176 were more abundant when grown in the amended and un-amended mixed hay soils.

**Quantitative relationships between damping-off and selected TRF.** The relevance of the several TRF described above to pre-emergence damping-off was assessed using non-parametric correlation analysis. Individual correlations were performed, on a per-pot basis, between the ranked data of the relative abundance of each TRF and the observed pre-emergence damping-off incidence (percent value). Correlations were calculated only when sufficiently replicated data (i.e. a minimum sample size of \( n \geq 5 \)) were available for analysis. Again, since not all of the TRF were present in all the samples of each treatment, and for some treatments stand was very low, the soilborne disease data were necessarily grouped into three distinct levels for the
purposes of these assessments. Specifically, for each experiment, data were grouped into low, medium and high disease incidence. Grouping was done independently for each bioassay, and disease levels reflect observed treatment separation after statistical analysis of damping-off incidence data (Baysal et al., 2008; Table 2.3). For the tomato bioassays, low disease was defined as 50-60%, intermediate as 60-70%, and high as 70-80% damping off. For the soybean greenhouse bioassay, low disease corresponded to 40-60% damping-off, intermediate to 60-80%, and high to > 80%. For soybean grown in the field, low corresponded to 70-80%, intermediate to 80-90%; and high to >90% damping off. The mixed hay plus compost transition strategy had the lowest damping-off in all experiments. In addition, the mixed hay and tilled fallow plus compost strategies contributed to lower damping-off for the tomato grown in the greenhouse and the field; and the mixed hay to lower damping-off of soybean grown in the greenhouse (Table 2.3).

Terminal restriction fragments that were more abundant in suppressive contexts (H and H+C) were as a group more negatively correlated with disease incidence. To facilitate the analysis and interpretation of the correlation results, the TRF were grouped into two categories. The first group corresponds to those TRF that were identified as being more abundant in the transition strategies with the lowest percentage of damping-off (Tables 2.2 and 2.3). The second group consisted of the TRF that were more abundant in transitions strategies with intermediate and high damping-off incidence, and those TRF with high factor loadings in the PCA that were not included in the first data set (Tables 2.1, 2.2 and 2.3). The first group of TRF (i.e. associated with low disease incidence) was negatively correlated with pre-emergence damping-off over 65% of the time (Table 2.4). This pattern was consistent for data taken on both crops and for both the soil and
rhizosphere data sets. Overall, these negative correlations occurred more frequently than expected by chance \((P < 0.05)\). The frequency of negative correlations with damping-off incidence (compared to the frequency of positive correlations) was also greater for the first group of TRF than for the second group (i.e. those more abundant in the intermediate and high disease situations) \((P = 0.055)\).

The individual correlation coefficients obtained for the TRF associated through the analysis of variance with low disease treatments are shown in Table 2.5. Of the 37 correlations between the relative abundance of individual TRF and the percent damping-off for each class of samples (i.e. those experiencing either low, medium, or high incidence of damping-off) in the rhizosphere samples, 25 were negative, but this proportion was not significantly different from that expected by chance \((P = 0.27)\). However, of the 44 correlations performed for the soil samples, 30 were negative, a number significantly greater than that expected by chance \((P = 0.057)\). If one considers the proportion of correlations generated from all the rhizosphere and soil samples, 55 out of the 81 measured correlation coefficients were negative with damping-off, a result with even greater statistical support \((P = 0.02)\). And, while only six of the 81 individual correlation tests were significant, five of those corresponded to negative correlation coefficients. The TRF showing negative correlations with damping-off at the three disease levels were M488 from the rhizosphere of soybean (greenhouse), M401 from the soils where tomato were grown (field), and M148 (greenhouse) and M137 (field) from the soils where soybean was grown.

**Probable bacterial sources of TRF associated with damping-off suppression.**

Taxonomic placement of a TRF was predicted based on comparisons to TRF size
expected for all of the different sequences present in publicly available databases. More than one bacterial species, however, can produce a terminal restriction fragment of the same size. Several tools are available in the World Wide Web for TRF analysis and identification. The Virtual Digest (ISPaR) option of the Microbial Community Analysis III software of the University of Idaho (MiCA 3; Shyu et al., 2007) allows for virtual digestion of a PCR product generated with a desired primer set, based on in silico PCR of sequences available on the Ribosomal Database Project II (RDPII, Release 9, Update 37, Bacterial SSU 16SrRNA). After virtual digestion with MspI of our desired amplicon (using 8F and 1492R primers), 7746 records were returned. The extended list of bacterial genera producing a terminal fragment of a size equivalent to the TRF showing association with disease suppression in this work is shown in Table 2.6. Several bacterial classes are represented within this list. In the range of 137 and 139 bp, mostly members of the Actinobacteria and Bacilli are present. Along the 141 bp range, the classes Actinobacteria, Bacilli, γ and β-proteobacteria are represented. Most of the genera included in the 159 bp group are Actinobacteria; and in the 401 bp size α-proteobacteria from the order Rhizobiales. Finally, the range of 488-489 bp includes bacterial genera from the γ and β-proteobacteria only.

DISCUSSION

In this work, we described a multi-pronged approach to assess the statistical associations between agricultural management practices, disease suppression and bacterial community structure revealed by T-RFLP analysis. Principal component analysis revealed differences in overall bacterial community structure in response to
transition strategy and compost amendment (Figures 2.1-2.4). In addition, the TRF M148 contributed substantially to the variation and separation between transition strategies along the first and second principal components (Table 2.1). We hypothesize that TRF with significant contribution to the variations along the principal components are somehow contributing to the differences observed in disease suppressiveness. Because the observed patterns of variation in bacterial communities were replicated in the different experimental contexts, we concluded that field history (i.e. transition strategy) can be a main factor in determining bacterial community structure in soil and the rhizosphere. That cropping sequence could affect microbial community structure was also shown by Larkin (2003) and Larkin and Honeycutt (2006), where the influence in community profiles assayed using lipid analyses were observed from immediate preceding crops. Similarly, responses of bacterial and fungal communities to amendment treatments were observed by Pérez-Piqué et al. (2006); however, in that work, they did not statistically relate such shifts to changes in crop health.

Here, a variable fraction of the analyzed TRF was associated with transitions strategies exhibiting different levels of disease suppression (Tables 2.2 and 2.3). This fraction ranged from 3-63%, depending of the experimental context. Field experiments showed a lower degree of significance of the selected TRF than greenhouse experiments, which might indicate that other variables contributed more to determining crop health status under those less controlled conditions. In addition, variation was observed between experimental contexts in the significance of specific TRF. Only TRF M137, M139, M141 and M148, were associated more than once with the mixed hay transitions strategy. From this we conclude that, while multiple populations may promote or respond to changes in
crop health status, these four TRF include bacteria that are more generally associated with the crop stands in this soil. Previous studies used an analysis of variance approach to identify responses of significant bacterial TRF to treatments (Lukow et al, 2000, McSpadden Gardener and Weller, 2001; Blouin-Bankhead et al, 2004). However, the problem of high sample to sample variation was handled in a unique way in this study. Because a certain degree of independent replication is required to obtain mean separation, our trimming of the data set to remove rarely observed TRF provided a more streamlined approach to identifying significant changes in community profiles (i.e. one requiring fewer ANOVA tests to be run).

From the studied transition strategies, the mixed hay resulted in greatest disease suppression in subsequent tomato and soybean crops in two experimental settings (greenhouse and field bioassays) (Table 2.3). The mixed hay transition strategy consisted of a combination of eight hay species, specifically rye fescue undersown with alfalfa, red and white clover, timothy, chicory, orchardgrass and plantain. Studies of mixtures of grasses and legumes as cover crops have demonstrated a benefit to agricultural systems. Such benefits may be conferred through the improvement of soil characteristics (Fageria et al., 2005) and suppression of weeds (Ross et al., 2001). Additionally cover crops can increase microbial biomass and activity (Mendes et al., 1999; Schutter and Dick, 2002) which may lead to increased levels of general disease suppression (Seigies and Pritts, 2006; Vilich, 1993). Differences in suppressiveness to pathogen growth have also been observed in long-term grassland fields in the Netherlands when compared to arable land under rotation or monoculture (van Elsas et al., 2002). In addition, these grasslands harbor higher diversity of *Pseudomonas* and *Burkholderia* species antagonistic to
Rhizoctonia solani in comparison to arable land (Garbeva et al., 2004b; Salles et al.,
2006). Our data indicate that the mixed hay strategy promoted shifts in specific bacterial
populations concomitant with improvements in crop health. While correlative, such an
association provides a reasonable criterion for targeting bacteria marked by M137, M139,
M141, and M148 for selection and analysis as biocontrol agents.

Differences in effectiveness of disease suppression by compost can be related to
amendment characteristics and soil type (Hoitink and Boehm, 1999). Here, the added
fertility provided by the composted dairy manure might have boosted the natural disease
pressure in the field, resulting in the noted inconsistency in the effects of compost
addition on disease (Table 2.3). Variations in disease suppressing capability of compost
amendments have been described for other pathosystems. For example, Scheuerell et al.
(2005) analyzed the suppressiveness of damping-off of cucumber of 36 compost sources.
Of these, 60% significantly suppressed Pythium irregulare and P. ultimum, but only 17%
suppressed R. solani. Similarly, Termorshuizen et al. (2006) assayed the suppressiveness
of 18 different composts on seven pathosystems, and only 54% of the bioassays
performed exhibited significant disease suppression. The latter studies also point out
variation in efficacy of compost on suppressing different pathogens. Therefore, the
structure of soilborne pathogen populations could be contributing to the inconsistent
effects of compost on disease suppression in this system.

Evidence provided in this work contrasts with observations from soils known to
exhibit specific disease suppression where a specific population is expected to be present
and identifiable in high abundance in suppressive scenarios exclusively (Weller et al.,
2002). For example, in the soils suppressive to the beet-cyst nematode, population levels
of *Dactylella oviparasitica* were almost 100 times higher than other fungal groups profiled in that study (Yin et al., 2003). In our study, several bacterial populations were more abundant in the more suppressive transition strategy and were negatively correlated with damping-off incidence (Tables 2.2 and 2.5). In addition, differences in abundance of TRF between treatments were of a smaller magnitude, typically less than 10 times higher in our profiles. The most prominent fragment in this study was M148, being present in 89% of the samples analyzed and the most abundant in the large majority of those profiles. M148 significantly contributed to the variation along the principal components, varied significantly between treatments in two of the eight experimental contexts, and was negatively correlated with disease incidence in several instances. Other members of the community potentially involved in disease suppression were those marked by M137, M139 and M141. Because these TRF were more abundant in samples obtained from the mixed hay treatment, but were not necessarily absent in the other samples, we propose that general disease suppression was promoted in the mixed hay transition strategy. In addition our results confirm previous observations that mixed crops (used as cover crops) increased the populations of beneficial organisms and reduced disease incidence (Mendes et al., 1999; Schutter and Dick, 2002).

In a recent review, Janvier et al. (2007) state the importance of not only describing differences in microbial communities between soils with different levels of disease suppression, but also identifying the microorganisms more likely involved in this phenomenon. In this study, two statistical analyses were used to screen for candidate bacterial TRF involved in disease suppression. Correlation analysis between the relative abundance of selected TRF and disease levels were performed to determine further
associations of bacterial TRF with disease suppression. Sequencing of specific TRF is required for correct identification of the TRF identified in this study. It is interesting, however, that from the *in silico* analysis, members of the genus *Burkholderia, Bacillus, Paenibacillus* and *Streptomyces* correspond to the sizes of the TRF consistently found in low disease transition strategies (M137, M139, M141; Tables 2.2 and 2.6) or significant for the separation of treatments along the principal components (M148; Table 2.1). Bacterial species from these groups are commonly found in the soil, and have been studied as potential biological control agents for plant pathogens and as plant growth promoting rhizobacteria (Weller et al., 2002; McSpadden Gardener, 2004; Salles et al., 2006; McSpadden Gardener et al., 2007).

This work represents the first step of a step-wise approach for identifying and confirming the role of bacterial populations in disease suppression in the studied mixed hay system. Previously Barnett et al. (2006) described, through a culture-based, multi-step approach, the interaction of three bacterial species involved in the suppression of *Rhizoctonia solani* on wheat. Other studies have focused on culture-independent methods. For example Yin et al. (2003) and Olantinwo et al. (2006) identified the fungus *D. oviparasitica* as antagonist of the beet-cyst nematode *H. shactii*. In both instances, a suppressive system was identified, multiple populations were screened and correlated with disease suppression, and suppressive activities of specific populations were independently confirmed. We have focused only on bacterial populations and their relationship with disease suppression. However, bacteria may not be the only cause of the differences in damping-off observed between transition strategies. In other systems, the analysis of fungal communities has revealed differences in antagonistic fungal
populations (Kuter et al., 1983; Yin et al., 2003; Olantinwo et al., 2006). In those studies, however, specific suppressiveness was implicated. This contrasts with the evidence presented of general suppression of soilborne fungal and oomycete pathogens that can cause damping-off diseases. Complex responses of pathogen populations to agricultural management strategies (Rousseau et al., 2006) also need to be considered on the assessment and application of disease suppressive strategies. Nevertheless, the subset of TRF associated with disease suppressive treatments, which have more negative correlations with pre-emergence damping-off, are candidates for future studies. The characterization of these TRF is presented in the subsequent chapters.

ACKNOWLEDGEMENTS

This work was supported by funds provided by the USDA CSREES Integrated Research, Extension, and Education, Organic Transitions Grants Program (Award Number 2002-51106-01935). The authors thank S. Walker, M. Sutter and their field crew for field site management. Thanks also to G. Agar, D. Chavez, G. Iriarte, R. Raudales, for assistance with sample collection, stand counts and DNA extractions.

A modified version of this chapter was published as:

REFERENCES


Garbeva P, van Veen JA, van Elsas JD. 2004b. Assessment of the diversity, and
antagonism towards *Rhizoctonia solani* AG3, of pseudomonas species in soil from

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS. 2003. Soil type is the primary
determinant of the composition of the total and active bacterial communities in arable

Hiddink GA, Termorshuizen AJ, Raaijmakers JM, van Bruggen AHC. 2005. Effect of
mixed and single crops on disease suppressiveness of soils. Phytopathology 95(11):1325-
1332.

Hoitink H, Boehm M. 1999. Biocontrol within the context of soil microbial communities:

Hoitink HAJ, Fahy PC. 1986. Basis for the control of soilborne plant-pathogens with

Soil health through soil disease suppression: Which strategy from descriptors to

media amended with composted hardwood bark suppressive and conducive to

Larkin RP. 2003. Characterization of soil microbial communities under different potato
cropping systems by microbial population dynamics, substrate utilization, and fatty acid

Larkin RP, Honeycutt CW. 2006. Effects of different 3-year cropping systems on soil

Liebhardt WC, Andrews RW, Culik MN, Harwood RR, Janke RR, Radke JK,
Riegersschwartz SL. 1989. Crop production during conversion from conventional to low-

Lukow T, Dunfield PF, 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

Lupwayi NZ, Rice WA, Clayton GW. 1998. Soil microbial diversity and community
structure under wheat as influenced by tillage and crop rotation. Soil Biol Biochem
30(13):1733-1741.


<table>
<thead>
<tr>
<th>Crop</th>
<th>Experiment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sample&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TRF with high factor loadings for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PC 1</td>
</tr>
<tr>
<td>Tomato</td>
<td>GH</td>
<td>S</td>
<td>M148&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>M148</td>
</tr>
<tr>
<td>Field</td>
<td>S</td>
<td>M148</td>
<td>M382, M385</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>M410, M501</td>
<td>M148, M151, M222, M262, M422, M464, M521, M541, M583, M602, M623, M643</td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>S</td>
<td>M148</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>M493</td>
</tr>
<tr>
<td>Field</td>
<td>S</td>
<td>M104, M176</td>
<td>M148, M151</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>M151, M402</td>
<td>M154</td>
</tr>
</tbody>
</table>

<sup>a</sup>GH: greenhouse.
<sup>b</sup>S: soil; R: rhizosphere.
<sup>c</sup>Size in base pairs of each terminal restriction fragment. Restriction fragments were generated after digestion of the 16S amplicon with MspI.

Table 2.1. 16S rDNA terminal restriction fragments (TRF) with high factor loadings (| x | ≥ 0.70) on the first three principal components (PC) for each experimental context.
TRF were obtained from soil (S) and rhizosphere (R) DNA samples from tomato and soybean crops grown in the greenhouse (GH) and in the field in soils previously exposed to different transition strategies.

a Relative abundance is expressed as the mean percentage of fluorescence of a TRF in relation to the total fluorescence of the sample.

b TRF were selected if present in 60% of the samples of at least one treatment. Only TRF with significant differences in response to transition strategies are shown. 29 - 83 TRF were observed per experimental context, and 4 - 23 of these were selected. Up to 12 TRF per experimental context were observed to show significance between treatments.

c Transitions strategies: TF, tilled fallow; H, mixed hay; FV, field vegetable; +C, compost amended treatment.

d Denotes size in base pairs of restriction fragments after digestion with MspI.

e Values from treatments followed by a different letter in each row show differences at $P<0.1$ using the Kruskal-Wallis test, followed by the Bonferroni-Dunn method for pairwise treatment comparison.

Table 2.2. Relative abundance of a selected subset of 16S rDNA terminal restriction fragments (TRF) showing significant differences between transition strategies, for each experimental context.
Crop | Experiment | Transition Strategies exhibiting\textsuperscript{a} | Low damping-off incidence | Intermediate damping-off incidence | High damping-off incidence |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>GH</td>
<td>H+C, TF+C</td>
<td>H, FV, FV+C</td>
<td>TF</td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>H+C, H</td>
<td>TF, TF+C</td>
<td>FV, FV+C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>H+C, H</td>
<td>TF+C, FV</td>
<td>TF, FV+C</td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>H+C</td>
<td>TF, H</td>
<td>TF+C, FV, FV+C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} N=12 pots in the greenhouse or n=8 field sites per treatment were analyzed for differences in damping-off under pathogen pressure. For the tomato, low is defined as 50-60% damping off; intermediate as 60-70%; and high, as 70-80%. For soybean in the greenhouse, low corresponds to 40-60% damping-off; intermediate 60-80%; and high > 80%. For soybean grown in the field, low 70-80%; intermediate, 80-90%; and high, >90%.

\textsuperscript{b} Abbreviations: TF, tilled fallow; H, mixed hay; FV, field vegetable; +C, compost amended treatment.

Table 2.3. Damping-off incidence levels observed in tomato and soybean grown in the greenhouse (GH) and the field, in soils previously cropped with different transition strategies.
<table>
<thead>
<tr>
<th>Crop</th>
<th>Sample&lt;sup&gt;c&lt;/sup&gt;</th>
<th>lowest damping-off incidence</th>
<th>mid/high damping-off incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato</td>
<td>R</td>
<td>0.67</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.61</td>
<td>0.50</td>
</tr>
<tr>
<td>Soybean</td>
<td>R</td>
<td>0.69</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.73</td>
<td>0.54</td>
</tr>
<tr>
<td>Tomato+Soybean</td>
<td>R</td>
<td>0.68</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>0.68</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>Tomato+Soybean</strong></td>
<td><strong>R+S</strong></td>
<td><strong>0.68</strong></td>
<td><strong>0.54</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Terminal restriction fragments generated by restriction digest of 16S rDNA amplicon with *MspI*.

<sup>b</sup> To calculate the correlation coefficient transition strategies were grouped into three levels of disease, based on % pre-emergence damping-off (see Table 2.3). Pair wise correlations on rank data were performed only if n≥5.

<sup>c</sup> R: rhizosphere; S: soil.

<sup>*</sup> Chi-square, *P* =0.055.

Table 2.4. Comparison of the proportion of negative correlations between percent pre-emergence damping-off and the relative abundance of individual 16S rDNA terminal restriction fragments (TRF) associated with transition strategies exhibiting different damping-off incidence levels, across multiple experiments.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Crop</th>
<th>Experiment</th>
<th>TRF</th>
<th>Correlation of TRF abundance with % damping-off at differing disease severity levels</th>
<th>No. of negative correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizosphere</td>
<td>Tomato</td>
<td>GH</td>
<td>M141</td>
<td>Low: -0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mid: -0.52</td>
</tr>
<tr>
<td>Field</td>
<td>M148</td>
<td>-0.57</td>
<td>NA</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>M488</td>
<td>-0.21</td>
<td>-0.47</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>M137</td>
<td>0.29</td>
<td>-0.15</td>
<td>0.12</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M139</td>
<td>-0.18</td>
<td>-0.13</td>
<td>0.43</td>
<td>2</td>
</tr>
<tr>
<td>Field</td>
<td>M141</td>
<td>-0.02</td>
<td>-0.11</td>
<td>0.55</td>
<td>2</td>
</tr>
<tr>
<td>Field</td>
<td>M148</td>
<td>-0.04</td>
<td>0.00</td>
<td>0.28</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M159</td>
<td>-0.32</td>
<td>0.15</td>
<td>-0.06</td>
<td>2</td>
</tr>
<tr>
<td>Field</td>
<td>M401</td>
<td>-0.35</td>
<td>0.41</td>
<td>-0.09</td>
<td>2</td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>M141</td>
<td>-0.41</td>
<td>NA</td>
<td>-0.35</td>
</tr>
<tr>
<td>Field</td>
<td>M148</td>
<td>0.24</td>
<td>0.58</td>
<td>0.58</td>
<td>0</td>
</tr>
<tr>
<td>Field</td>
<td>M489</td>
<td>-0.17</td>
<td>-0.48</td>
<td>-0.35</td>
<td>3</td>
</tr>
<tr>
<td>Soil</td>
<td>Tomato</td>
<td>GH</td>
<td>M137</td>
<td>-0.06</td>
<td>-0.49</td>
</tr>
<tr>
<td>Field</td>
<td>M139</td>
<td>NA</td>
<td>NA</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>M141</td>
<td>0.56</td>
<td>-0.45</td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M148</td>
<td>0.29</td>
<td>0.03</td>
<td>-0.27</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M159</td>
<td>NA</td>
<td>NA</td>
<td>-0.35</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M148</td>
<td>-0.36</td>
<td>0.00</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M180</td>
<td>-0.17</td>
<td>NA</td>
<td>0.11</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M401</td>
<td>-0.44</td>
<td>-0.15</td>
<td>-0.63</td>
<td>3</td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>M137</td>
<td>-0.20</td>
<td>0.00</td>
<td>-0.24</td>
</tr>
<tr>
<td>Field</td>
<td>M139</td>
<td>0.12</td>
<td>-0.72</td>
<td>-0.29</td>
<td>2</td>
</tr>
<tr>
<td>Field</td>
<td>M141</td>
<td>-0.08</td>
<td>0.00</td>
<td>0.19</td>
<td>1</td>
</tr>
<tr>
<td>Field</td>
<td>M148</td>
<td>-0.25</td>
<td>-0.26</td>
<td>-0.24</td>
<td>3</td>
</tr>
<tr>
<td>Field</td>
<td>M401</td>
<td>-0.58</td>
<td>-0.64</td>
<td>0.61</td>
<td>2</td>
</tr>
</tbody>
</table>

Overall: 55

<sup>a</sup> Terminal restriction fragments generated by restriction digest with *MspI*.

<sup>b</sup> Transition strategies were grouped into three levels of disease, based on % pre-emergence damping-off (Table 2.3).

<sup>c</sup> GH: greenhouse.

<sup>d</sup> Denotes TRF size in base pairs

<sup>e</sup> Spearman (ρ) correlation coefficients calculated with n≥5

<sup>f</sup> NA: not applicable, small sample size (n<5).

Table 2.5. Prevalence of negative correlations between percent pre-emergence damping-off and the relative abundance of individual 16S rDNA terminal restriction fragments (TRF) associated with transition strategies exhibiting the lowest damping-off incidence (i.e. TRF more abundant in mixed hay transition strategy).
Expected *MspI* TRF size (bp)*a* | Bacterial genera
---|---
137 | *Paenibacillus, Thiocapsa*
139 | *Actinocorallia, Bacillus* (2)*b*, *Chitinimonas, Leptothrix, Micromonospora* (6), *Nonomuraea, Ottowia, Paenibacillus, Parastreptomyces, Rhodococcus* (2), *Salinispora* (9), *Streptacidiphilus*
148 | *Actinomyces, Aerococcus* (8), *Bacillus* (2), *Cylindrospermopsis, Halobacillus, Ochrobactrum, Phyllobacterium, Rhodopseudomonas* (3)
489 | *Achromobacter, Bordetella, Delftia, Kinetoplastibacterium, Leeuwenhoekella, Pseudoalteromonas* (7), *Pseudomonas* (5), *Thiobacillus, Variobacter, Zobellia*

*a* TRF size was predicted based on *in silico* amplification with 8F and 1492R primers, followed by virtual digestion with *MspI* of the available 16S rDNA sequences available on the ribosomal database project tool using the MiCA 3 web-based software for microbial community analysis (Shyu et al., 2007).

*b* Number in parenthesis indicates the number of isolates of the genus that could generate that same fragment size, based on the database sequence information.

Table 2.6. Bacterial genera, based on *in silico* analysis, predicted to generate a terminal restriction fragment (TRF) corresponding to the size of the TRF associated with damping-off suppression.
Figure 2.1. Bacterial community profiles obtained from the rhizosphere of tomato grown in soils previously exposed to different transition strategies. Ordination plots for the first two principal components (PC) are shown. Plots were generated from the mean principal component scores for each transition strategy, with their corresponding standard error bars. The principal components analysis was performed using the relative abundance data of MspI generated 16S rDNA terminal restriction fragments. Tomatoes were grown in the greenhouse and the field in soils previously exposed to the following transition strategies: tilled fallow (TF), mixed hay (H) and field vegetables (FV), with (+C) and without compost amendment.
Figure 2.2. Bacterial community profiles obtained from the rhizosphere of soybean grown in soils previously exposed to different transition strategies. Ordination plots for the first two principal components (PC) are shown. Plots were generated from the mean principal component scores for each transition strategy, with their corresponding standard error bars. The principal components analysis was performed using the relative abundance data of *MspI* generated 16S rDNA terminal restriction fragments. Soybeans were grown in the greenhouse and the field in soils previously exposed to the following transition strategies: tilled fallow (TF), mixed hay (H) and field vegetables (FV), with (+C) and without compost amendment.
Figure 2.3. Bacterial community profiles obtained from soils where tomatoes were grown. Tomatoes were grown in the greenhouse and the field in soils previously exposed to different transition strategies. Ordination plots for the first two principal components (PC) are shown. Plots were generated from the mean principal component scores for each transition strategy, with their corresponding standard error bars. The principal components analysis was performed using the relative abundance data of MspI generated 16S rDNA terminal restriction fragments. Abbreviations, tilled fallow (TF), mixed hay (H) and field vegetables (FV), with (+C) and without compost amendment.
Figure 2.4. Bacterial community profiles obtained from soils where soybeans were grown. Soybeans were grown in the greenhouse and the field in soils previously exposed to different transition strategies. Ordination plots for the first two principal components (PC) are shown. Plots were generated from the mean principal component scores for each transition strategy, with their corresponding standard error bars. The principal components analysis was performed using the relative abundance data of MspI generated 16S rDNA terminal restriction fragments. Abbreviations, tilled fallow (TF), mixed hay (H) and field vegetables (FV), with (+C) and without compost amendment.
CHAPTER 3

ASSOCIATION OF MULTIPLE FUNGAL AND OOMYCETE POPULATIONS WITH DAMPING-OFF DISEASE INCIDENCE IN SOILS PREVIOUSLY MANAGED UNDER DIFFERENT ORGANIC TRANSITION STRATEGIES

ABSTRACT

Fungal and oomycete populations likely contributing to damping-off disease incidence of tomato and soybean, in a transitional organic experiment, were identified through terminal restriction fragment length polymorphism (T-RFLP) of the internal transcribed spacer region (ITS). Previously, soils from three transition strategies were observed to differ in damping-off incidence of subsequent tomato and soybean crops. Principal component analysis (PCA) of the T-RFLP data revealed distinct fungal and oomycete community composition in response to transition strategies. To better visualize the associations between management, microbial communities and disease, ordination plots were generated with samples arranged according to disease incidence. Six terminal restriction fragments (TRF) consistently contributed to the variation within the first three PC across experimental contexts. In addition, non-parametric analysis of variance revealed a small subset of TRF significantly more abundant ($P < 0.1$) in the transition strategies with high disease incidence. From these TRF two, H116 and H312, were
observed to be significant in more than one experimental context. Pair-wise correlations between the abundance of individual TRF and damping-off incidence revealed that three (H99, H118 and H128) and five (H116, H128, H316, H323 and H329) TRF were positively correlated with damping-off incidence of tomato and soybean, respectively. Terminal restriction fragment H128 appeared positively associated with damping-off in both crops. Fungal and oomycete isolates were obtained from soils of the same field site and their T-RFLP profiles were determined. From the isolates analyzed, genera that match in size the TRF associated with disease incidence in this study include the fungi *Fusarium* (H116, H118, H316), *Chaeotomium* (H128) and *Alternaria* (H329), and the oomycetes *Pythium* (H99) and *Phytophthora* (H316). Species within these genera are well known soilborne pathogens of tomato and soybean. Therefore, members from these genera likely contributed to damping-off of tomato and soybean in the studied system. In addition, their contributions to disease incidence appear to not only differ in response to crop but also to site of experiment. The results of this study substantiate the utility of T-RFLP analyses for the study of soilborne diseases caused by multiple pathogen populations.

**INTRODUCTION**

Fungi and oomycetes, as well as bacteria, perform several functions in the soil ecosystem, which can directly or indirectly affect plant growth. In spite of their distinct phylogeny and cell structural differences (Gunderson et al., 1987), fungal and oomycete communities share not only growth pattern similarities (as seen from their mycelial type
of growth) but also some ecological roles in soil and their associations with plants. For instance, through their saprophytic growth fungal and oomycete species participate in organic matter decomposition and contribute to nutrient cycling. Hyphae and mycelia in soil contribute to aggregate formation and soil structure. Many species are opportunistic or obligate plant pathogens. On the other hand, some fungal species form close mutualistic relationships with plants (i.e. mycorrhizae and endophytes) (Park, 1963; Agrios, 1997; Finlay, 2007). In addition, some fungal and oomycete species are well-studied plant pathogen antagonists or mycoparasites and are known to induce systemic resistance in plants (Al-Rawahi and Hancock, 1998; Harman, 2006).

Together with bacteria, fungal and oomycete populations in soil are responsive to nutrient input, crop diversity, and other disturbances. Studies aimed at understanding fungal and oomycete responses to stimuli in soil and their associations with plant species have utilized multiple methods. These include for example culture-based enumeration and identification of isolates or propagules in soil, fungal biomass, or metabolic activity measurements, phospholipid fatty acids analysis, (e.g. Larkin and Honeycutt, 2006; Wakelin et al., 2008), DNA or RNA based community profiles (e.g. Wu et al., 2007; Wu et al., 2008) and arrays (e.g. Tambong et al, 2006; Zhang et al., 2008), and clone library analysis (e.g. O’Brien et al., 2005). Changes in fungal populations have been described through molecular and/or phospholipid fatty acid profiles, between agricultural land management and in response to compost amendments (Kuter et al., 1983; van Elsas et al., 2002; Buckley and Schmidt 2003; Pérez-Piqueres et al., 2006; Garbeva et al., 2006; Wu et al., 2008), as well as an increase in fungal biomass when comparing improved with unimproved grasslands (Kennedy et al., 2005). Tillage has a negative impact in fungal
and oomycete populations. For example, a reduction in fungal biomass (Follett and Schimel, 1989), as well as in isolate recovery (Workneh et al., 1998) has been observed in intense till systems compared to less tillage-intense systems. Similarly, tillage has shown to affect the structure and diversity of mycorrhiza colonization in maize roots (Jansa et al., 2003). Though sometimes interspersed within fungal community studies, examples are available regarding oomycete community structure dynamics (Arcate et al., 2006), with a greater number of studies focusing on populations of individual plant pathogenic genera and their population structure within hosts (e.g. Martin and Loper, 1999; Garzón et al., 2005; Broders et al., 2007a).

Culture-independent studies of microbial communities rely on the analysis of DNA or RNA sequences such as the small subunit of the rDNA (16S and 18S for prokaryotes and eukaryotes, respectively) and the internal transcribed spacer region (ITS), found between the small and large rDNA subunits (Anderson and Cairney, 2004). The small subunit of the rDNA region has been widely used for phylogenetic purposes, since its use for defining the tree of life by Carl Woese and collaborators (Fox et al., 1980; Woese et al., 1990), and provides resolution up to the genus level. The ITS region, in contrast, has been used to resolve phylogeny up to species and subspecies levels, due to greater sequence variability and greater evolutionary rates (White et al., 1990). Both the 18S rDNA and ITS regions were used previously for fungal, and other eukaryote, diversity studies, with both regions presenting similar limitations (Anderson et al., 2003). Compared to bacteria, culture-independent methodologies have been less intensively exploited for profiling of whole fungal and oomycete communities (Anderson and Cairney, 2004). Instead these high throughput methodologies tend to be targeted to either
specific functional groups or environments. For instance, the use of terminal restriction fragment length polymorphism (T-RFLP) of ITS amplicons from fungal populations has been widely directed towards the study and identification of mycorrhizae in soils (Dickie and FitzJohn, 2007). Similarly, ITS T-RFLP was used to study fungal populations in spruce wood (Allmer et al., 2006) and a high throughput fingerprinting technique (oligonucleotide fingerprinting of rRNA genes) was applied to study fungal populations within the nematode cyst to identify nematode-antagonist populations (Yin et al., 2003).

An organic transition strategy experiment was established to assess biological, agronomical and economical aspects associated with the transition from conventional to organic farming. In that system, one of the four transition strategies studied resulted in the consistent suppression of damping-off incidence of tomato and soybean seedlings (Baysal et al., 2008). Baysal et al. (2008) observed subtle differences in soil chemistry between the studied transition strategies. Significant changes, however, were observed mainly in response to compost application in contrast to the main transition effects. These observations suggest a greater contribution of microbial populations to the suppression differential. Bacterial community analysis of soils and rhizosphere samples of tomato and soybean seedlings grown in these soils revealed multiple populations associated with damping-off suppression induced by the mixed hay strategy (Benítez et al., 2007). Changes in bacterial populations, however, may not be the only cause of the differences observed in damping-off between the transition strategies. The analysis of fungal and oomycete communities can provide information regarding the pathogen populations involved in the studied system. In addition, fungal and oomycete populations could also contribute to pathogen antagonism and disease suppression. Finally, the interactions
between fungal and oomycete populations with management practices, and other biotic components of soil can also be assessed. Studies on individual pathogen populations, such as *Sclerotinia* stem rot of soybean, indicated pathogen responses to multiple components of agricultural systems, such as management and microbial communities (Rousseau et al., 2006). In order to more fully understand the agroecosystem under study, in this chapter we focused on the analysis of fungal and oomycete community profiles, generated through terminal restriction fragment length polymorphism (T-RFLP) of the ITS region. We hypothesized that transition strategy results in variation between fungal and oomycete community profiles. In addition, we hypothesized that the abundance of individual fungal and oomycete populations responds to transition strategy and compost amendment. And, lastly that individual fungal and oomycete population abundance in conducive contexts positively correlates with damping-off incidence of subsequent crops.

**MATERIALS AND METHODS**

**The system of study.** An organic transition field experiment was established at the Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH to address economic, environmental and biological impacts of multiple management strategies during the transition period. Four transition strategies were considered, tilled fallow (TF), mixed hay species (H), low intensity open field vegetables production (FV) and high tunnel vegetables production (HT). Split-plots within the main transition strategy plots were arranged to address amendment effects during this period. Within this experiment, damping-off suppressiveness bioassays were performed for tomato and soybean in the greenhouse and the field, in soils previously exposed to the stated
transition strategies. More detailed description of the organic transition field experiment and the damping-off suppressiveness bioassays is presented on Baysal et al. (2008) and the first chapter of this dissertation. Soybean and tomato seedlings from multiple damping-off bioassays were sampled not only to assess differences in disease incidence and seedling vigor, but also to further study microbial communities associated to the studied transition strategies. The roots of one seedling per pot/field site and its adhering soil, after shaking (rhizosphere), were sampled for DNA extraction. After plant removal and rhizosphere sampling, soil was mixed and homogenized by hand; and any remaining visible plant material was removed. Soil from each pot/field site was also sampled for DNA extraction. Rhizosphere and soil samples were stored at -20°C until processing. Microbial community studies excluded information of the high tunnel transition strategy due to greater environmental variation between this and the open field plots.

**Fungal and oomycete community profiling.** Differences in fungal and oomycete community structure in response to transition strategy were determined from terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified internal transcribed spacer region (ITS, between the 18S rRNA and 28S rRNA). The Ultra-Clean soil DNA extraction kit (MoBio) was used for DNA extraction starting from 0.5 g of soil and 0.3 g of rhizosphere samples. 1:25 dilutions of soil and rhizosphere DNA were used for PCR amplification of the ITS region with the ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') forward and reverse primers, respectively, based on those described by White (et al., 1990). The ITS5 primer was labeled with the fluorescent WellRED dye D4 (Sigma, Proligo) for further visualization of the terminal restriction fragments (TRF). Amplification was carried out
in 25 µl reactions containing 1X Mg-free buffer (Promega Corp.), 1.8 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, (Sigma, Molecular Biology Reagent), 0.8 pmol each primer, 0.04 mg RNase A, 0.06 U GoTaq DNA polymerase (Promega), and 2.5 µl template. Amplification was performed with a PTC-200 Thermocycler (MJ Research Inc.). The cycling program consisted of a 5 min initial denaturation step at 95°C followed by 32 cycles of 94°C for 60 s, 52°C for 45 s, and 70°C for 2 min; and an 8 min final extension step at 70°C. Amplification products were separated on 1.5% agarose gels in 50% Tris-borate-EDTA buffer and visualized by ethidium bromide staining (1 mg l⁻¹). Prior to digestion, PCR products were purified by precipitation with 0.1X volume sodium acetate pH 5.2 and 2.5X volume ice-cold 95% ethanol. Samples were incubated for 30 min at -80°C and centrifuged at maximum speed for 15 min at 4°C. The pellet containing the DNA was rinsed twice with ice-cold 70% ethanol followed by a 2 min centrifugation step. Dried pellets were resuspended in water.

Restriction digestion of PCR products was performed in 10 µl reactions containing 0.3 U HhaI enzyme (Promega), 0.5X enzyme Buffer C and 3.5 µl PCR product. Samples were incubated for 4 h at 37°C followed by 20 min at 65°C for enzyme inactivation. 3.5 µl of the digestion reaction were diluted into 7 µl of water and sent to the Molecular and Cellular Imaging Center of the OARDC, Wooster, OH. There, 0.1 µl of sample were mixed with 0.5 µl 600 bp size standard (CEQ DNA size standard kit 600) and 40 µl formamide (loading solution). Terminal fragments were loaded and separated on the CEQ 8800 Genetic Analysis System (Beckman Coulter) and individual profiles were analyzed with the CEQ fragment analysis software (CEQ 8000 Genetic Analysis System).
**T-RFLP data analyses.** T-RFLP data were analyzed based on peak height of individual TRF. Individual profiles were generated for all samples and the profiles from each suppressiveness bioassays were analyzed separately, with a total of eight experimental contexts (2 crops in 2 sites, and soil and rhizosphere samples for each). Fluorescence peaks were considered in the analysis if fragment size ≥ 90 bp and fluorescence intensity (peak height) ≥ 300 fluorescence units. Binning was defined as ± 1 nt for TRF ≤ 300 bp, and ± 2 nt for TRF ≥ 300 bp.

Statistical analyses on T-RFLP data were performed according to Benítez (et al., 2007). First, the relative abundance of each TRF was expressed as the proportion of fluorescence of a TRF per total fluorescence of the sample. Ordination through principal components analysis (PCA) on covariance matrices was used to determine the relationship between microbial community structure, transition strategies and damping-off incidence levels. For PCA each TRF was considered as a different variable and data from each experimental context was analyzed separately. All statistical analyses were run using JMP (v7.0. SAS Inc.) statistical software package. Ordination plots were generated with Microsoft Excel from the mean principal component scores for each damping-off incidence level. For this, transition strategies were grouped into three damping-off incidence levels (high, medium and low) based on data from Baysal (et al., 2008), and as described in chapter 1 (Table 1.8). In addition factor loadings were analyzed to determine the relative influence of each TRF on the variation observed along the first three PC, according to Benítez et al. (2007) guidelines. The rank-based Kruskall-Wallis test was used to determine treatment differences in relation to the relative abundance of the selected TRF. For this analysis TRF were selected based on reproducibility within a
treatment, according to Benítez et al. (2007). Pair-wise treatment comparisons were performed based on the Wilcoxon 2-sample test. In addition, richness (total observed TRF per sample per treatment) and diversity (Shannon-Weiner index, H) measures were estimated for each treatment within each experimental context. Diversity (H) was calculated as follows: $H' = -\sum (p_i)(\log_e p_i)$, where $p_i$ is the proportion of an individual peak height relative to the sum of all peak heights in a sample. The quantitative associations between percent damping-off and the relative abundance of selected TRF (>60% criteria) were also determined. The Spearman correlation coefficients ($\rho$) were calculated on a per pot/field site basis. For this, samples were grouped according to disease incidence levels and correlations were run only if $n \geq 5$. To determine differences in the prevalence of negative and positive correlations, one-tailed paired t-tests were performed when considering groups of TRF. For individual TRF the probability of the distribution of positive and negative correlations being different than chance was tested according to a one-tail (directional) binomial test. Finally, quantitative associations ($\rho$) between fungal and oomycete ITS TRF and bacterial 16S TRF (from Benítez et al., 2007) were determined on a per pot/field site basis.

**Fungal and oomycete cultures.** Representative isolates of known soybean and tomato damping-off and root pathogens, and biocontrol agents, included, *Pythium aphanidermatum*, *Phytophthora capsici* and *Trichoderma harzianum* provided by S. Miller (OARDC); *Pythium ultimum* and *P. sylvaticum*, *Phytophthora sojae* and *Rhizoctonia solani* provided by A. Dorrance (OARDC); *Fusarium graminearum* provided by P. Paul (OARDC) and *Alternaria solani* Mg23 and *Fusarium oxysporum* Ft25 (Gutierrez Chapin et al., 2006). In addition, fungal and oomycete isolates were
obtained from soils of the organic transitions field experiment. Soil samples from summer 2006 were air-dried and stored at 12°C. Before sawing, soils were watered thoroughly with distilled water. Tomato and soybean seeds were planted in multiple pots in soils from different transition strategies. Isolates were obtained from seedling roots and shoots, rotten seeds, and soil. Initial isolations were performed on water agar, and incubated at room temperature in the dark. Pure cultures were obtained through hyphal tip transfers and cultures were stored in 1/5 potato dextrose agar (PDA, Difco-BD).

**Analysis of individual isolates.** Isolates of either, known soybean and tomato damping-off and root pathogens, or isolates obtained from field soils were analyzed to determine their respective ITS TRF size. For this, fungal and oomycete isolates were grown on either 1/10 trypticase soy (TS, Difco-BD) agar or 1/5 PDA. A mycelia suspension in water was prepared by picking mycelia with a toothpick and placing it into a 1.5 ml microcentrifuge tube prefilled with 500 µl sterile distilled water. After vigorous vortexing, 200 µl of the suspension were transferred into a 48-welled PCR plate and stored at -80°C. Prior to the PCR reaction setup, mycelia suspensions were thawed at 80°C for 5 min and stored at 4°C until use. The ITS PCR reaction for the region was setup as described above, with 2.5 µl freeze-thawed mycelia suspension as template. Parallel PCR reactions were run with samples for T-RFLP and for sequencing. Samples for T-RFLP were processed as described above for DNA samples. The sequence of the ITS region was used for classification purposes. For sequencing, ITS PCR products from individual isolates were treated with ExoSAP-IT (USB) according to manufacturer’s recommendations. PCR products were sequenced directly with ITS 5 primer. Sequencing was performed at the Molecular and Cellular Imaging Center of the OARDC (Wooster,
OH) in an ABI Prism 3100xl genetic analyzer system using 3’-BigDye dideoxynucleotide triphosphates labeling chemistry.

RESULTS

Principal component analysis on T-RFLP profile data. Principal component analysis (PCA) was performed to determine associations between transition strategies, damping-off disease incidence, and the presence and abundance of ITS terminal restriction fragments (TRF). For this analysis all TRF present in a profile were considered, and each TRF represented a different variable. In addition, each experimental context (crop, experimental site and sample – rhizosphere or soil) was analyzed separately. Ordination plots were generated for the first three principal components, from the mean principal component scores for each damping-off disease level (based on Baysal et al., 2008 data, as shown in Table 1.8). This information summarized the ITS T-RFLP profile data according to the relationship between transition strategy and disease incidence. Previously it was shown that the studied transition strategies impacted in different ways damping-off incidence of tomato and soybean (Baysal et al., 2008). The observed variation explained by the first two principal components ranged between 35-94% in all bioassays and including soil and rhizosphere samples. The third principal component contributed between 4-13% of the observed variation.

Fungal and oomycete community profiles appeared to differ according to disease incidence level observed between transition strategies. Samples originating from low disease incidence transition strategies separated from samples from high disease incidence in multiple scenarios; though most of the separation was observed along the
second and third PC, rather than the first. For tomato, separation between high and low
disease incidence samples was observed for soil samples from the field (along PC2; Figure 3.1), and rhizosphere samples from the greenhouse and the field (along PC3 in both scenarios; Figure 3.2). For soybean, high disease incidence separated from low
disease incidence in greenhouse and field soil samples (PC1 and 3, and PC3 respectively; Figure 3.3), and greenhouse rhizosphere samples (PC1 and 2; Figure 3.4). Samples corresponding to intermediate disease not necessarily differentiated from low and high
disease incidence samples.

As indicated by differences in community profile by disease incidence level, fungal and oomycete communities were influenced by transition strategy and compost amendment. For all experimental contexts, at least one compost-amended strategy differentiated from its un-amended counterpart along one of the first three PC (data not shown). Individual transition strategies differentiated among them only three out of the eight experimental contexts (in the absence of compost), whereas at least one amended transition strategy separated from the other two in six out of the eight contexts (data not shown). Overall, greatest separation was observed along the second and third principal component and compost effects were more evident than transition strategy effects.

The relative contribution of individual TRF to the separation of treatments and disease incidence levels along the first three principal components is shown in Table 3.1. A small subset of the TRF population, 2 – 11 from 40 – 101 TRF present per experimental context, had a factor loading of $|x| \geq 0.70$ for at least one of the first three principal components. Terminal restriction fragment H108/109 contributed to the variation along the first PC in all of the tomato scenarios and soybean field soil. In these
contexts, however, disease incidence levels did not differentiate along PC1, suggesting TRF H108/109 presence and abundant was equivalent for most of the samples. Terminal restriction fragment H118/119 had high factor loadings on the tomato soil contexts and all of the soybean scenarios. For soybean greenhouse and field soils, H118/119 loading factor corresponded with the separation between high and low disease incidence with the same direction as the latter (sign of the loading factor). Though separation was not evident in the other scenarios, the correlation between direction of H118/119 loading factor and low disease incidence was maintained. Other TRF contributing with high loading factors to the variation along the first three PCs were H128 and H131 in rhizosphere samples, H99/100 in greenhouse soil samples and H326 in field soil samples. Of these, no consistent association between TRF and separation of disease incidence levels was observed across different contexts.

**Transition strategy effects on TRF abundance and diversity.** Transition strategy influenced the relative abundance of fungal and oomycete TRF. Overall, the sum of the total fluorescence signal for the fungal/oomycete ITS T-RFPL samples was lower in the mixed hay compared to the field vegetables and tilled fallow, if comparing between transition strategies (Figure 3.5-3.6). This was observed five out of the eight times for the un-amended strategies and three out of the eight experimental contexts for the amended counterparts. Significance, however (Kruskal-Wallis, $P<0.1$), was observed only for the amended soybean rhizosphere samples and the un-amended tomato field soil samples. This pattern implied that as a whole the fungal and oomycete populations, as indicated by total fluorescence signal, were smaller in the disease suppressive context (i.e. mixed hay transition strategy). Similarly, though no significance was observed in most experimental
contexts, richness and diversity of TRF appeared to be consistently low in the mixed hay, at least compared to one of the other two analyzed transition strategies (Tables 3.2 – 3.3).

Consistent with the previous observations of total fluorescence data, a subset of TRF were significantly less abundant in the mixed hay compared to the tilled fallow and the field vegetables (Table 3.4). Because of the variability in TRF incidence between samples, the data set was sifted to select TRF occurring with a certain degree of replication. Thus, for each experimental context, TRF present in ≥ 60% of the samples of at least one treatment were selected for further analysis. The number of TRF selected varied between bioassays, and overall the selected TRF represented the majority of the fluorescence of each bioassay. The proportion of significant observations varied according to experimental context, and only two TRF were significant in two different contexts (Table 3.4). For the soils from tomato grown in the greenhouse, six TRF were selected from 62, and from these, TRF H116 and H131 were more abundant, in terms of proportion of fluorescence, in the un-amended and amended tilled fallow compared to the mixed hay. In the soils from tomato grown in the field, six out of 67 possible TRF were selected; from which TRF H116 was significantly higher in the un-amended field vegetables, and H329 in the amended mixed hay. Ten from 75 TRF were selected for the soils from soybean grown in the greenhouse. Five of these fragments were significantly higher for a treatment or set of treatments. H307, H312, H316 and H654, were more abundant in either the tilled fallow or field vegetables, compared to the mixed hay. Terminal fragment H323, in contrast, was significantly more abundant in the soils from the un-amended mixed hay. In the soybean field soils, 18 out of 101 TRF were selected and nine showed significant differences. From these, H122, H287, H312 and H326 were
more abundant in either (or both) the tilled fallow or field vegetables, compared to the mixed hay. Terminal fragments H99 and H101 were more abundant in the amended mixed hay than the tilled fallow, but similar in the field vegetables. Terminal restriction fragments H131, H172 and H323 were high in the un-amended mixed hay, but except for H172, were not necessarily significantly different than field vegetables and tilled fallow. Though the overall fluorescence signal in the rhizosphere samples was greater than the signal in the soil samples (Figure 3.5-3.6), the total number of TRF present per samples was lower in the rhizosphere. Likewise, the number of TRF with significant difference in abundance per transition strategy was lower. For tomato greenhouse samples, from 42 TRF three were selected and none showed significant differences between treatments. In the field, six out of 67 TRF were selected with no significant differences observed between treatments. From the soybean greenhouse samples, five out of 40 TRF were selected, from which only H99 was significantly higher in the amended tilled fallow. Also six TRF were selected out of 48 present in the soybean rhizosphere field samples, three of which showed any differences. Terminal restriction fragment H128 was more abundant when plants were grown in the amended field vegetables, and H136 and H175 were less abundant in the amended mixed hay soils.

**Quantitative relationships between damping-off incidence and selected TRF.**

To further quantify the relationship between the relative abundance of selected TRF and damping-off incidence, pair-wise correlation analysis was performed. The Spearman correlation coefficient was calculated in a per-pot/field site basis. As described previously, to ensure a sample size of n ≥ 5, and due to the high disease incidence observed in certain experimental contexts, data were analyzed according to disease
incidence levels (Table 1.8). Individual pair-wise correlations were performed for those TRF that contributed to the variation observed along the first three PC in at least two experimental contexts (H99/100, H108/109, H118/119, H128, H131, H316, H326), TRF that were significantly less abundant in the mixed hay in at least two experimental contexts (H116, H312), and TRF more abundant in the mixed hay in at least one context (H172, H323 and H329).

We hypothesized that TRF less abundant in the mixed hay, and contributing to separation (and direction), as shown by PCA, of high disease incidence levels, had a greater proportion of positive correlations between the relative abundance of an individual TRF and damping-off incidence. The prevalence of positive correlations with damping-off incidence of each individual TRF, considering all experimental contexts, was not different than the expected by chance (paired t-test, one-tail, $P>0.1$). Terminal restriction fragment H99, however had a greater number of positive associations with damping-off incidence (paired t-test, one-tail, $P=0.03$; Table 3.5) for tomato samples only. Similarly, TRF H329 had a greater number of positive associations with damping-off incidence for soybean samples (paired t-test, one-tail, $P=0.04$; Table 3.6). Other TRF with higher number of positive correlations were H118 and H128 for tomato and H116, H128, H316 and H323 for soybean ($P>0.1$; Tables 3.5-3.6). In contrast, for tomato samples, TRF H108 and H316 had a greater number of negative associations with damping-off incidence (paired t-test, one-tail, $P<0.09$).

**Quantitative relationships between ITS TRF and 16S TRF.** As described in Benítez (et al. 2007), a similar approach as the presented in this chapter was followed to determine the effects of transitional organic managements in damping-off incidence and
bacterial community structure. In that work, multiple TRF were consistently associated with the mixed hay transition strategy, which resulted in the lowest damping-off incidence. Here, the associations between the relative abundance of bacterial 16S TRF and fungal/oomycete ITS TRF was explored through pair-wise correlation analysis. Spearman correlations were performed as described previously, both for individual TRF or TRF grouped according to their positive or negative relationship with damping-off incidence.

The most consistent association observed between 16S and ITS TRF relative abundance, was a positive correlation between abundance of these two TRF populations. This pattern was observed for total 16S fluorescence signal versus total ITS fluorescence signal, were a greater proportion of positive correlations, greater than expected by chance, was observed ($P=0.02$) (Table 3.7). A similar pattern, of greater number of positive correlations ($P=0.075$), was observed between all selected bacterial TRF against all selected fungal and oomycete TRF. For individual bacterial TRF, only TRF M148 and M141, showed greater number of positive correlations with total ITS TRF signal ($P=0.007$ and 0.06). This was more evident for tomato ($P=0.01$ for both M148 and M141) compared to soybean samples ($P>0.1$). For tomato samples, TRF 139 also showed greater number of positive correlations ($P=0.08$). No differences in patterns, or significance in prevalence of a direction of the correlation, were observed if the analysis was made for the disease associated ITS TRF as a group (from Tables 3.5 and 3.6). For individual 16S TRF versus ITS TRF, correlations between TRF M139 and H99, H128, H329 tended to be positive ($P<0.04$). Similarly, correlations between TRF M141 and H99, H116, H118, H128, H131 and H323 were also predominantly positive ($P<0.06$). In
contrast, for 16S TRF M400 correlations with ITS TRF were predominantly negative, especially against H99, H131 and H329 ($P<0.02$).

**T-RFLP analysis of individual isolates.** A collection of 46 isolates was generated from tomato and soybean samples, grown in soils from the Peri-Urban field experiment (Summer 2006 field soil). Isolates from this collection, were classified, based on ITS sequence and sequence comparison with GenBank database entries. In addition, the individual T-RFLP profile for each isolate was obtained. The TRF of each individual isolate was compared to the TRF size of populations positively correlated with damping-off incidence in this work (Tables 3.5 – 3.6) as an attempt to classify these populations. Table 3.8 summarizes the fungal and oomycete genera, from our isolate collection, matching in size the TRF from this study. Fungal and oomycete populations producing one of the TRF associated with disease in this study corresponded to the genera *Pythium*, *Phytophthora*, *Fusarium*, *Alternaria* and *Chaetomium*. Since it was possible that more than one genera produced a TRF of a specific length, then it was probable that the TRF observed in our profiles came from a different organism not identified in our collection. The genus most commonly isolated from these soils was *Pythium*, representing 60 % of the samples (with 50 % likely corresponding to *P. ultimum*), followed by *Fusarium* (20 % of samples). *Pythium* and *Fusarium* isolates were obtained from soil samples corresponding to the three transition strategies. Though the sample size was small for characterization of isolate diversity, there was a difference in genotype richness (based on ITS TRF size) of isolates obtained from soils of each transition strategy, with greater richness in the most conducive transitions (TF=4, TF+C=8, FV=7, H=2). This observation was consistent with previous results described above were overall the fungal
and oomycete T-RFLP signal and individual TRF were less abundant in the mixed hay transition strategy compared to the tilled fallow and field vegetables.

DISCUSSION

In this work, distinct fungal and oomycete populations were distinguished, from whole community profile analysis, for their association with damping-off disease incidence. Principal component analysis (PCA) of the T-RFLP profile data revealed differences in community structure of samples originating from transition strategies differing in damping-off incidence (Figure 3.1-3.4). These differences are a reflection of transition strategy effects on both microbial communities and disease. Transition strategy effects in bacterial community structure were demonstrated previously (Benítez et al., 2007). Principal component analysis, and other multivariate ordination and cluster analyses are widely applied on T-RFLP data and other microbial community profiling methodologies. Compared to richness or diversity measures (Tables 3.2 - 3.3), ordination or cluster analysis provides better differentiation between management/treatment effects. This was shown for instance by Hartmann and Widmer (2006) who compared the use of diversity indices versus cluster analysis for bacterial T-RFLP data from three agricultural management practices in long-term plots in Switzerland. Similarly, when using diversity and richness measures of fungal community profiles (length-heterogeneity PCR) Wu et al (2007) could not differentiate to the same extent the effects of management on fungal communities, than when using cluster analysis. Diversity indexes based on T-RFLP data could be underestimated in situations were two or more species generate a TRF of the same size (Hartmann and Widmer, 2006). On the other hand, overestimation could result
from the formation of pseudo-terminal restriction fragments (Egert and Friedrich, 2003) or hypervariability within the gene of study (Avis et al., 2006). From the analysis of PC structure (based on loading values) it is possible to determine which variables, in this case TRF, contribute the greatest to the variation encompassed by each PC (McGarigal et al., 2000). This information allows generating hypothesis regarding association with disease incidence levels. Similar to the observations for bacterial communities (Benítez et al., 2007), only a small subset of the whole TRF population highly contributed to the variation along the first three PCs. Two individual TRF consistently contributed to the variation across six out of the eight experimental contexts (H108/109 and H118/119).

Multiple examples are available in the literature where plant species or agricultural management affect the structure of fungal and oomycete communities, as shown by culture-independent analyses (e.g. Gomes et al., 2003; Edel-Hermann et al., 2004; Klamer and Hedlund, 2004; Girvan et al., 2004; Pérez-Piqueres et al., 2006; Wu et al., 2007; Wu et al., 2008). The relative contribution of individual members of the community to the variation, however, is addressed only in a fraction of those studies (e.g. Gomes et al., 2003; Wu et al., 2007; Wu et al., 2008).

The fungal and oomycete populations associated to the mixed hay transition strategy appear to be less diverse and less abundant, than populations associated to the field vegetables and tilled fallow. This pattern is observed by analyzing on the one hand total fluorescence signal (Figure 3.5-3.6) and the T-RFLP richness and diversity measures (Shannon-Weiner) per transition strategy (Tables 3.2 – 3.3), and on the other hand from the results of non-parametric analysis of variance tests for individual TRF (Table 3.4). Though as stated above, T-RFLP-based diversity measures are constrained
within the limitations of this methodology. For most of the overall summary measures per transition strategy, significant difference between transition strategies are not observed, however the pattern of less abundance in the mixed hay compared to either the field vegetables or the tilled fallow is consistent across experiments. In addition, most of the individual TRF exhibiting a significant response to transition strategy were more abundant in either the field vegetables or tilled fallow compared to the mixed hay (Table 3.4). The fungal biomass and diversity, as well as the fungal/bacterial ratio found in grasslands or pastures, is influenced by fertility level and plant species composition (Grayston et al., 2004). Grayston et al. (2004) described fertile and more managed pastures (e.g. reseeded) as less fungal-dominated environments, compared with less fertile un-managed grasslands. As shown by the soil chemical analyses described in Baysal et al. (2008) and chapter 1 (Table 1.1) the mixed hay transition strategy had higher % C and % N compared to the field vegetable and tilled fallow. Therefore our observations of less fungal and oomycete T-RFLP abundance, support Grayston’s (et al., 2004) hypothesis of fertile pastures being less fungal-dominated. Contrary to these observations, previous studies comparing grasslands and agricultural land under different rotations described greater fungal biomass and activity in grasslands (van Elsas et al 2002, Garbeva et al., 2006), and Hiddink et al. (2005) reported no differences in microbial communities in response to mixed cropping systems compared to single cropping.

The use of multiple statistical analyses on fungal and oomycete ITS T-RFLP data allowed the association of individual fungal and oomycete TRF with disease incidence. In addition to the hypothesis generated by PCA described above, a small subset of the
total TRF population, 0 – 9 % of the TRF present in an experimental context, significantly differed in abundance between transition strategies. From these 17 distinct TRF (Table 3.4) only three showed to be significantly more abundant in the mixed hay compared to the other two transition strategies. The other 14 were more predominant in the field vegetables and/or tilled fallow, with TRF H116 and H312 showing significance in two out of the eight experimental contexts. In contrast, eight bacterial TRF (out of 19 distinct TRF significantly more abundant in one treatment) were observed to associate with the mixed hay transition strategy, with at least four TRF associated with suppression in multiple contexts (Benítez et al., 2007). Individual pair-wise correlations indicated an association of certain TRF with disease incidence levels observed in either tomato or soybean (Tables 3.5-3.6.). Tables 3.5 and 3.6 represent a summary of the frequency of observations of positive and negative correlations across experimental contexts. In contrast to bacterial populations there was not a consistent trend between crops or groups of TRF associated with suppression or disease suppression. In addition, those TRF that associated with disease incidence in soybean tend to have a reverse association for tomato samples, further indicating a crop effect in these subset of fungal and oomycete populations.

The probable identity of TRF associated with differences in disease incidence levels in this study was determined through direct comparison with fungal and oomycete isolates. Though further study is needed to confirm the identity of these TRF, some insight is given by the analysis of individual isolates of known tomato and soybean pathogens, or isolates obtained from soils of the studied system (Table 3.8). For instance, isolates of the soybean-associated TRF-matching genera have been previously associated
with disease. *Fusarium* (some species corresponding to H116 and H118), *Phytophthora* (some species corresponding to H316) and *Alternaria* (some species corresponding to H329) cause blight, wilt, root rot, damping-off and seed infestation (Hartman et al., 1999) of soybean, and the incidence of *Fusarium* and *Phytophthora* in soybean in Ohio soils is known to be high (Dorrance et al., 2003; Broders et al., 2007b). For tomato, *Pythium* (some species corresponding to H99) may cause seed rot, damping-off, stem rot; and *Fusarium* (some species corresponding to H118) causes crown and root rot or wilt (Jones et al., 1991). Non-pathogenic species, however, have also been described for each of the genera mentioned above, including endophytes and soil saprophytes, some of which can antagonize plant pathogen growth (Larkin and Fravel, 1998; Al-Rawahi and Hancock, 1998). An isolate obtained from tomato grown in field vegetable soils matched the 128 nt TRF size, suggesting that TRF H128 in our profiles, could correspond to the genus *Chaetomium*. *Chaetomium* species have been shown to be associated with seed infestation (Lima et al., 1997) and to reduce quality of soybean seed (Jordan et al., 1986). In addition, some species are endophytes of soybean roots (Mueller et al., 1985) or pathogenic to avocado and castor bean (Lima et al., 1997; Violi et al., 2007). Some endophytic and saprophyte isolates, however, are antagonists of soilborne pathogens such as *R. solani* (Gao et al., 2005) and *P. ultimum* (DiPrieto et al., 1992). The greenhouse bioassays discussed in this work included the addition of pathogen inocula in the soil. For soybean the inoculum was a mixture of *P. ultimum* and *P. sojae*, which generated an *HhaI* TRF of 99 and 315 nt, respectively. Terminal restriction fragments of both sizes (±1) were detected in all soybean contexts, except for the field rhizosphere samples. Both TRF sizes were statistically associated with disease incidence, even in the field assays.
without added inocula. Though these pathogens most likely contribute to damping-off in this system, it is not clear what was the contribution of the added inocula compared to the native populations. Tomato greenhouse bioassays were inoculated with *P. ultimum* and *P. aphanidermatum* (*Hha* TRF of 580 nt). H99 was detected in all tomato rhizosphere and soil samples, whereas we failed to detect TRF H580 in any of the tomato profiles.

This study represents, to our knowledge, the first use of T-RFLP for the association and identification of individual fungal and oomycete TRF with damping-off disease incidence. In this work we targeted both fungal and oomycete populations based on the previous knowledge of species from both groups being common soilborne damping-off pathogens. In addition, this work provides an alternative methodology to be used for studies of complex pathogen populations, such as those causing damping-off. DNA-based macroarray systems, with the ability to detect multiple pathogen populations from one sample, allowing for high throughput analyzes of microbial populations, had been described (Zhang et al., 2008; Tambong et al., 06). Macroarray development, however, relies on *a-priori* sequence information, hence limiting the assessment of non-yet described populations. We were able to not only recover TRF associated to pathogens introduced to the soil, but also identify other pathogens that could be contributing to damping-off in the studied soils. Data provided in this work is consistent with the notion that seedling damping-off is caused by a pathogen complex. In addition, differences observed in TRF associations with disease in greenhouse versus field bioassays might result of different pathogens responding to different environmental conditions. Though other T-RFLP based studies demonstrate changes in fungal and oomycete community structure in a disease suppressive context (Pérez-Piqueres et al., 2006; Laurent et al.,
2008), statistical associations with individual TRF or a subset of the community are lacking. Terminal restriction fragment length polymorphism of fungal populations has been extensively used for the study and identification of mycorrhizae, but even within this less diverse group identification of isolates based on T-RFLP profile has not been successful (Dickie and FitzJohn, 2007). Few examples were found in the literature concerning T-RFLP community profiling of oomycetes. In two studies at an apple replant disease site oomycete T-RFLP communities differentiate between rootstocks varying in susceptibility and yield, and between sites with different levels of disease severity (Rumberger et al., 2007; Laurent et al., 2008). Terminal restriction fragment length polymorphism, as any other PCR-based culture-independent technique has certain limitations. First of all the relative abundance of each TRF (expressed as proportion of fluorescence of that TRF in relation to total fluorescence of the sample) is a quantitative value of the abundance of that individual TRF within each PCR amplification reaction. PCR amplification from environmental samples might be proportional to the amount of template in a sample, but other bias such as specificity of PCR primers and gene copy number can affect overall amplification (Osborn, et al 2000). Applicable to certain fungal populations, the efficiency of extraction of spores and sporocarps compared to hyphae will affect TRF richness measures (Avis et al., 2006). The nature of the ITS region, where intraspecific variation has been observed, will also influence the number of TRF per sample and hence T-RFLP profile based isolate identification. More than one TRF size could be produced by the same species, and more than one species can produce the same TRF size (Avis et al., 2006). In spite of these limitations, T-RFLP is a robust, reproducible and sensitive methodology (Osborn et al., 2000; Edel-Hermann et al., 2004).
In this study, the level of replication and the observation of patterns across experiments aid to overcome some of the described limitations of T-RFLP. Follow-up work, including isolate and TRF clone library analysis is necessary for classification purposes and further confirmation of function of TRF associated with disease incidence in the studied system.

ACKNOWLEDGEMENTS

This work was supported by the OARDC SEEDS Graduate Student Enhancement Program (2007093) to M.S. Benítez.

REFERENCES


Data were analyzed separately for each experimental context; each experimental context being a crop sample (S, soil or R, rhizosphere) at a specific experiment site (Field or GH, greenhouse).

Size in base pairs of each restriction fragment. Restriction fragments were generated after digestion of the ITS amplicon with *HhaI*.

Restriction fragments in bold correspond to those providing greatest discrimination between high and low disease incidence samples for each experimental context.

### Table 3.1. Fungal and oomycete ITS terminal restriction fragments (TRF) with high factor loadings (|x| ≥ 0.70) on the first three principal components (PC) for each experimental context

<table>
<thead>
<tr>
<th>Crop</th>
<th>Experiment</th>
<th>Sample</th>
<th>TRF with high factor loadings for</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>S</td>
<td>H100, H119f</td>
<td>H316</td>
<td>H128, H131</td>
<td>H118</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>H100, H119f</td>
<td>H316</td>
<td>H128, H131</td>
<td>H118</td>
<td>H326</td>
</tr>
<tr>
<td>Field</td>
<td>S</td>
<td>H109</td>
<td>H118</td>
<td>H131, H139</td>
<td>H118, H316, H514</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>H109</td>
<td>H118</td>
<td>H131, H139</td>
<td>H118, H316, H514</td>
<td></td>
</tr>
</tbody>
</table>

f Data were analyzed separately for each experimental context; each experimental context being a crop sample (S, soil or R, rhizosphere) at a specific experiment site (Field or GH, greenhouse).

f Size in base pairs of each restriction fragment. Restriction fragments were generated after digestion of the ITS amplicon with *HhaI*.

f Restriction fragments in bold correspond to those providing greatest discrimination between high and low disease incidence samples for each experimental context.
Terminal restriction fragments of the amplified ITS region were generated after digestion with \textit{HhaI}.

Analyzed samples were obtained from damping-off suppressiveness bioassays of soils previously exposed to different transition strategies under two amendment levels (Baysal et al., 2008). TF, tilled fallow; H, mixed hay; FV, field vegetables.

Data were analyzed separately for soybean and tomato samples grown in the greenhouse (GH) and the field.

Values correspond to the median richness values for each transition strategy (n= 2 – 8, depending on experimental context and damping-off incidence data).

Values followed by a different letter are significantly different within amendment regimes (smaller case, un-amended; large case, amended) at $P<0.05$ (Kruskal-Wallis followed by Wilcoxon 2-sample test).

<table>
<thead>
<tr>
<th>Crop</th>
<th>Experiment</th>
<th>Sample</th>
<th>Richness of ITS TRF(^a) within transition strategies(^b) under different amendment regimes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amended</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TF</td>
</tr>
<tr>
<td>Tomato</td>
<td>GH</td>
<td>S</td>
<td>7.0(^d)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>2.5</td>
</tr>
<tr>
<td>Field</td>
<td></td>
<td>S</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>22.5</td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>S</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>9.0</td>
</tr>
<tr>
<td>Field</td>
<td></td>
<td>S</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(^a\)Terminal restriction fragments of the amplified ITS region were generated after digestion with \textit{HhaI}.

\(^b\)Analyzed samples were obtained from damping-off suppressiveness bioassays of soils previously exposed to different transition strategies under two amendment levels (Baysal et al., 2008). TF, tilled fallow; H, mixed hay; FV, field vegetables.

\(^c\)Data were analyzed separately for soybean and tomato samples grown in the greenhouse (GH) and the field.

\(^d\)Values correspond to the median richness values for each transition strategy (n= 2 – 8, depending on experimental context and damping-off incidence data).

\(^e\)Values followed by a different letter are significantly different within amendment regimes (smaller case, un-amended; large case, amended) at $P<0.05$ (Kruskal-Wallis followed by Wilcoxon 2-sample test).

Table 3.2. Average richness of fungal and oomycete ITS terminal restriction fragments obtained from samples originating of tomato or soybean roots grown in soils previously exposed to the different transition strategies.
Terminal restriction fragments of the amplified ITS region were generated after digestion with HhaI.

Analyzed samples were obtained from damping-off suppressiveness bioassays of soils previously exposed to different transition strategies under two amendment levels (Baysal et al., 2008). TF, tilled fallow; H, mixed hay; FV, field vegetables.

Data were analyzed separately for soybean and tomato samples grown in the greenhouse (GH) and the field.

Values correspond to the median diversity index for each transition strategy (n= 2 – 8, depending on experimental context and damping-off incidence data).

Values followed by a different letter are significantly different within amendment regimes (smaller case, un-amended; large case, amended) at P<0.05 (Kruskal-Wallis followed by Wilcoxon 2-sample test).

<table>
<thead>
<tr>
<th>Crop</th>
<th>Experiment</th>
<th>Sample</th>
<th>Diversity of ITS TRF within transition strategies under different amendment regimes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amended</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TF</td>
</tr>
<tr>
<td>Tomato</td>
<td>GH</td>
<td>S</td>
<td>1.62&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>0.36</td>
</tr>
<tr>
<td>Field</td>
<td>S</td>
<td>1.79</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.02</td>
<td>1.04</td>
</tr>
<tr>
<td>Soybean</td>
<td>GH</td>
<td>S</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>1.31</td>
</tr>
<tr>
<td>Field</td>
<td>S</td>
<td>2.27</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>0.23</td>
<td>0.24</td>
</tr>
</tbody>
</table>

a Terminal restriction fragments of the amplified ITS region were generated after digestion with HhaI.
b Analyzed samples were obtained from damping-off suppressiveness bioassays of soils previously exposed to different transition strategies under two amendment levels (Baysal et al., 2008). TF, tilled fallow; H, mixed hay; FV, field vegetables.
c Data were analyzed separately for soybean and tomato samples grown in the greenhouse (GH) and the field.
d Values correspond to the median diversity index for each transition strategy (n= 2 – 8, depending on experimental context and damping-off incidence data).
e Values followed by a different letter are significantly different within amendment regimes (smaller case, un-amended; large case, amended) at P<0.05 (Kruskal-Wallis followed by Wilcoxon 2-sample test).

Table 3.3. Average diversity (Shannon-Weiner index) of fungal and oomycete ITS terminal restriction fragments obtained from samples originating of tomato and soybean grown in soils of different transition strategies.
Mean relative abundance, expressed as the percentage of total fluorescence of a TRF within each individual sample.

TRF were selected if present in 60% of the samples of at least one treatment. 40 - 101 TRF were observed per experimental context, and 3 - 20 of these were selected under the specified criteria. Only TRF exhibiting significance at least in one amendment level are shown.

Terminal restriction fragments of the amplified ITS region were generated after digestion with *HhaI*.

Transitions strategies: TF, tilled fallow; H, mixed hay; FV, field vegetable. For each transition a split-plot was included to address effects of composted dairy manure amendment.

TRF were obtained from DNA samples of multiple experimental contexts. Soil (S) and rhizosphere (R) samples of tomato and soybean grown in the greenhouse (GH) and in the field in soils previously exposed to different transitions strategies were analyzed.

Size in base pairs of ITS amplicons TRF after digestion with *HhaI*.

Median relative fluorescence values are shown.

Values within amendment type, followed by a different letter are significantly different at $P<0.1$, using the Kruskal-Wallis test followed by the Wilcoxon 2-sample test.

---

**Table 3.4. Relative abundance of a selected subset of fungal and oomycete ITS terminal restriction fragments (TRF) showing significant differences between transition strategies**
<table>
<thead>
<tr>
<th>TRF</th>
<th>Direction of correlation</th>
<th>Proportion of correlations with damping-off incidence for TRF associated with conducive strategies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tomato</td>
</tr>
<tr>
<td>H99</td>
<td>-</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.83 **</td>
</tr>
<tr>
<td>H118</td>
<td>-</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.70</td>
</tr>
<tr>
<td>H128</td>
<td>-</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.67</td>
</tr>
<tr>
<td>Sum of 3 TRF</td>
<td>-</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.74 ***</td>
</tr>
</tbody>
</table>

* Spearman pair-wise correlations were calculated in a per pot/field plot basis. Correlations were run by grouping transition strategies into three damping-off incidence levels (Table 1.8) and were calculated only if n≥5.

** See Table 1.8.

** Size in base pairs of ITS TRF after digestion with *HhaI*.

*** Significant paired t-test (number of positive correlations vs. number of negative correlations) at *P*<0.05 or **P**<0.001.

Table 3.5. Proportion of positive and negative correlations between percent damping-off and the relative abundance of individual fungal/oomycete ITS terminal restriction fragments (TRF) associated with transition strategies exhibiting high disease incidence in tomato seedlings
<table>
<thead>
<tr>
<th>TRF</th>
<th>Direction of correlation</th>
<th>Proportion of correlations with damping-off incidence for TRF associated with conducive strategies&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tomato</th>
<th>Soybean</th>
<th>Tomato+Soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H116&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td></td>
<td>0.83</td>
<td>0.20</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.17</td>
<td>0.80</td>
<td>0.45</td>
</tr>
<tr>
<td>H128</td>
<td>-</td>
<td></td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.67</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>H316</td>
<td>-</td>
<td></td>
<td>0.63</td>
<td>*</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.38</td>
<td>0.57</td>
<td>0.47</td>
</tr>
<tr>
<td>H323</td>
<td>-</td>
<td></td>
<td>0.50</td>
<td>0.33</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.50</td>
<td>0.67</td>
<td>0.60</td>
</tr>
<tr>
<td>H329</td>
<td>-</td>
<td></td>
<td>0.80</td>
<td>0.13</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.20</td>
<td>0.88</td>
<td>** 0.62</td>
</tr>
<tr>
<td>Sum of 5 TRF</td>
<td>-</td>
<td></td>
<td>0.67</td>
<td>0.28</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.33</td>
<td>0.72</td>
<td>* 0.55</td>
</tr>
</tbody>
</table>

<sup>a</sup> Spearman pair-wise correlations were calculated in a per pot/field plot basis. Correlations were run by grouping transition strategies into three damping-off incidence levels (Table 1.8) and were calculated only if n≥5.

<sup>b</sup> See Table 1.8.

<sup>c</sup> Size in base pairs of ITS TRF after digestion with HhaI.

** Significant paired t-test (number of positive correlations vs. number of negative correlations) at P<0.05 or * P<0.1.

Table 3.6. Proportion of positive and negative correlations between percent damping-off and the relative abundance of individual fungal/oomycete ITS terminal restriction fragments (TRF) associated with transition strategies exhibiting high disease incidence in soybean seedlings.
<table>
<thead>
<tr>
<th>Direction of correlation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proportion of correlations between 16S TRF and ITS TRF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tomato</td>
</tr>
<tr>
<td>-</td>
<td>0.30</td>
</tr>
<tr>
<td>+</td>
<td>0.70</td>
</tr>
</tbody>
</table>

<sup>a</sup> Correlations were run between total fluorescence signal of 16S *MspI* generated T-RFLP data from (Benítez et al., 2007) and ITS *HhaI* T-RFLP data generated in this study. Spearman pair-wise correlations were calculated in a per pot/field site basis. Correlations were run by grouping transition strategies into three disease incidence levels (Table 1.8) and are summarized herein. Correlations were calculated only if \( n \geq 5 \).

** Paired t-test (number of positive correlations vs. number of negative correlations per experimental context), \( P<0.05 \)

Table 3.7. Proportion of positive and negative correlations between total 16S T-RFLP fluorescence signal and total ITS T-RFLP fluorescence signal
<table>
<thead>
<tr>
<th>TRF size (bp)</th>
<th>Fungal/oomycete genera</th>
<th>Isolate origin</th>
<th>Sample</th>
<th>Transition strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>99</td>
<td><em>Pythium</em>[^b,c]</td>
<td>soybean, soil</td>
<td>TF[^e], FV, H</td>
<td></td>
</tr>
<tr>
<td>116</td>
<td><em>Fusarium</em>[^a,b]</td>
<td>tomato</td>
<td>TF</td>
<td></td>
</tr>
<tr>
<td>118</td>
<td><em>Fusarium</em>[^a,b]</td>
<td>tomato</td>
<td>TF</td>
<td></td>
</tr>
<tr>
<td>128</td>
<td><em>Chaetomium</em>[^b]</td>
<td>tomato</td>
<td>FV</td>
<td></td>
</tr>
<tr>
<td>316</td>
<td><em>Phytophthora</em>[^a], <em>Fusarium</em>[^b]</td>
<td>soybean</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>323</td>
<td>ND[^d]</td>
<td>NA[^e]</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>329</td>
<td><em>Alternaria</em>[^a]</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

[^a]: *HhaI* generated ITS TRF  
[^b]: Terminal restriction fragment size determined for known pathogenic isolates  
[^c]: Terminal restriction fragment size determined for individual fungal and oomycete isolates obtained from soil and rhizosphere samples of tomato and soybean grown in soils from different transition strategies. Fungal and oomycete genera shown correspond to the highest BLAST hits (>98% ID and >98% coverage) within the NCBI Genbank database. Reported TRF size as ±1 nt.  
[^d]: ND, not determined since no isolate found matching the corresponding TRF size  
[^e]: NA, not applicable

Table 3.8. Identity of fungal and/or oomycete isolates that generate a terminal restriction fragment (TRF) corresponding in size to TRF associated with disease incidence of tomato and soybean seedlings.
Figure 3.1. Separation of ITS-based community profiles based on variation in damping-off disease levels of soils where tomatoes were grown. Tomato seedlings were grown in the greenhouse (A) and the field (B) in soils previously exposed to different transition strategies. Ordination plots for the first three principal components (PC) are shown. The PC analysis was performed using fungal and oomycete T-RFLP data of *HhaI* digests of ITS amplicons. Plots were generated from the mean principal component score for each disease incidence levels, with their corresponding standard error bars. Data were organized into disease incidence levels according to Table 1.8.
Figure 3.2. Separation of ITS-based community profiles from the rhizosphere of tomato seedlings based on variation in damping-off disease levels of soils where tomatoes were grown. Tomato seedlings were grown in the greenhouse (A) and the field (B) in soils previously exposed to different transition strategies. Ordination plots for the first three principal components (PC) are shown. The PC analysis was performed using fungal and oomycete T-RFLP data of HhaI digests of ITS amplicons. Plots were generated from the mean principal component score for each disease incidence levels, with their corresponding standard error bars. Data were organized into disease incidence levels according to Table 1.8.
Figure 3.3. Separation of ITS-based community profiles based on variation in damping-off disease levels of soils where soybeans were grown. Soybean seedlings were grown in the greenhouse (A) and the field (B) in soils previously exposed to different transition strategies. Ordination plots for the first three principal components (PC) are shown. The PC analysis was performed using fungal and oomycete T-RFLP data of *HhaI* digests of ITS amplicons. Plots were generated from the mean principal component score for each disease incidence levels, with their corresponding standard error bars. Data were organized into disease incidence levels according to Table 1.8.
Figure 3.4. Separation of ITS-based community profiles from the rhizosphere of soybean seedlings based on variation in damping-off disease levels of soils where soybeans were grown. Soybean seedlings were grown in the greenhouse (A) and the field (B) in soils previously exposed to different transition strategies. Ordination plots for the first three principal components (PC) are shown. The PC analysis was performed using fungal and oomycete T-RFLP data of *HhaI* digests of ITS amplicons. Plots were generated from the mean principal component score for each disease incidence levels, with their corresponding standard error bars. Data were organized into disease incidence levels according to Table 1.8.
Figure 3.5. Mean total fluorescence signal of ITS- terminal restriction fragments obtained from tomato samples grown in soils previously exposed to different transition strategies. Samples are grouped by transition strategy within compost amendment regime. C, amended and NC, un-amended. FV, field vegetables; H, mixed hay; TF, tilled fallow.
Figure 3.6. Mean total fluorescence signal of ITS-terminal restriction fragments obtained from soybean samples grown in soils previously exposed to different transition strategies. Samples are grouped by transition strategy within compost amendment regime. C, amended and NC, un-amended. FV, field vegetables; H, mixed hay; TF, tilled fallow.
CHAPTER 4

LINKING SEQUENCE TO FUNCTION IN SOIL: SEQUENCE-DIRECTED ISOLATION OF NOVEL BACTERIA CONTRIBUTING TO SOILBORNE PLANT DISEASE SUPPRESSION

ABSTRACT

Microbial community profiling of samples differing in a specific ecological function, e.g. soilborne plant disease suppression, can be used to mark, recover, and ultimately identify the bacteria responsible for that specific function. Previously, several terminal restriction fragments (TRF) of 16S rDNA were statistically associated with damping-off disease suppression. This work presents the identification, isolation and functional characterization of bacteria giving rise to those TRF. Multiple sequences matching TRF M139 and M141 were cloned and displayed identity to multiple database entries in the Genera Incertae Sedis and Comamonadaceae of the Burkholderiales. A sequence-directed culturing strategy was developed using TRF-derived markers and media reported to be selective for the genera identified. Using this approach we identified novel Mitsuaria and Burkholderia species with high levels of sequence similarity to the targeted M139 and M141 TRF, respectively. As predicted, these Mitsuaria and
*Burkholderia* isolates displayed the targeted function by reducing fungal and oomycete plant pathogen growth *in vitro*, and reducing disease severity of infected tomato and soybean seedlings. This work represents the first successful example of the use of T-RFLP-derived markers to direct the isolation of microbes with disease-suppressive activities, and it establishes the power of low-cost molecular screening to identify and direct the recovery of functionally important microbes, such as these novel biocontrol strains.

**INTRODUCTION**

One of the challenges microbial ecology faces is to understand the associations between microbial populations and ecosystem functions. Understanding these links is important from an ecological and evolutionary perspective, and also for the application and use of microorganisms or their products for medical, agricultural, environmental and industrial purposes (Daniel, 2005; Maron et al., 2007; Torsvik and Ovreas, 2002; Van Lanen and Shen, 2006). Multiple approaches can be used to describe such associations, ranging from genomic comparative studies (e.g. Martin et al., 2006) to culture-based screens for specific activities (e.g. Adesina et al., 2007; Miller et al., 2005). The identification of microorganisms associated with plant disease suppression is important for the development of sustainable disease management strategies that employ natural or inoculated biocontrols (Borneman and Becker, 2007; Mazzola, 2004). We have focused on identifying a differential in a specific function mediated by microorganisms in soil, i.e. soilborne plant disease suppression, and then used analyses of microbial community
fingerprints of the differential to mark, recover, and ultimately identify the bacteria responsible for the specific function.

The identification of microorganisms associated with plant disease suppression is important for the development of sustainable disease management strategies, either through the direct use of microorganisms as biological control agents or through cropping systems that favor their populations (Borneman and Becker, 2007; Mazzola, 2004). The microbial nature of plant-pathogen suppression in soil was first described in the 1930’s, but it was not until the late 1960’s that these concepts were actively explored for their application in the control of plant diseases (Baker, 1987). The microbial components of suppressiveness have been described for multiple pathosystems, especially for those involving a pathogen and a specific microbial antagonist. Examples of this include take-all decline in wheat mediated by antibiotic producing Pseudomonas fluorescens strains; suppression of potato scab by non-pathogenic Streptomyces and suppressiveness of the beet cyst nematode by the fungus, Dactylella oviparasitica (Borneman and Becker, 2007; Weller et al., 2002). Uncovering microorganisms associated with general soilborne disease suppression, i.e. that mediated by the activities of multiple microbial populations (Weller et al., 2002), is more challenging. However, some success has been achieved with damping-off suppression mediated by compost-associated microorganisms (Mazzola 2004) and through correlations with the abundance of microbial populations with known suppressive activities (e.g. Garbeva et al., 2006).

In previous work, a functional differential was observed across an array of different organic transition management strategies (Baysal et al., 2008). In that study, a transitional strategy of perennial mixed hay cropping resulted in soils that were
significantly more suppressive to damping-off of tomato and soybean seedlings. The analysis of community profiles of samples in that study revealed a positive association between multiple bacterial populations (marked by different terminal restriction fragments, TRF) and plant disease suppression (Benítez et al., 2007), an indication that general suppression was at work in the studied system. We hypothesized that the bacteria giving rise to the TRF linked to damping-off suppression were generally able to contribute to that suppression (Benítez et al., 2007).

The primary aims of this study were to i) identify the bacteria giving rise to TRF previously associated with disease suppression (i.e. *MspI* generated TRF of 139, 141 and 148 nt), ii) obtain multiple isolates of those bacteria with corresponding *MspI* generated TRF and iii) characterize the disease suppressive capacities of those isolates to further corroborate their association with general disease suppression in the studied system.

**MATERIALS AND METHODS**

**Cloning of *MspI* generated 16S rDNA TRF.** The procedure for cloning and sequencing of TRF was modified from Widmer et al. (2006). The 16S rDNA was amplified and digested with *MspI* (Promega) from multiple soil and rhizosphere DNA samples of tomato and soybean (from Benítez et al., 2007). A double stranded asymmetric adapter was ligated into the *MspI* site of the TRF. Double stranded adapter was prepared by combining 5µM *MspI*-adapters 1 (5′-CGGTACTCAGGACTCAT-3′) and 2 (5′-GACGATGAGTCCTGAGTAC-3′) (Widmer et al., 2006) in 1x Buffer C (Promega) and incubating 10 min at 65°C, 10 min at 37°C, 10 min at 25°C and 10 min at 4°C. For ligation, 2 µl of the digested amplicon were mixed with 1 µl double-stranded
adapter, 4.5 U T4 ligase (Promega) and 1X ligase buffer (Promega) in a 10 µl reaction. The reaction was incubated 12h at 16°C. Following ligation, TRF were size selected from a portion of the agarose gel corresponding to 90 to 160 bp in length and purified using UltraClean GelSpin DNA Purification Kit (MoBio). The purified DNA was used to enrich the samples with 16S rDNA TRF of the target sizes. PCR was performed using 16S primer 8F (5’-AGAGTT TGATCCTGGCTCAG-3’, Benitez et al., 2007) in combination with MspI-adapter primer (5’-GATGAGTCCTGAGTACCG-3’, Widmer et al., 2006). Amplification was carried out in 25 µl reactions containing 1X Mg-free buffer, 1.8 mM MgCl₂, 0.2 mM dNTPs, 1 pmol µl⁻¹ each primer, 0.04 mg ml⁻¹ RNase A (Novagen), 0.06 U µl⁻¹ GoTaq Flexi DNA polymerase (Promega), and 2.5 µl template. The cycling program consisted of a 5 min denaturation at 95°C followed by 26 cycles of 94°C for 45 s, 54°C for 45 s, and 70°C for 45 s; and an 8 min final extension at 70°C. The double stranded adapter was removed by digestion with MspI and the TRF-enriched samples were ligated into pGEM-T Easy Vector (Promega) prior to introduction into E. coli JM109 competent cells (Promega). A total of 56 transformants were selected for sequencing, based on insert size. Sequencing of this and other samples were performed at the Molecular and Cellular Imaging Center of the OARDC (Wooster, OH) in an ABI Prism 3100xl genetic analyzer system using 3'-BigDye dideoxynucleotide triphosphates labeling chemistry.

**Extension of target 16S rDNA TRF.** The cloned TRF sequences overlap with the first variable loop region between *E. coli* positions 69-101 bp (Cannone et al., 2002), www.rna.icmb.utexas.edu). Sequence alignments were used for designing variable loop-specific primers M139F (5’-TAACGCGGGCAACCTGGGGA-3’) and M141F (5’-
CAGCACGGGAGCAATCCTGGTGG (Figure 4.1). These primers were used independently in combination with universal primer 518R (primer 2 from Muyzer et al., 1993; 5’-ATTACCGCGCTGCTGG-3’) to generate extended amplicons from multiple samples, with the following variations in the cycling program: 30 cycles of 94°C for 1 min, 65°C for 45 s, and 70°C for 45 s. Amplicons from two independent samples were cloned, as described above, and 16 transformants were selected for sequencing.

**Culture-based screening for M139 and M141 positive isolates.** A bacterial collection was generated from the rhizosphere of hay grown in soils previously described as suppressive (Baysal et al., 2008; Benítez et al., 2007). The hay mix contained Festulolium duo (36% v/v), alfalfa (14%), Starfire red clover (11%), Jumbo white clover (9%), Tekapo orchard grass (9%), Tuukka timothy (9%), Lancelot plantain (6%) and chicory (6%). The hay was grown in the greenhouse during the spring of 2007, with temperatures for the period ranging from 23°C to 31°C. Roots and soils were thoroughly mixed, and five grams of the mixture were sampled and diluted in 50 ml of sterile water (SW). The suspension was vortexed (1 min), sonicated (1 min), and vortexed again (15 sec), and serially diluted in SW and spread-plated in *Leptothrix* strain medium (LM; per liter: 5g peptone, 0.2g magnesium sulfate heptahydrate, 0.15g ferric ammonium citrate, 0.05g calcium chloride, 0.01g ferric chloride anhydrous, 0.01g manganese sulfate monohydrate, 15g agar; Atlas, 1997), Yeast agar van Niel’s (YAN; per liter: 10g yeast extract, 1g dipotassium phosphate, 0.5g magnesium sulfate heptahydrate, 15g agar; Atlas, 1997), Nutrient agar buffered (NB; per liter: 4g peptone, 4g sodium chloride, 2g yeast extract, 1g beef extract, 0.45g monopotassium phosphate, 1.78g dissodium hydrogen phosphate heptahydrate, 15g agar; Atlas, 1997) and R2A (Difco BD) plates. These
culture media were previously reported to support the growth of various Burkholderiales species, including members of the Comamonadaceae (R2A and NB; Atlas, 1997; Kampfer et al., 1996; Massa et al., 1998) and Genera *Incertae Sedis* (R2A, LM, YAN; Atlas, 1997; Kampfer, 1997; Spring and Kampfer, 2005). Plates were incubated for 48h at room temperature (RT) in the dark. From each plate eight colonies were picked and transferred into a 96-well plate pre-filled with 200 µl well\(^{-1}\) of corresponding liquid medium. A total of 11 mixed hay pots were sampled, resulting in a collection of 704 isolates. Liquid cultures were pooled (eight per well), prior to DNA isolation performed with the Wizard Genomic DNA purification kit (Promega). DNA-pools (1:100 dilution) were PCR-screened for the presence of M139 and M141-like sequences as described above, with a 25 cycles amplification program. The primer and amplification protocol for M141 was modified (M141F2-primer: 5’-GGAGCAATCCTGGTGGCGA-3’; amplification reaction with final 1.0 mM MgCl\(_2\)) to maximize recovery of isolates matching the targeted variable loop sequence. Individual amplifications were performed from individual cultures present in PCR-positive pools only. Colony-PCR was performed with the 8F and 1492R primer combination (Benítez et al., 2007). 16S amplicons were purified with ExoSAP-IT (USB), and sequenced. Consensus sequences for each isolate were constructed using Sequencher 4.7 (Gene Codes Corporation).

**In vitro inhibition of pathogen growth.** Pathogen growth inhibition was tested in multiple contexts. For *Mitsuaria* isolates, assays were performed on R2A, LM and 1/10 TS agar (TSA). For *Burkholderia* isolates, R2A, LM and 1/3 King’s Medium B (KB, 1X: for 1 liter, 20g proteose peptone, 1.2g dipotassium phosphate, 1.5g magnesium sulfate heptahydrate, 10ml glycerol, 15g agar; Atlas, 1997) were used. Bacteria from 48h-old
culture plates were resuspended in SW, and a 10 µl drop was placed on a plate with a test pathogen in the center. Plates were incubated at root temperature and growth inhibition was scored between 4 -10 days, depending on the pathogen. In vitro inhibition was scored as positive or negative, though phenotypes scored as positive varied somewhat depending on the pathogen and media combination used. Positive scores reflected the formation of clear inhibition zones between the pathogen and the bacteria, diminished total growth of pathogen as compared to the control, melanization or morphology change in pathogen colony, and/or bacterial swarming over the pathogen culture. In vitro inhibition tests were performed against Pythium aphanidermatum isolate 349, and Phytophthora capsici provided by S. Miller (OARDC); Pythium sylvaticum 134, Phytophthora sojae race 25 and Rhizoctonia solani AG4 provided by A. Dorrance (OARDC); F. graminearum provided by P. Paul (OARDC); and Alternaria solani Mg23 and Fusarium oxysporum Ft25 (Gutierrez-Chapin et al., 2006).

**Seedling disease bioassays.** Soybean and tomato seeds were surface sterilized and germinated on water agar (WA; 7.5 g agar l⁻¹) at RT in the dark. After four days three seedlings were transferred to Petri-plates containing WA (tomato: 100 x 15mm; soybean 150x15mm). A 5mm pathogen plug was placed in the center of the plate and seedlings were treated with ~ 10⁷ cell ml⁻¹ seedling⁻¹, in ≤ 100 µl volume. Inoculum was prepared from 24h cultures in 1/10X TS broth, collected by centrifugation, and washed twice with SW. Control plates with water-treated seedlings with and without pathogen inoculum were also prepared. Each plate was prepared in triplicate. Seedling disease was scored after 4 and 5 days for soybean and tomato, respectively. For each seedling (n ≥ 9), total seedling length and lesion length were measured, and disease severity was expressed
as the percent of the seedling that showed a lesion. Three bacterial isolates of each recovered genus were selected for analysis based on their independent isolation from different hay-containing pots. For *Mitsuaria* isolates, soybean assays were run against *P. aphanidermatum*, *P. sojae* and *R. solani* and for tomato against *P. aphanidermatum* and *R. solani*. For *Burkholderia* isolates soybean and tomato assays were run against *R. solani* only. All experiments were run at least twice.

**Test for chitinolytic activity.** *Mitsuaria* and *Burkholderia* isolates were assayed for chitinolytic activity, as a possible mechanism of action for pathogen growth inhibition. For each isolate tested 7 µl of bacterial suspension (in SW) were spotted on 1/10 TS agar plates amended with 0.2% colloidal chitin. The protocol for preparation of colloidal chitin was modified from Rodriguez-Kabana et al. (1983) and Shimahara and Takiguchi (1988). Briefly, 20 ml of 10N HCl were added to 0.5g of chitin (Sigma C8908) and stirred constantly for 2h. The colloidal chitin was thoroughly washed with water, with three overnight steps. When suspension reached pH 6.0 colloidal chitin was resuspended in 200 ml water and stored at 10° C until use. Inoculated plates were incubated at room temperature in the dark and observations were recorded at 2, 5, 7 and 9 days after inoculation. *Pseudomonas fluorescens* (strain wood1R) was used as a negative control of chitinolytic activity.

**Sequence data analysis.** Sequence data were trimmed either manually or using Sequencher 4.7 (Gene Codes Corporation). Sequences were aligned and pair-wise comparisons calculated with ClustalW2 (EMBL-EBI Tools). Graphic alignments were prepared using Jalview (v 2.3) alignment editor (Clamp et al., 2004). Shorter sequences were compared to the Ribosomal Database Project (Release 10.0 Beta; Cole et al., 2007)
using the Sequence Match program to determine the best match to isolate data only. Full-length sequences were compared also to the non-redundant nucleotide collection NCBI database (nr/nt) using BLAST (blastn program). Database searches include data as of June 10, 2008. Phylogenetic analyses were performed using MEGA 4 (Tamura et al., 2007). Trees were generated using the Neighbor-Joining algorithm from distances calculated using the Maximum Composite Likelihood Method, with the complete deletion option (all positions containing gaps and missing data were eliminated from the dataset). The bootstrap test was performed with 1000 replicates to determine the percentage of replicate trees in which data cluster together. For comparison of topology between different algorithms, trees were also generated with Maximum Parsimony Method using the Close-Neighbor-Interchange algorithm. Both sets of tree topologies were equivalent (data not shown).

**Statistical analyses.** All analyses were performed using JMP v7.0 (SAS Institute Inc.). The Kruskall-Wallis test was used to determine differences in disease severity (expressed as % root length marked by a lesion). Five treatment levels were considered: three bacterial isolates in the presence of pathogen and water treated seedlings with or without pathogen. Bacterial isolates were chosen based on their isolation of independent hay pots. Treatment-level differences were determined with the non-parametric Kruskall-Wallis test. Pair-wise comparisons were performed between individual bacteria and water treated seedlings (plus pathogen) with Wilcoxon-2-sample test. Contrast analysis (Wilcoxon-2-sample test, one tail) was performed to determine overall effect of bacterial treatment compared to water treated seedlings (plus pathogen).
Nucleotide sequence accession numbers. Sequences generated within this study were deposited in GenBank under accession numbers EU14905-EU714956.

RESULTS

Classification of 16S eubacterial sequences corresponding in size to a target TRF. The identity of bacteria giving rise to MspI generated TRF associated with disease suppression in the microbial community profiles (Benítez et al., 2007) was first assessed by cloning TRF of the selected size range. Of 56 clones sequenced, 20 were confirmed as a targeted TRF (seven to M139, eight to M141 and five to M148). These sequences were compared to bacterial isolate sequences only, available at the Ribosomal Database Project (Release 10.0). Table 4.1 summarizes the best sequence match data for each individual TRF clone. Five M139 clones shared > 90% sequence identity with one another, and likely arise from β-Proteobacteria; and, of these, four, recovered from three independent samples, shared sequence identity (> 0.75 S_ab Sequence Match score) to database members of the order Burkholderiales not assigned to a named family (i.e. Genera Incertae Sedis) and to a member of the Comamonadaceae. Similarly, four M141 clones derived from independent samples showed a high degree of similarity to one another and were classified as Burkholderiales. These clones, however, had a higher number of sequence matches to members from multiple families and genera (Table 4.1). These four M141 clones were recovered from two independent samples yet still displayed 98-100% sequence identity. Other M141 clones differed substantially from this group (68-82% sequence identity) and among themselves (66-78% sequence identity) and might belong to the divisions Firmicutes, Actinobacteria, Proteobacteria and/or Spirochaete. The
greatest sequence variation was observed within the sampled population of M148 clones, which shared only 46-71% sequence identity with each other. Two clones matched Proteobacteria, one matched Planctomycete sequences, and the other three matched sequences of members from multiple divisions. Within each cloned TRF subset, at least one did not show any significant match, as reflected from matches to multiple divisions. These data further support our initial hypothesis that multiple novel bacterial populations are associated with the suppressive activity developing from the hay-based transition strategy.

The cloned M139 and M141 TRF were used to recover longer and more phylogenetically informative sequences from the suppressive soils. Among these, over half of the TRF likely arose from novel bacterial species not previously associated with plant disease suppression (i.e. Burkholderiales, Genera *Incertae Sedis*). Sequence alignments of known Burkholderiales species and M139 and M141 clones revealed sequence variation within the first variable loop of the 16S rRNA (Cannone et al., 2002), and this data were used to design M139- and M141-specific primers (Figure 4.1). These primers were used in combination with eubacterial primer 518R to generate extended amplicons from two DNA samples from Benítez et al. (2007). Four of the M139-extended sequences showed similarity to bacteria of the Genera *Incertae Sedis* (up to three genera) and three M141 matched Comamonadaceae (up to 2 genera) (Table 4.2).

Sequences from both cloning steps were aligned to assemble consensus sequences (Figure 4.2). For M139, three different consensus sequences with 100% identity over a 76 nt overlap were constructed. Based on approximately 520 nt, the three M139 constructed sequences exhibited similarity to database entries of Genera *Incertae Sedis: Leptothrix,*
Ideonella, Methylibium, Rubrivivax, Schlegelella and Azohydromonas. In addition, one M141 consensus sequence was constructed (97% sequence identity on a 78 nt overlap) which matched to database entries of the Comamonadaceae (Ramlibacter and Curvibacter, Table 4.2). Sequence analysis revealed the presence of an MspI recognition site that will produce a TRF of 139 bp in the three Genera Incertae Sedis-like assembled sequences. The Comamonadaceae-like sequence, however, lacked the MspI site to produce the expected 141 bp TRF. It is unclear if this lack of consistency reflects a high degree of sequence diversity amongst the bacteria giving rise to the targeted TRF in our samples or amplification artifacts.

**Culture-collection screening for M139 and M141 isolates.** Because no isolates with 100% sequence identity to the cloned markers had been previously identified, efforts were made to recover bacteria giving rise to the M139 and M141 markers. To do so, culture media favoring growth of Burkholderiales species related to the genera described above were selected. The isolates were obtained from the mixture of hay species that had resulted in damping-off suppression, and a 2-step PCR-based approach was used to screen the collection, first from pooled samples and then individually. Of the 704 isolates examined, eight, all isolated from Leptothrix strain medium (10^{-2} dilution plates) had an exact sequence match to the M139 variable loop. The highest BLAST hit to a named species for all eight isolates was to Mitsuaria chitosanitabida (98-99% identity), followed by Roseateles depolymerans and Pelomonas aquatica or P. saccarophila (>97% identity), all belonging to the Genera Incertae Sedis. Sequence identity within the isolates ranged from 98-100%, and their phylogenetic relationships to representative type strains of Genera Incertae Sedis (Burkholderiales) are shown in Figure 4.3. The type strain most
closely related to the isolates retrieved from the mixed species hay soils is *M. chitosanitabida* 3001 (Amakata et al., 2005), but there is a clear distinction between known *Mitsuaria* species and the isolates from this study (Figure 4.4).

While the novel *Mitsuaria* isolates recovered from the disease-suppressive soil were found to have 16S sequences similar to the initial M139 clones, they were not identical. The isolates shared just 99% identity to a *Mitsuaria*-like extended sequence clone. Furthermore, *Mitsuaria* species do not produce an M139 *in vitro* or *in silico*. In contrast, the *MspI* TRF for the isolates was 487 nt (488 nt expected from sequence). Interestingly, M488 and M489 TRF were common in the TRF profiles of the studied soils, and positive associations between M488 and M489 and soilborne disease suppression were observed in two of the studied contexts (Benítez et al., 2007). Variation in TRF size could relate to amplification artifacts resulting from sampling complex mixtures of closely related bacteria, as well as to the presence of pseudo-terminal restriction fragments in the samples (Hurst et al., 2007). Given the sequence similarity between *Mitsuaria* isolates and M139 clones it seems likely that these represent bacteria that are very closely related to those giving rise to the M139 TRF associated with disease suppression.

A similar isolation strategy led to the recovery of eight pure cultures from R2A media (dilution 10^{-4}) with an M141-like amplification profile. The 16S sequences amplified from these isolates shared 24 of the 26 nt of the M141-derived variable loop sequence. The highest BLAST hit for all eight was to unclassified *Burkholderia* spp. (i.e. 99% identity to GenBank AY238505, AB025790, and AB298718). Sequence identity within the eight isolates was > 99%, but was only 96% identical to the type strain of the
genus, *B. cepacia* (GenBank U96927). The isolates from this study form a phylogenetically-distinct cluster within the genus (Figure 4.5), with their closest relatives being *Candidatus Burkholderia* spp., non-cultured endosymbionts from leaf galls (Van Oevelen et al., 2002; Van Oevelen et al., 2004; 97% identical). Sequence analysis revealed 97% identity between our *Burkholderia* isolates and the initial M141 clones, but only 72-88% sequence identity with clones of the ~450 nt extended sequences. Still, the observed 16S rDNA MspI TRF for the isolates was a 139/141 bp double-peak, indicating that at least one group of bacteria with an M141 TRF was successfully isolated.

**Characterization of pathogen inhibition and disease suppressive activities.**

The association of the M139 and M141 TRF with *in situ* soilborne disease suppression (Benítez et al., 2007) led us to hypothesize that the novel *Mitsuaria* and *Burkholderia* isolates obtained would express antagonistic activities towards diverse soilborne pathogens. Initially, the capacity of the isolates to reduce pathogen growth *in vitro* against multiple fungal and oomycete plant pathogens was assayed. For the *Mitsuaria* isolates, inhibition was observed regardless of the pathogen tested (Figure 4.6A), with the greatest frequency of inhibition expressed against *Pythium aphanidermatum Phytophthora sojae, Rhizoctonia solani, and Alternaria solani*, and the least against *Pythium sylvaticum*. All of the *Mitsuaria* isolates from this study have chitinolytic activity *in vitro* (Figure 4.7), which can relate to the broad-spectrum inhibition observed against the various fungi, but other mechanisms must be involved in the inhibition of the oomycetes which do not harbor chitin as a major component in their cell walls (Bartnicki-Garcia, 1968). Similar assays were performed with other *Mitsuaria* spp. including multiple chitosan-degrading strains isolated from soils in Japan (ATCC type strain *M.*
chitosanitabida 3001, strain 12 and strain 13; Amakata et al., 2005) and gallic acid degrading strains associated with freshwater plants (Mit\(\text{suaria}\) spp.: FBTS 25 and FBTS 19, (Muller et al., 2007). Of these, chitosan-degrading strains 12 and 13 showed a similar spectrum of inhibition; whereas the type strain 3001 gave a positive inhibition in only about half of the assays. While the sequence identity with the tested Japanese strains was \(\geq 98\%\), the antagonistic phenotype of our isolates was less variable. The Mit\(\text{suaria}\) strains recovered from freshwater plants expressed no pathogen inhibition in most cases. Among the Bur\(\text{kholderia}\) isolates, \(\text{in vitro}\) pathogen inhibition was less frequent and more variable (Figure 4.6B). Significant variation in the expressed inhibitory capacities was observed among isolates, with six isolates inhibiting at least three pathogens, but none of these inhibited the same three pathogens. In contrast to Mit\(\text{suaria}\) isolates, all eight Bur\(\text{kholderia}\) isolates tested negative for chitinolytic activity.

Seedling diseases were suppressed by inoculation with the novel Mit\(\text{suaria}\) isolates (Tables 4.3-4.4). All the tested isolates reduced disease severity in soybeans challenged with \(P.\ aphanidermatum\) (\(P=0.03\) and 0.005) (Table 4.3) and in tomato challenged with \(P.\ aphanidermatum\) (\(P=0.0003\) and 0.002) and \(R.\ solani\) (\(P=0.27, 0.02\) and 0.0007) (Table 4.4). Although not significant for most experiments, the lesion severity caused by \(R.\ solani\) was also reduced by the Mit\(\text{suaria}\) isolates in three separate assays (Tables 4.3-4.4). In addition, lesion severity was also reduced in soybean seedlings treated with Mit\(\text{suaria}\) isolates when challenged with \(P.\ sojae\), though only once was a disease differential observed between challenged and non-challenged control plants (Table 4.3). Overall, disease severity reductions ranged from 5 to 20 percent. In 7 out of the 11 tests, treatment with Mit\(\text{suaria}\) isolate H24L5A resulted in lower disease severity than the
water treated control, whereas isolates H23L1 and H29L1B resulted in disease severity reduction in 4 out of the 11 tests, with greatest variation observed in the soybean bioassays (Tables 4.3-4.4).

Similarly, seedling disease severity, caused by \textit{R. solani} was reduced on soybean (Table 4.5) and tomatoes (Table 4.6) inoculated with \textit{Burkholderia} isolates. As a group, disease severity was reduced by at least 15% on soybean \((P=0.0001\) and 0.0005) and 20\% on tomato seedlings \((P<0.0001\) for both tests) (Tables 4.5-4.6). For the \textit{Burkholderia} isolates tested, no apparent variation in their ability to reduce lesion severity was observed. Overall these data support the hypothesis that multiple isolates of novel \textit{Mitsuaria} and \textit{Burkholderia} species contribute to the general soilborne disease suppression induced by the mixed hay cropping system.

\textbf{DISCUSSION}

We successfully used molecular profile data of bacterial community structure to direct the isolation of novel and ecologically important microbial populations. Specifically, we recovered two novel groups of rhizosphere bacteria whose TRF had been associated with the ecologically important function of soilborne plant disease suppression. Based on our culturing method, these bacteria represented <1\% of the sampled population and were isolated based on sequence identity to TRF clones. General soilborne disease suppression involves the activities of multiple members of the community through multiple mechanisms of action (Weller et al., 2002). Therefore, it is likely that the two isolated populations are just a subset of the whole involved in the suppression differential described previously (Baysal et al., 2008; Benítez et al., 2007).
This result is consistent with our hypothesis that multiple bacteria whose TRF were associated with damping-off suppression would be involved in disease suppression in the studied system.

The *Mitsuaria* isolates described in this work represent the first association of this genus, and closely related species of the Genera *Incertae Sedis* (Figure 4.3) of the Burkholderiales (such as *Roseateles* and *Pelomonas*), with plant disease suppression. *Mitsuaria* strains have been described from soils in Japan (Amakata et al., 2005; Yun et al., 2005) and the rhizosphere of a freshwater plant (Muller et al., 2007). These earlier studies, however, focused on exploring specific substrate degradation (i.e. chitosan and gallic acid). And, while bacteria with sequences similar to *Mitsuaria* were isolated from the rhizoplane of oil seed rape (Kaiser et al., 2001) and hemodialysis fluid (Gomila et al., 2005), no function was ascribed to them. Bacteria of the Genera *Incertae Sedis* are known to exhibit a range of metabolic activities, including nitrogen fixation, photosynthesis and metal oxidation (Gomila et al., 2008; Malmqvist et al., 1994; Siering and Ghiorse, 1996; Xie and Yokota, 2005). Some sequences associated with these genera have been found in the rhizosphere and phyllosphere of crops (Coelho, 2008; Kadivar and Stapleton, 2003; Roesch et al., 2008) but were not previously associated with disease suppression. Other metabolic activities, besides chitin degradation, might be involved in the observed pathogen suppressing phenotype. Further characterization of *Mitsuaria* isolates from this study will be required to better understand their metabolism, their contributions to plant disease suppression, and their potential utility as biological control agents. Work in progress has shown the ability of these isolates and culture filtrates to inhibit growth of
other plant pathogens *in vitro*, in addition to those presented in this study, including plant pathogenic bacteria (Raudales and McSpadden Gardener, unpublished data).

The other isolates described in this study likely represent a new species of the genus *Burkholderia*. These isolates share 99% sequence identity with several strains isolated from soils (Hayatsu et al., 2000; Macur et al., 2007) and insect guts (Kikuchi et al., 2007). However, within validly named species, the closest relatives are plant symbiotic *Candidatus Burkholderia* species (Figure 4.5). Because the prefix *Candidatus* has been adopted for the description of non-cultured bacteria with defined phylogeny and some phenotypic description (Murray and Stackebrandt, 1995), our isolates make further systematic work on these organisms possible for the first time. The *Burkholderia cepacia* complex is the major phylogenetic cluster associated with human diseases, with *B. multivorans* and *B. cenocepacia* (*B. cepacia* genomovars II and III, respectively) being most frequently isolated from cystic fibrosis patients (Coenye and Vandamme, 2003). But our *Burkholderia* isolates share only 96% sequence identity with *B. cepacia* and are part of a distinct clade within the genus separate from the *B. cepacia* complex (Figure 4.5). This lack of similarity may assuage some of the concerns about using these strains as microbial biopesticides, a problem that has limited the commercial interest of other species of the genus (Parke and Gurian-Sherman, 2001). Antibiotic production, nitrogen fixation and production of indol acetic acid have been described for some *Burkholderia* strains (Caballero-Mellado et al., 2007; el-Banna and Winkelmann, 1998; Perin et al., 2006; Salles et al., 2006a; Salles, et al., 2006b), and this study adds to the list of species with the potential for plant health promotion. Further analysis of the *Burkholderia*
isolates described in this study aim to understand the mechanisms involved in plant pathogen suppression.

To our knowledge, this work represents the first direct connection of TRF-derived molecular markers to an ecologically important microbial function. T-RFLP has been used extensively for comparing microbial community structure (Hurst et al., 2007), including in plant disease suppression contexts (Bluoin-Bankhead et al., 2004). Yet, to date, no group has reported the use of such a technique to direct the recovery of novel disease suppressive microbes. Methods used to classify microbes that generate specific TRF include the analysis of isolates or clone collections for matching TRF sizes, as well as sequencing of selected TRF clones (e.g. McSpadden Gardener and Weller, 2001; Nakanishi et al., 2006; Widmer et al., 2006). T-RFLP has been used to guide the isolation of methanogen achaea (Cadillo-Quiroz et al., 2008) and biodegraders (Jeon et al., 2003), but those investigations monitored solely for TRF size and did not consider sequence. Sequence-directed isolation of microorganisms involved in plant disease suppression was performed previously for fungal species, using data generated from oligonucleotide fingerprinting of rRNA (Olatinwo et al., 2006; Yin et al., 2003). In that system, however, disease suppression was specific, and caused by a parasite of nematode cysts. For that same system, bacterial populations were also analyzed and correlations with suppressiveness were observed (Valinsky et al., 2002), but suppressiveness was demonstrated only for the fungi D. oviparasitica and Fusarium oxysporum (Olatinwo et al., 2006). These studies can lead to the discovery of novel microorganisms expressing a specific function by first coupling them to sequence data associated to that function. Because of this initial in situ association, it seems likely that the microbes recovered
using this approach will have a much higher probability of being able to perform effectively when reintroduced through inoculation. Studies of novel microorganisms associated with plant disease suppression will provide insight into the diversity of metabolisms involved in this type of interaction (Leveau, 2007). Our approach not only led to the discovery of two novel sets of bacteria that likely contribute to general soilborne disease suppression in the field, it further establishes the validity of using this approach to better link structure and function of diverse microbial communities via sequence-directed isolations.

ACKNOWLEDGEMENTS
This work was supported by the USDA CSREES Integrated Research, Extension, and Education, Organic Transitions Grants Program (Award Number 2002-51106-01935) and by the OARDC SEEDS Graduate Student Enhancement Program (2007093) to M.S. Benítez. We thank Dr. E. Gross (Universität Konstanz, Germany) and Dr. M. Kawamukai (Shimane University, Japan) for kindly providing Mitsuaria isolates for comparison. Thanks also to R. Raudales, S. Park, A. Sánchez, C. Merry and W. Pipatpongpinyo for assistance in this work.

REFERENCES


Target *Msp*I TRF of the 16S rDNA gene were previously associated with general disease suppression (Benitez et al., 2007)

Identifiers for GenBank accession numbers for clones of 16S rDNA TRF sequences obtained in this study were compared to the Ribosomal Database Project (Release 10.0 Beta) isolate data only using SeqMatch. The number of genera per family are shown only for those samples matching sequences within the same order (corresponding to SeqMatch hits with > 0.75 S_ab scores).

Table 4.1. Classification of 16S rDNA clones that match the size (bp) of targeted *Msp*I-generated terminal restriction fragments (TRF)

<table>
<thead>
<tr>
<th>TRF size (bp)</th>
<th>Clone ID</th>
<th>Higher rank taxonomy</th>
<th>Order</th>
<th>Family</th>
<th>No. of matching genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>139</td>
<td>S101D</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera Incertae</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SB42G</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>S102A</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera Incertae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB81G</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SB11G</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera Incertae</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>SB12G</td>
<td>α-Proteobacteria</td>
<td>Rhodocycales</td>
<td>Comamonadaceae</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SB41H</td>
<td>Actinobacteria</td>
<td>Firmicutes Nitrospira</td>
<td>Genera Incertae</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>SB141</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB41E</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera Incertae</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SB41G</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB42F</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera Incertae</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SB71E</td>
<td>β-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB31A</td>
<td>Firmicutes Actinobacteria</td>
<td>Proteobacteria</td>
<td>Genera Incertae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>S102G</td>
<td>Proteobacteria</td>
<td>Firmicutes Actinobacteria</td>
<td>Burkholderiales</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>S101F</td>
<td>Proteobacteria</td>
<td>Actinobacteria</td>
<td>Comamonadaceae</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>SB31C</td>
<td>Proteobacteria</td>
<td>Actinobacteria</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td>148</td>
<td>SB82F</td>
<td>α-Proteobacteria</td>
<td>Caulobacteriales</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB71D</td>
<td>Sphingobacteria</td>
<td>Rhizobiales</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB42C</td>
<td>Bacteroidetes</td>
<td>Proteobacteria</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB21H</td>
<td>Planctomycete</td>
<td>Comamonadaceae</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>SB32F</td>
<td>γ-Proteobacteria</td>
<td>Xanthomonadaceae, Methylcoccales</td>
<td>Comamonadaceae</td>
<td>3</td>
</tr>
</tbody>
</table>

a Target *Msp*I TRF of the 16S rDNA gene were previously associated with general disease suppression (Benitez et al., 2007)

b Identifiers for GenBank accession numbers for clones of 16S rDNA TRF sequences obtained in this study were compared to the Ribosomal Database Project (Release 10.0 Beta) isolate data only using SeqMatch. The number of genera per family are shown only for those samples matching sequences within the same order (corresponding to SeqMatch hits with > 0.75 S_ab scores).
Target \( Msp-I \) TRF of the 16S rDNA gene were previously associated with general disease suppression (Benitez et al., 2007).

Identifiers for GenBank accession numbers for clones of 16S rDNA TRF extended sequences obtained in this study.

Sequences were compared to the Ribosomal Database Project (Release 10.0 Beta) isolate data only using SeqMatch. The number of genera per family are shown only for those samples matching sequences within the same order (correponding to SeqMatch hits with > 0.75 \( S_{ab} \) scores).

Matching 16S rDNA \( Msp-I \) TRF of 139 or 141 nt respectively, based on sequence information.

<table>
<thead>
<tr>
<th>TRF target group(^a)</th>
<th>Clone ID(^b)</th>
<th>Higher rank taxonomy</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Matching TRF size(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M139</td>
<td>3971G ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera incertae</td>
<td>Methylibium, ( \beta )-Proteobacteria</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3971H ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera incertae</td>
<td>Leptothrix, ( \beta )-Proteobacteria</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3972H ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera incertae</td>
<td>Methylibium, Roseateles, ( \beta )-Proteobacteria</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3972G ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera incertae</td>
<td>Methylibium, Leptothrix, ( \beta )-Proteobacteria</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3912A ( \beta )-Proteobacteria</td>
<td>Rhodocyclales</td>
<td>Rhodocyclaceae</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3911B ( \gamma )-Proteobacteria</td>
<td>Chromatiales, Thiomicrobiales</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3912A ( \gamma )-Proteobacteria</td>
<td>Chromatiales, Thiomicrobiales</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>139cons1 ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera incertae</td>
<td>Ideonella, Leptothrix, Rubrivivax, Schlegella, Azohydromonas</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>139cons2 ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera incertae</td>
<td>Ideonella, Leptothrix, Methylibium</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>139cons3 ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Genera incertae</td>
<td>Ideonella, Leptothrix, Rubrivivax, Mitsuaria, Azohydromonas</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>M141</td>
<td>4112A ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>Varioroxax</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4172H ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>Ramlibacter, Curvibacter</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4111H ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>Ramlibacter, Curvibacter</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4171G ( \beta )-Proteobacteria</td>
<td>Nitrospomonadaceae</td>
<td>Nitrospomonadaceae</td>
<td>Nitrospira</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4172C Bacteroidetes</td>
<td>Sphingobacteriales</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4112H Bacteroidetes</td>
<td>Proteobacteria</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4111A Bacteroidetes</td>
<td>Proteobacteria</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4171C Bacteroidetes</td>
<td>Proteobacteria</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>141cons1 ( \beta )-Proteobacteria</td>
<td>Burkholderiales</td>
<td>Comamonadaceae</td>
<td>Ramlibacter, Curvibacter</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Target \( Msp-I \) TRF of the 16S rDNA gene were previously associated with general disease suppression (Benitez et al., 2007).

\(^b\) Identifiers for GenBank accession numbers for clones of 16S rDNA TRF extended sequences obtained in this study.

\(^c\) Sequences were compared to the Ribosomal Database Project (Release 10.0 Beta) isolate data only using SeqMatch. The number of genera per family are shown only for those samples matching sequences within the same order (correponding to SeqMatch hits with > 0.75 \( S_{ab} \) scores).

\(^d\) Matching 16S rDNA \( Msp-I \) TRF of 139 or 141 nt respectively, based on sequence information.

Table 4.2. Classification of 16S rDNA clones, representing extended sequences of M139 and M141 TRF
Median values are reported for \(^a\) \(n = 9\) (one outlier removed from H23L1), \(^b\) \(n = 12\) (one outlier removed from H29L1B), \(^c\) \(n = 9\) \(^d\) \(n = 16\) \(^e\) \(n = 9\) (one outlier removed from H23L1) \(^f\) \(n = 9\).

Non-parametric Kruskall-Wallis test was used to assess differences among all five treatments.

Significant pairwise comparisons between treatment and pathogen only control at ** \(P < 0.05\) and \(* P < 0.1\) (Wilcoxon 2-sample test).

Table 4.3. Lesion severity in soybean seedlings treated with *Mitsuaria* isolates and challenged with *Pythium aphanidermatum, Rhizoctonia solani* and *Phythophthora sojae*.
<table>
<thead>
<tr>
<th>Mitsuaria isolate</th>
<th>Seedling disease severity (% lesion length in relation to total seedling length)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aphanidermatum</td>
</tr>
<tr>
<td></td>
<td>Exp. 1</td>
</tr>
<tr>
<td>H23L1</td>
<td>51.9***</td>
</tr>
<tr>
<td>H24L5A</td>
<td>37.1***</td>
</tr>
<tr>
<td>H29L1B</td>
<td>55.0**</td>
</tr>
<tr>
<td>Pathogen only</td>
<td>100</td>
</tr>
<tr>
<td>No Pathogen</td>
<td>13.1</td>
</tr>
<tr>
<td>K-W test&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$P&lt;0.0001$</td>
</tr>
</tbody>
</table>

<sup>a</sup> Median values are reported for n = 12
<sup>b</sup> Non-parametric Kruskall-Wallis test was used to assess differences among all five treatments
<sup>c</sup> Significant pairwise comparisons between treatment and pathogen only control at ***$P < 0.01$ , **$P < 0.05$ and *$P < 0.1$ (Wilcoxon 2-sample test).

Table 4.4. Lesion severity in tomato seedlings treated with *Mitsuaria* isolates and challenged with *Pythium aphanidermatum* and *Rhizoctonia solani*
<table>
<thead>
<tr>
<th>Burkholderia isolate</th>
<th>Seedling disease severity* (% lesion length in relation to total seedling length)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
</tr>
<tr>
<td>R2C2</td>
<td>36.1***</td>
</tr>
<tr>
<td>R2G3</td>
<td>34.5***</td>
</tr>
<tr>
<td>R4F2</td>
<td>34.2**</td>
</tr>
<tr>
<td>Pathogen only</td>
<td>56.1</td>
</tr>
<tr>
<td>No Pathogen</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td><strong>K-W test</strong></td>
</tr>
<tr>
<td></td>
<td>$P=0.003$</td>
</tr>
</tbody>
</table>

* Median values are reported for n = 12  
* Non-parametric Kruskall-Wallis test was used to assess differences among all five treatments  
* Significant pairwise comparisons between treatment and pathogen only control at ***$P < 0.01$ and **$P < 0.05$ (Wilcoxon 2-sample test).

Table 4.5. Lesion severity in soybean seedlings treated with *Burkholderia* isolates and challenged with *Rhizoctonia solani*
<table>
<thead>
<tr>
<th><strong>Burkholderia</strong> isolate</th>
<th><strong>Seedling disease severity</strong>&lt;sup&gt;a&lt;/sup&gt; (% lesion length in relation to total seedling length)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
</tr>
<tr>
<td>R2C2</td>
<td>46.2***&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>R2G3</td>
<td>42.3***</td>
</tr>
<tr>
<td>R4F2</td>
<td>48.3***</td>
</tr>
<tr>
<td>Pathogen only</td>
<td>63.6</td>
</tr>
<tr>
<td>No Pathogen</td>
<td>45.2</td>
</tr>
</tbody>
</table>

**K-W test**<sup>b</sup>  

|                      | P=0.002 | P=0.0005 |

---

<sup>a</sup> Median values are reported for n = 12

<sup>b</sup> Non-parametric Kruskall-Wallis test was used to assess differences among all five treatments

<sup>c</sup> Significant pairwise comparisons between treatment and pathogen only control at ***P < 0.01 (Wilcoxon 2-sample test)

<sup>d</sup> For Exp. 2 R2C2, one missed data point

Table 4.6. Lesion severity in soybean seedlings treated with *Burkholderia* isolates and challenged with *Rhizoctonia solani*
Figure 4.1. DNA-sequence alignment showing the position and variation of the first variable region of the 16S rRNA of representative species within the order Burkholderiales and clones generated in this study. *E. coli* sequence is shown as a reference, with the variable loop between positions 69 and 101. Primers were designed for this region. Primer sequences and overlap are shown below the alignment.
Figure 4.2. Sequence alignments showing overlap region between TRF clones and extended TRF sequence clones. Alignments show only partial sequence (up to 178 nt) of extended TRF clones. Each nucleotide is highlighted with a different color, and the variable loop-specific sequence is shown within the blue box in each alignment.
Figure 4.3. Classification of M139-associated isolates (◇) as *Mitsuaria* sp. based on 16S rDNA sequence analyses. Included in the dendrogram are the sequence of the type strains representative of other species of *Genera incertae* of the order Burkholderiales. The phylogenetic relationships among taxa were inferred from ~1200 bp of the 16S rDNA gene, using the Neighbor-Joining method from distances computed by the Maximum Composite Likelihood algorithm. Bootstrap values > 60% (1000 replicates) are shown next to the branches. Accession numbers for each sequence are shown in parenthesis. Scale bar: number of base substitutions per site.
Figure 4.4. Classification of *Mitsuaria* isolates (◇) identified in this study in relation to the other known members of the genus. The phylogenetic relationships among taxa were inferred from ~800 bp of the 16S rDNA gene, using the Neighbor-Joining method from distances computed by the Maximum Composite Likelihood algorithm. Bootstrap values > 60% (1000 replicates) are shown next to the branches. Accession numbers for each sequence are shown in parenthesis. Scale bar: number of base substitutions per site.
Figure 4.5. Classification of M141-associated isolates (◊) as a novel *Burkholderia* sp. based on 16S rDNA sequence analyses. Included in the dendrogram are the sequence of the other 22 named *Burkholderia* species. The phylogenetic relationship among taxa was inferred from ~1300 bp of the 16S rDNA gene, using the Neighbor-Joining method from distances computed by the Maximum Composite Likelihood algorithm. Bootstrap values > 60% (1000 replicates) are shown next to the branches. Accession numbers for each sequence are shown in parenthesis. Scale bar: number of base substitutions per site.

* *Candidatus Burkholderia* species with no cultured isolate.
Figure 4.6. Frequency of positive *in-vitro* inhibition activity of *Mitsuaria* (A) and *Burkholderia* (B) isolates identified in this study against multiple fungal and oomycete tomato and soybean pathogens. *In-vitro* inhibition activity was tested for eight isolates of each genus on three different media and was scored as positive or negative. TSA, tryptase soy agar; R2A, R2A media for growth of heterotrophic organisms; KB, King’s medium B; LM, *Leptothrix* strain medium.
Figure 4.7. Chitinolytic activity of Mitsuaria isolates. Observations were recorded at A) 2, B) 5, C) 7 and D) 9 days after inoculation. 1: H24LB; 2: H23L1; 3: H24L1C; 4: H24L2C2; 5: H29L1B; 6: H24L5A; 7: H24L6B; 8: H24L3B; 9: Pseudomonas fluorescens (Ps., strain wood1R).
CHAPTER 5

CONCLUSIONS

Microbial populations in soil contribute to ecosystem function through roles that include nutrient cycling, pathogenicity and mutualism with plant species (Atlas and Bartha, 1998). Within agricultural ecosystems, a major focus of study has been the contributions of microbial populations to plant disease suppression. Multiple authors have reviewed concepts related to soil microbial diversity within this context (Garbeva et al., 2004; Mazzola, 2004; Weller et al., 2002). These authors have focused on the factors that affect microbial population abundance and community composition in soil. Soil physico-chemical characteristics, plant species and plant community composition, and land management regime interact at different scales, and the level of influence of each on microbial populations may vary from one field to the next. For example, as reviewed in McSpadden Gardener (2007), plant species or cultivar, soil type, fertility, temperature and moisture, affect the abundance and incidence of rhizosphere-colonizing antibiotic producing Pseudomonas populations. In spite of this, Rotenberg et al. (2007) when comparing multiple field sites under different management strategies (i.e. tillage, rotation, amendment and seed treatments), observed a certain degree of consistency in the contribution of these populations to crop health, as well as in response to management.
Certain methodological and statistical approaches have been also suggested in the literature for a better description and understanding of the associations between disease suppression, presence and abundance of microbial communities, and soil characteristics affected by management (Weller et al. 2002; van Bruggen et al., 2006; Borneman et al., 2007; Borneman and Becker, 2007; Janvier et al., 2007). This dissertation describes various effects of agricultural management strategies on microbiotic characteristics of soil and their association with plant health, including the identification of novel species with biological control potential. This was accomplished by following a step-wise approach, starting from the identification of a disease suppressive system and ending in the confirmation of disease suppressive activity by novel bacterial isolates obtained from the suppressive soils. The details of the step-wise approach followed in this work are shown in Figure 5.1.

The greenhouse and field bioassays presented in Baysal et al. (2008) and summarized in chapter 1 represent the observational corner stone from which to build upon microbial community information. The reproducibility of the results, where soil from the mixed hay transition strategy were suppressive to damping-off of tomato and soybean in both greenhouse and field bioassays, indicated not only a certain degree of durability of the suppressive phenomenon, but also that a more general mechanism of suppression was in play. Soils from the mixed hay transition strategy were found to contain more carbon and nitrogen, compared to the tilled fallow and the field vegetables. The degree to which these added nutrients contributed to the observed suppression is unclear; however, in the other chapters of this work evidence is presented regarding the microbial basis of the observed disease incidence differential. The composition and
abundance of the studied microbial communities are likely affected by other soil factors not considered in this analysis, and finding these linkages require further studies. Compost amendment significantly affected soil chemical characteristics, at a greater magnitude than by the hay alone. The added fertility in the system was consistent with greater seedling biomass and vigor (Baysal et al., 2008), as well as yield of a subsequent organic tomato crop (Kleinhenz et al., unpublished data); but in this study, compost amendment did not show an effect on damping-off suppression, compared with the main transition strategies. Variability in compost-based disease suppression has been described previously (e.g. Termorshuizen et al., 2006; Scheurell et al., 2005) and most likely depends on fertility levels and biotic characteristics of soils prior to amendment, as well as compost’s ability to sustain microbial growth and activity. When amended, the mixed hay resulted in positive yield responses of a subsequent organic tomato crop (total yield, considering all fruit maturity stages, $P < 0.07$) (Kleinhenz et al., unpublished data), likely providing other benefits besides damping-off suppression.

Follow up questions regarding this experimental system include, for example, how durable is the suppressiveness effect observed in the field. In other words, how many seasons of, for instance tomato or other crop, will result in the loss the suppressiveness? Also, how many years of a rotation with an equivalent mixed hay cropping system will be required to restore suppressiveness. These types of questions could help address the need and utility of incorporating into a rotation scheme a suppressive system, such as the mixed hay.

Based on the well-established concept that microbial communities are responsive to soil type, management practices and vegetation cover, we expected to observe
microbial community variations between the different transition strategies. However, the extent to which we would be able to detect those variations and differentiate individual populations favored by each transition strategy was unclear. Terminal restriction fragment length polymorphism has proved to be a robust, reproducible and sensitive method for microbial community analysis (Edel-Hermann et al., 2004; Osborn et al., 2000). Yet, without an appropriate approach to T-RFLP data analysis, the power of this methodology might be underestimated. The combined used of multiple statistical approaches, including principal component analysis, non-parametric analysis of variance and pair-wise correlations, provided us with information from which to generate hypotheses regarding the relationship between microbial population structure and function. In other words, in this work individual taxonomic groups (bacteria, fungi and oomycetes) were associated with disease suppression (chapter 2) or disease expression (chapter 3). The relative abundance of these populations in soil, and the proportion of the community that they represent might be contributing factors to the differences in suppressiveness observed between the studied transition strategies.

Other components of this system that were not considered in this study, since they did not represent the focus of our work, include individual microbial functional groups, such as mycorrhizal fungi or nitrogen fixers; as well as invertebrates such as nematodes or earthworms, or aboveground microbial populations. Powell (1971) and more recently Back et al (2002) reviewed, for instance, interactions or disease complexes between nematode and soilborne pathogens. Synergistic interactions have been described in the presence of both groups of plant pathogens, occurring for example through the predisposition of seedling infection due to plant parasitic nematode damage. A parallel
study targeting nematode populations at the same field site was presented by Briar (2007). Briar (2007) observed the greatest differences in nematode guild groups in response to compost amendments, including the suppression of some plant parasitic genera in response to compost. Another interesting observation was the number of fungivorous nematodes being high in the mixed hay strategies. The integration of concepts arising from the analysis of both nematode (from Briar, 2007) and microbial populations (presented in this work) could provide a broader ecological perspective to understand the processes driving the differences observed between the studied transition strategies.

The analysis of bacterial T-RFLP profiles resulted in the identification of multiple members of the community (expressed as individual terminal restriction fragments) that consistently associated with damping-off suppression. On the one hand, the T-RFLP data supported the hypothesis of a general suppression mechanism being induced by the mixed hay transitions strategy; on the other hand, it provided molecular information about bacteria that could potentially be used as biological control agents. The work presented in chapter 4 has multiple aspects to highlight. First of all, it is the first successful application of T-RFLP profiling of microbial communities to direct the identification and isolation of potential biological control agents. Second, both sets of isolates obtained in this work most likely represent newly identified bacterial species. Third, it is the first association of the genus *Mitsuaria* (*Genera Incertae Sedis, Burkholderiales*), with plant disease suppression. And fourth the ability of both species, isolated from the mixed hay soils, to suppress tomato and soybean pathogen growth and disease were demonstrated. Based on the T-RFLP profile information, it is highly
probable that the two sets of isolates presented in this work represent only a subset of the whole community that is involved in suppression. Nevertheless, they represent good candidates for future studies on general disease suppression and they may represent good candidates for biopesticide development.

Further research is required to understand the full potential of these isolates as biological control agents. As stated by Thomas (1999), “by increasing our basic understanding of how individual pest control methods act and interact, new opportunities for improving pest control can be revealed”. The effectiveness of these bacteria within the mixed hay strategy could either be additive to the effect of multiple populations or dependent on interactions with other species. Field bioassays with these bacteria applied as seed treatments can provide information first about the ability to colonize and establish in the rhizosphere of crops, as well as their contribution to disease suppression in the field. Comparisons can be made, individually or in combination, in terms of damping-off suppression efficacy when inoculated in soils previously exposed to the hay transition strategy or to the field vegetables and tilled fallow. Furthermore, the efficacy of disease suppression observed at the original field site can be compared to inoculations performed at foreign sites (to understand site-specific interactions). Understanding the relationships between these isolates and other management strategies (different from the mixed hay) can lead to the development of methods to augment the abundance of these indigenous populations in soil, for example, by inclusion of a rotation or cover crop that favors their abundance. Tests concerning plant host range, spectrum of pathogen inhibition as well as successful bioassays in field settings are another set of questions that need to be answered
before the utility of applying such bacteria (or hay-based amendments) as disease suppressive treatments can be fully realized.

The direct application and commercialization of microorganisms (or their products) for plant disease control in the greenhouse and the field require the development of biopesticide formulations. The understanding of mechanisms of action involved in pathogen growth inhibition and disease suppression, as well as environmental conditions that can enhance this phenotype provide information for biopesticide development. For example, if the suppressive phenotype is mediated by a secreted product, bacterial cultures could be managed to favor these secretions under controlled conditions. Biopesticide development require mass production of the agent of interest or its derived products. Hence, research involving density dependent growth regulation or secondary metabolism production (e.g. through quorum sensing mechanisms) is also necessary. The genomic sequence of *Pseudomonas fluorescense* Pf5, a known biocontrol agent, provided greater insight associated to the mechanisms involved with biological control and associations with its environment. Including the description of novel secondary metabolite genes, as well as genes involved in siderophore mediated iron sequestration and a broad range of catabolic potential (Loper et al., 2007).

The overall accomplishments of this work result from the integration of knowledge generated at the multiple steps of the project. These include the observation of a phenomenon in the field, the description of characteristics (i.e. microbial population markers) associated to the phenomenon and finally experimentation to prove function. The approach followed in this work can be certainly applied to other systems, to identify the microbial factors that are involved in a specific, but measurable, function. Terminal
restriction fragment length polymorphism has also been used for profiling microbial communities associated to the human gut (Wang et al., 2004). The diversity and composition of a healthy adult gut microbiota appears to be stable over time, however diseases can more certainly affect the composition of this community (Zoetendal et al., 2006). Therefore providing a differential for comparison and an avenue to understand how “normal” human gut microbiota (as a community or as individual populations) contribute to homeostasis. Further refinements of the methodology might be used to not only overcome PCR bias limitations, but also to achieve a higher through-put and maximize the amount of data generated from each sample. For instance, the possibility of multiplexing T-RFLP for combined analysis of microbial populations can provide with community analyses targeted to multiple populations (and/or multiple functions) all in one experimental run (Singh et al., 2006). Nonetheless, this work provides a proven roadmap to further discovery of the relationships between microbial community structure and function.

REFERENCES


175


Figure 5.1. Step-wise approach followed in this dissertation for the understanding of the of microbial populations associated with disease suppression and disease in an organic transition field experiment


