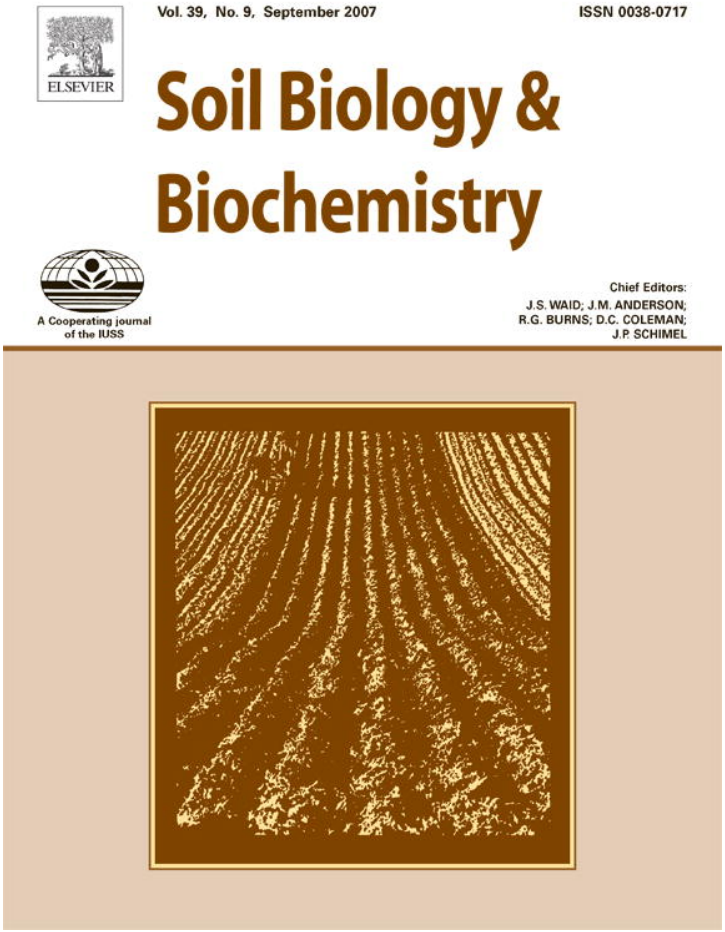

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Soil Biology & Biochemistry 39 (2007) 2289–2301

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Multiple statistical approaches of community fingerprint data reveal bacterial populations associated with general disease suppression arising from the application of different organic field management strategies

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Received 7 December 2006; received in revised form 9 March 2007; accepted 23 March 2007
Available online 30 April 2007

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 (H), tilled fallowing and open-field vegetables production) were used during the transition from conventional to organic farming, with and without compost amendment. The H 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, nonparametric 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 H transition strategy in multiple experimental contexts. Subsequent correlation analyses revealed that TRF associated with disease suppressive treatments (i.e. H 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. Interestingly, *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.

Keywords: Bacterial communities; Damping-off; Disease suppression; Terminal restriction fragment length polymorphism; Transition strategy

1. 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 differences in communities associated to soil types compared to crop species. Tillage

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doi:10.1016/j.soilbio.2007.03.028

(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-diacetylphloroglucinol (DAPG), were described by Rotenberg et al. (2007). The abundance of DAPG-producing *Pseudomonas*, which have previously been implicated in soilborne disease suppression (Weller et al., 2002, McSpadden Gardener, 2007), in the rhizosphere of corn were positively correlated with stand and yield 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 resource competition 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 10 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 encompassed in 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 3-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 (MacRae 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-Tustas et al., 2006). 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 approach consisted of a combination of multivariate and nonparametric univariate procedures that provide different perspectives on the extensive data sets generated by microbial community fingerprinting techniques.

2. Materials and methods

2.1. 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 (H) species, and low-intensity open-field vegetables (FV) production. 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 × 17.1 m long. After the 3-year transition period (2006) the field was certified as organic, and the whole field was planted to tomato.

2.2. 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 $10 \times 10 \times 9\,cm^3$ 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 h 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 in soybean, respectively. In field, bioassays were performed in the summer of 2006. Twenty-four tomato seeds and 16 soybean seeds were planted in two $45 \times 45\,cm^2$ 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 from the greenhouse bioassays.

2.3. 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/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 to V1) were recorded and are reported elsewhere (Baysal-Tustas et al., 2006, in preparation). The roots of one plant 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 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.

2.4. 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. About 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 10× Mg-free buffer (Promega Corp.), 2 or 1.5 µl (for soil and rhizosphere samples, respectively) 25 mM $MgCl_2$, 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).

Matrixes 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 $\geqslant 100$ bp, fluorescence intensity $\geqslant 200$ fluorescence units (peak height). Binning was defined as ± 1 nt for TRF $\leqslant 300$ bp, and ± 2 nt for TRF $\geqslant 300$ 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.

2.5. 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 "select TRF" were observed in $\geqslant 60\%$ of the samples of at least one treatment. For comparison and correlation analyses, nonparametric procedures were used because the abundance of TRF are 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 select TRF. Analyses were run on MINITAB (v14.2, Minitab, Inc., State College, PA) and pairwise 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/field site basis, using MINITAB.

3. Results

3.1. Principal component analysis on data from T-RFLP profiles

PCA was performed to determine the overall effect of transition strategies on the observed population of 16S rDNA 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. The observed variation explained by the first two principal components ranged between 58% and 86% among all bioassays and soil and rhizosphere samples. And, 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 nonamended transition strategies was observed in the greenhouse and the field (Fig. 1). In the greenhouse,

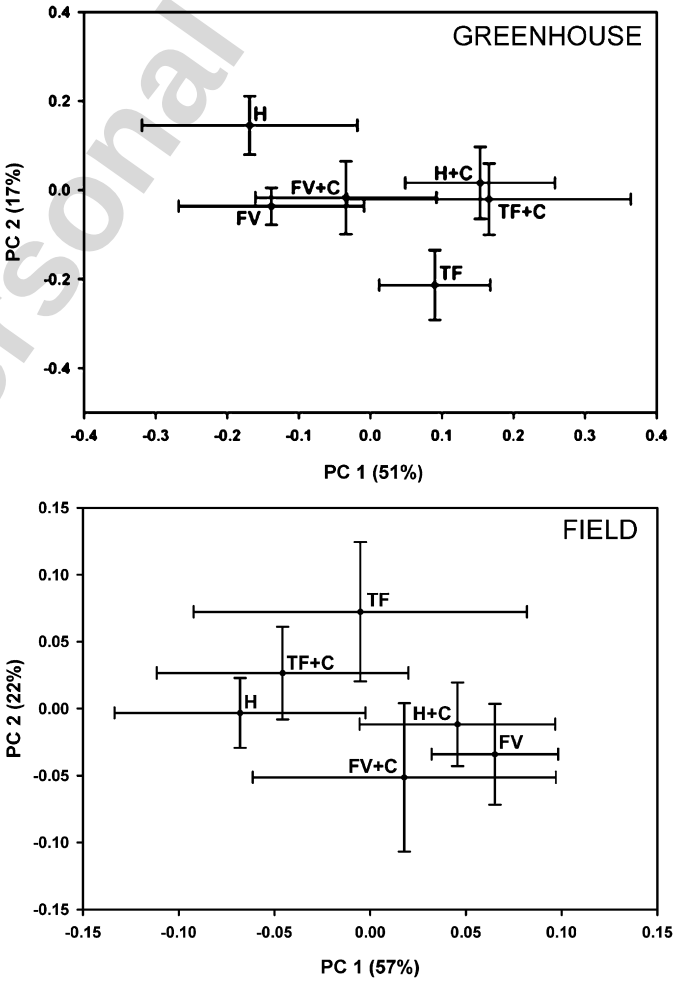

Fig. 1. Effects of transition strategy and compost amendment in bacterial community structure in the rhizosphere of tomato. Ordination plots from the first two principal components (PC) are shown. Plots were generated from the mean principal component scores for each treatment, with their corresponding standard error bars. The PCA was performed using the 16S rDNA terminal restriction fragment (*MspI*) relative abundance data obtained from the rhizosphere of tomato 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.

the H separated from the TF along the first PC; whereas, the three transition strategies separated on the second PC. In the field, the H and 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 (Fig. 2). In the greenhouse, the H and FV differed from each other especially along the second PC. All three transitions strategies were distinguished in the field, with greater variation along the first PC, and separation between the H and 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 (Figs. 1 and 2). In the greenhouse experiments, the nonamended H and TF differed from their amended (+C) equivalent in tomato and soybean. In the field tomato, effects from compost amendment were observed only for the H. In contrast, for the field soybean compost amended subplots of the TF and FV differed from the nonamended 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 (PCAs) of T-RFLP profiles from the soil fraction of both crops also depict differences due to transition strategy and in response to compost amendment. For tomato, the H separate from the FV in the greenhouse and the field; and for soybean, differences were observed between the three transition strategies (ordination plots not shown). Effects from compost amendment were observed in all three transition strategies for tomato greenhouse and field soils, and for the TF 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. TRF with a factor loading of $|x| \geqslant 0.70$ for each of the first three principal components are summarized in Table 1. The first three principal components explain between 64% and 92% of the variation along the 8 different experimental contexts. In four out of the eight scenarios, TRF M148 greatly contributed to the variation in PC 1 (tomato greenhouse soil and rhizosphere, tomato field soil and soybean greenhouse soil); and in two to the variation in PC 2 (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 $|x| \geqslant 0.70$ were in the analyses of the TRF obtained from the rhizosphere of soybean (in the

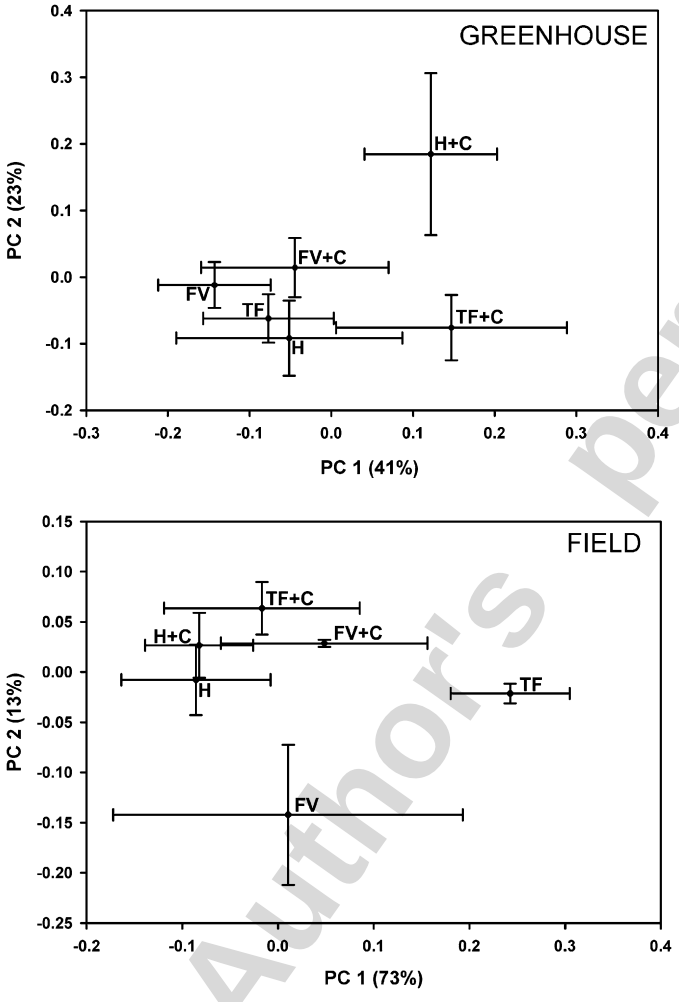

Fig. 2. Effects of transition strategy and compost amendment in bacterial community structure in the rhizosphere of soybean. Ordination plots from the first two principal components (PC) are shown. Plots were generated from the mean principal component scores for each treatment, with their corresponding standard error bars. The PCA was performed using the 16S rDNA terminal restriction fragment (*MspI*) relative abundance data obtained from the rhizosphere of soybean 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.

Table 1
16S rDNA terminal restriction fragments with high factor loadings on the first three principal components (PC) for each experimental context

Crop	Experiment[a]	Sample[b]	Principal component		
			PC 1	PC 2	PC 3
Tomato	GH	S	148[c]	109	192
		R	148	104, 488	
	Field	S	148	382, 385	
		R	410, 501	148, 151, 222, 262, 422, 464, 521, 541, 583, 602, 623, 643	302
Soybean	GH	S	148	490, 494	501, 516
		R	493	489	
	Field	S	104, 176	148, 151	106, 157
		R	151, 402	154	425

[a]GH: greenhouse.
[b]S: soil; R: rhizosphere.
[c]Size in base pairs of each restriction fragment, with a factor loading of $|x| \geqslant 0.70$. Restriction fragments were generated after digestion of the 16S amplicon with *MspI*.

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.

3.2. Treatment differences in the relative abundance of individual terminal restriction fragments

As a second approach to identifying important differences in bacterial community structure, the nonparametric 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, TRF present in $\geqslant 60\%$ 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.

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 H (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 H 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 H and H plus compost. Terminal fragments M127, M204, M490 and M494, were significantly higher in the soils from the FV and TF treatments, and M162, M282 and M382 for the TF 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 TF and FV; and M123 and M136 in amended soils from TF and FV.

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 H plus compost and TF plus compost transitions strategies; and TRF M182 was significantly higher in the H plus compost and the FV 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 FV (M122), the TF (M403), and the amended H (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 TF; and M159 and M176 were more abundant when grown in the amended and nonamended H soils.

3.3. Quantitative relationships between damping-off and selected TRF

The relevance of the several TRF described above (see Section 3.2) to pre-emergence damping-off was assessed using nonparametric 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 percent of pre-emergence damping-off. Correlations were calculated only when sufficiently replicated data (i.e. a minimum sample size of $n\geqslant 5$) 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 was 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 (Table 3) (Baysal-Tustas et al., 2006, in preparation). For the tomato bioassays, low disease is defined as 50–60%, intermediate as 60–70%, and high as 70–80% damping off. For the soybean greenhouse bioassay, low disease corresponds to 40–60% damping-off, intermediate to 60–80%, and high to $>$ 80%. For soybean grown in the field, low corresponds to 70–80%, intermediate to 80–90%; and high to $>90\%$ damping off. The H plus compost transition strategy had the lowest

Table 2
Relative abundance[a] of a selected subset[b] of 16S rDNA terminal restriction fragments (TRF) showing significant differences between transition strategies

Crop	Experiment	Sample	TRF	Transition strategy[c]											
				TF		TF+C		H		H+C		FV		FV+C	
Tomato	GH	S	137[d]	0.6	cd[e]	4.5	ab	7.5	a	2.9	bc	2.4	bcd	0	d
			139	0.7	cd	0.8	bcd	5.8	a	2.8	bc	2.7	b	0	d
			141	0.3	c	2.8	bc	9.4	a	6	b	2	c	3.3	bc
	Field	S	180	2	b	0	c	5.7	b	7.9	a	2	b	1.2	b
		R	141	4.5	a	3.3	b	3.2	b	4.9	ab	1.5	c	1.6	c
			182	0.9	b	0.8	bc	0.2	c	1.5	a	2	a	1.6	a
Soybean	GH	S	127	5.8	ab	5.4	bc	1	de	0	e	7.1	a	3.5	cd
			137	1.2	b	1	b	6.7	a	6.5	a	1.2	b	1.8	b
			141	2.2	b	1.5	b	6.8	a	9.8	a	1.8	b	1.5	b
			148	15.6	c	20.3	bc	49.9	a	52.1	a	25.4	b	25.8	b
			162	2.4	a	0.7	bc	0.4	bc	0.2	c	0.5	bc	1.4	b
			202	0.7	b	0.5	b	0.2	b	0.7	b	0.5	b	6	a
			204	0	d	0.2	cd	0.7	bc	0.8	bcd	5.6	a	2.3	ab
			282	2.4	a	0.9	bc	1	bc	0	d	0.6	cd	1.9	ab
			382	2.1	a	0.6	bc	0.2	bc	0	c	0.4	bc	1.1	b
			401	4.3	a	3.9	b	5.2	a	0.5	c	1.6	bc	2	b
			490	6.3	ab	7.8	ab	0.6	c	0.7	c	10.5	a	5.4	b
			494	8.1	ab	6	ab	0.3	c	0	c	9.1	a	7.4	b
		R	122	0	b	0.6	b	0	b	0	b	0.3	b	6	a
			403	13.3	a	1.9	bc	2.3	bc	0	c	9.7	b	1.5	bc
			489	13.2	c	21.3	b	13.4	c	45.4	a	12.8	bc	16.7	bc
	Field	S	106	5.4	a	0.2	bc	0	c	0	c	2.6	ab	2.9	bc
			123	0.3	b	1	a	0.4	b	0	b	0.1	b	1.4	a
			136	0.3	b	1.4	a	0.4	b	0	b	0.5	b	2	a
		R	139	0	c	3.2	ab	0	c	1.32	bc	0	c	4.7	a
			148	1.4	b	7.7	a	7.5	a	6.7	a	0	b	8.9	a
			159	0	b	0.3	b	3.4	a	2.2	ab	0	b	0	b

TRF were obtained from soil (S) and rhizosphere (R) DNA samples from tomato and soybean 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. About 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]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 3
Damping-off levels observed in tomato and soybean grown in the greenhouse (GH) and the field, in soils previously cropped with one of the studied transition strategies

Crop	Experiment	% Damping-off[a]		
		Low	Intermediate	High
Tomato	GH	H+C[b], TF+C	H, FV, FV+C	TF
	Field	H+C, H	TF, TF+C	FV, FV+C
Soybean	GH	H+C, H	TF+C, FV	TF, FV+C
	Field	H+C	TF, H	TF+C, FV, FV+C

[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%.

[b]TF, tilled fallow; H, mixed hay; FV, field vegetable; +C, compost amended treatment.

damping-off in all experiments. In addition, the H and TF plus compost strategies contributed to lower damping-off for the tomato grown in the greenhouse and the field; and the H to lower damping-off of soybean grown in the greenhouse (Table 3).

TRF 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 and 3). The second group consisted of the TRF that were more abundant in transitions strategies with intermediate and high levels of damping-off, and those TRF with high factor loadings in the PCA that were not included in the first data set (Tables 1–3). The first group of TRF (i.e. associated with low disease

Table 4
Comparison of the proportion of negative correlations[a] between % pre-emergence damping-off and relative abundance of individual 16S rDNA terminal restriction fragments[b] (TRF) associated with transition strategies exhibiting lowest damping-off and TRF associated with transition strategies with intermediate or high disease incidence

Crop	Sample[c]	TRF associated with transitions strategies exhibiting	
		Lowest % damping-off	Mid/high % damping-off
Tomato	R	0.67	0.58
	S	0.61	0.50
Soybean	R	0.69	0.50
	S	0.73	0.54
Tomato + soybean	R	0.68	0.56
	S	0.68	0.52
Tomato + soybean	R + S	0.68	0.54*

*χ^2, $P = 0.055$.
[a]To calculate the correlation coefficient transition strategies were grouped into three levels of disease, based on % pre-emergence damping-off (see Table 3). Pairwise correlations on rank data were performed only if $n \geqslant 5$.
[b]Terminal restriction fragments generated by restriction digest with *MspI*.
[c]R: rhizosphere; S: soil.

incidence) was negatively correlated with pre-emergence damping-off over 65% of the time (Table 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 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 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 levels 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.

3.4. 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., 2002) 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 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. Within 148 bp, mainly members of the class Bacilli and α-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.

4. 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. PCA analysis revealed differences in overall bacterial community structure in response to transition strategy and compost amendment (Figs. 1 and 2). In addition, the TRF M148 contributed substantially to the variation and separation between transition strategies along the first and second principal components (Table 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

Table 5
Prevalence of negative correlations between % pre-emergence damping-off and the relative abundance of individual 16S rDNA terminal restriction fragments[a] (TRF) associated with transition strategies exhibiting the lowest damping-off incidence[b]

Sample	Crop	Experiment	TRF	Damping off incidence			No. of negative correlations
				Low[c]	Mid	High	
Rhizosphere	Tomato	GH[d]	141	−0.22[e]	−0.52	NA[f]	2
			148	−0.57	−0.18	NA	2
			488	−0.21	−0.47	NA	2
		Field	137	0.29	−0.15	0.12	1
			139	−0.18	−0.13	0.43	2
			141	−0.02	−0.11	0.55	2
			148	−0.04	0.00	0.28	1
			159	−0.32	0.15	−0.06	2
			401	−0.35	0.41	−0.09	2
	Soybean	GH	141	−0.41	NA	−0.35	2
			148	0.24	0.58	0.58	0
			489	−0.17	−0.48	−0.35	3
		Field	139	NA	NA	−0.35	1
			148	−0.70	−0.58	0.20	2
			159	NA	−0.45	NA	1
Soil	Tomato	GH	137	−0.06	−0.49	NA	2
			139	NA	−0.22	NA	1
			141	0.56	−0.45	NA	1
			148	0.29	0.03	−0.27	1
		Field	139	NA	−0.09	0.50	1
			148	−0.36	0.00	0.25	1
			180	−0.17	NA	0.11	1
			401	−0.44	−0.15	−0.63	3
	Soybean	GH	137	−0.20	0.00	−0.24	2
			139	0.12	−0.72	−0.29	2
			141	−0.08	0.00	0.19	1
			148	−0.25	−0.26	−0.24	3
			401	−0.58	−0.64	0.61	2
		Field	137	−0.45	−0.22	−0.38	3
			139	0.89	−0.41	−0.48	2
			141	NA	−0.43	−0.54	2
			148	0.49	−0.37	−0.09	2
Overall							55

[a]Terminal restriction fragments generated by restriction digest with *MspI*.
[b]TRF more abundant in mixed hay transition strategies (Tables 2 and 3).
[c]Transition strategies were grouped into three levels of disease, based on % pre-emergence damping-off (Table 3).
[d]GH: greenhouse.
[e]Spearman correlation coefficients ($n \geqslant 5$).
[f]NA: not applicable, not enough samples for performing correlations ($n < 5$).

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-Piqueres et al. (2006); however, in that work, they did not relate quantitatively 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 and 3). This fraction ranged from 3% to 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 H transitions strategy. From this, we conclude that, while multiple populations may promote or respond to changes in crop health status, these four TRFs include bacteria that are more generally associated with the health 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; Bluoin-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

Table 6
Bacterial genera predicted to generate a terminal restriction fragment (TRF) corresponding to the size of the TRF associated with disease suppression

TRF expected 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*
141	*Actinomadura*, *Burkholderia* (11), *Dehalobacter*, *Frankia* (3), *Leptothrix*, *Mycobacterium*, *Pseudomonas*, *Pseudonocardia* (5), *Ruania*, *Schlegella* (2), *Streptomyces*
148	*Actinomyces*, *Aerococcus* (8), *Bacillus* (2), *Cylindrospermopsis*, *Halobacillus*, *Ochrobactrum*, *Phyllobacterium*, *Rhodopseudomonas* (3)
159	Actinomycetales (5), *Actinoplanes* (2), *Amycolatopsis*, *Bacillus*, *Dethiosulfovibrio*, *Dietzia*, *Halobacillus*, *Herbidospora* (2), *Kineosporia* (2), *Kitasatospora* (2), *Kribbella*, *Moorella*, *Mycobacterium* (13), *Nocardia* (28), *Nocardiopsis* (2), *Pseudonocardia*, *Rhodococcus* (43), *Saccharothrix* (5), *Streptacidiphilus*, *Streptomyces* (141), *Tsukamurella*, *Xylanibacterium*
401	*Agrobacterium* (3), *Azorhizobium*, *Brucella* (3), *Hoeflea*, *Ochrobactrum* (16), *Rhizobium* (29), *Sinorhizobium* (26), *Mesorhizobium* (6),
488	*Acidithiobacillus* (5), *Acidovorax* (6), *Comamonas* (10), *Delftia*, *Diaphorobacter* (6), *Halomonas* (2), *Hydrogenophaga* (2), *Malikia*, *Pseudoalteromonas* (15), *Pseudomonas* (2), *Thiomonas* (79)
489	*Achromobacter*, *Bordetella*, *Delftia*, *Kinetoplastibacterium*, *Leeuwenhoekiella*, *Pseudoalteromonas* (7), *Pseudomonas* (5), *Thiomonas*, *Variovorax*, *Zobellia*

[a]TRF size was predicted based on *in silico* amplification and digestion of the available 16S rDNA sequences on the virtual digest tool of the MiCA 3 web-based software for microbial community analysis (Shyu et al., 2002).
[b]Number in parenthesis indicates the number of organisms of that genus that could generate that same fragment size, based on the database sequence information.

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 H resulted in greatest disease suppression in subsequent tomato and soybean crops in two experimental settings (greenhouse and field bioassays) (Table 3). The H 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 grasslands 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 the arable land (Garbeva et al., 2004b; Salles et al., 2006). Our data indicate that the H strategy promoted shifts in specific bacterial populations concominant with improvements in crop health. While correlative, such an association provides a reasonable criteria 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 relatee 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 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 *D. 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 and 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 TRF 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 H treatment, but were not necessarily absent in the other samples, we propose that general disease suppression was promoted in the H 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 with the sizes of the TRF consistently found in low disease transition strategies (M137, M139, M141; Tables 2 and 6) or significant for the separation of treatments along the principal components (M148; Table 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 plant growth promoting rhizobacteria (Weller et al., 2002; McSpadden Gardener, 2004; Salles et al., 2006; McSpadden Gardener, 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 H system. Previously Barnett et al. (2006) described, through a culture-based, multi-stepped approach, the interaction of three bacterial species involved in the suppression of *R. solani* on wheat. Other studies have focused on culture-independent methods, for example Yin et al. (2003) and Olatinwo 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; Olatinwo 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) need also 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. Further characterization of these TRF is underway.

Acknowledgments

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.

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