Applied Soil Ecology 80 (2014) 44–50

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Fungal and oomycete pathogen detection in the rhizosphere of organic tomatoes grown in cover crop-treated soils

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ARTICLE INFO

Article history:
Received 3 September 2013
Received in revised form 10 March 2014
Accepted 12 March 2014
Available online 25 April 2014

Keywords:
Cover crops
Suppressive soils
Microbial communities
Soilborne pathogens
Macroarray
Terminal restriction fragment length polymorphisms

ABSTRACT

Soil management practices, including the use of cover crops, affect soil and plant health through varied mechanisms. Impacts on microbial communities are known to be important, but are not well understood. Various techniques are used to measure the effect of treatments on microbial communities, but rarely are the results of more than one technique compared. This field study examined the impacts of a single-season application of cover crops on detection of pathogen species in the tomato crop rhizosphere. The study took place in Maryland, New York and Ohio (MD, NY and OH) in the summers of 2010 and 2011, with a total of 260 plots tested using both macroarray and T-RFLP analyses. The macroarray used in this study was specifically designed to detect thirty-one pathogens of solanaceous crops and had not previously been used for such a field study. The results of T-RFLP analysis, which is a common tool for examining microbial communities, were compared to the macroarray results and the limitations and benefits of each are presented. While not a quantitative measure, the macroarray was able to detect certain fungi with much greater sensitivity than T-RFLP. Our findings suggest that the results of PCR-based techniques used for microbial community studies should be compared to other methods to verify sensitivity.

1. Introduction

Plant type, soil type and management practices all affect the microbial community structure of the soil ecosystem. Plants induce changes in soil microbial communities because the rhizosphere of plants encourages diverse and abundant microbial communities due to chemical exudates, mucilage production, improved aeration and moisture retention (Angers and Caron, 1998). Likewise, microbial populations in the rhizosphere influence plant health, both directly and through interactions with other soil microbes (Kim et al., 2011; Whipps, 2001). Different plant species have been found to encourage distinct microbial species populations in the rhizosphere after only four weeks of plant growth (Grayston et al., 1998).

The interaction of microorganisms with each other and with plants both result in differences in plant growth and in the extent to which disease can be suppressed in the agroecosystem (Garbeva et al., 2004). Suppressive soils are soils that limit the survival, growth or disease causing activity of plant pathogens. Suppression can be general or specific. General suppression reduces fungal, oomycete and nematode damage. Mechanisms of action are unclear and dynamic, however, suppression often appears to be due to total microbial biomass (Weller et al., 2002). Cover crops have been found to increase soil microbial biomass, which could allow for general suppression (Mendes et al., 1999; Schutter and Dick, 2002). Research supports the role of enhanced microbial diversity in the disease suppression exhibited by cover crops (Abawi and Widmer, 2000; Mazzola, 2004; Van Bruggen and Semenov, 1999).

Abbreviations: T-RFLP, terminal restriction fragment length polymorphisms; GLM, generalized linear model; CCT, cover crop treatment; HSD, Honestly Significant Differences; TRF, terminal restriction fragment; Aa, *Alternaria alternata*; Fo, *Fusarium oxysporum*.

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http://dx.doi.org/10.1016/j.apsoil.2014.03.012
0929-1393/

However, while research supports this association, the profile of microbial communities involved and their role in soilborne disease control are not well understood. Benitez et al. (2007) observed that disease suppression of damping-off on tomato and soybean increased following a mixed-species hay cover crop. Furthermore, terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial communities was used to examine the rhizosphere of crop plants and correlate suppression to members of the genera *Burkholdaria*, *Bacillus*, *Paenibacillus*, and *Streptomyces*, all genera previously found to contain beneficial species. Larkin and Griffin (2007) observed suppression of various soilborne pathogens of potato in the field. The suppression occurred for all species of cover crop tested, which implies a role for microbial communities in the soil. A study of the short-term effects of an oat-vetch mixture on the growth of damping off pathogens, *Pythium aphanidermatum* (Edson) Fitzp. and *Rhizoctonia solani* (J.G. Kühn) suggests that cover crop incorporation leads to a suppressive effect *in vitro* (Grünwald et al., 2000).

In order to examine green manure impacts on soilborne pathogens in the crop rhizosphere, this project implemented macroarray and T-RFLP analyses. The macroarray detects thirty-one different fungal and oomycete pathogens of solanaceous crops common in the Northeastern region of the US, even at extremely low inoculum levels (Zhang et al., 2008). The limit of detection for the macroarray was determined to be 0.04 pg (Zhang et al., 2008). This technique was developed as a diagnostic tool for diseased plant samples and has not previously been used in a field study.

T-RFLP analysis has been used to determine the profile of microbial communities and identify potentially-beneficial bacteria (Benitez et al., 2007). Subsequent application of the technique revealed both beneficial and detrimental populations of fungi (Benitez, 2009). As T-RFLP was previously used successfully in the above field study (Benitez, 2009) to profile diverse species of microorganisms, this study tested the potential for detection of specific pathogens. The limit of detection of specific organisms by T-RFLP was not determined for this study. However, quantitative real-time PCR has been used in conjunction with T-RFLP in other studies to allow the quantification of targeted template in environmental samples (Yu et al., 2005). T-RFLP is relatively less expensive and more time efficient than macroarray. While T-RFLP can be used to identify specific organisms through *in silico* correlation with terminal restriction fragment lengths, the true accuracy of these assignments is not verifiable given the high degree of microbial diversity present in the soil. However, it is unknown if significant interference would occur with such assignments made from assays of root samples which would be expected to harbor less diversity by volume. Because specific species are able to be accurately targeted by macroarray, this experiment provided a unique opportunity to assess the relative detection power of the two techniques.

In this study, the single-season impacts of mixed-species cover crops on organic tomato (*Solanum lycopersici* L.) crop rhizosphere pathogen detection was evaluated in three states with distinct soilborne disease pressure and repeated over two field seasons. Two PCR-based molecular techniques, macroarray and T-RFLP, were used to detect species of fungal and oomycete pathogens. The results of each were used to evaluate and compare the efficacy of each technique. Finally, macroarray analysis was used to assess cover crop treatment effects on pathogen presence.

2. Materials and methods

2.1. Transplant production

Tomato cultivar Celebrity (Johnny's Select Seed, Winslow, ME) was used. This cultivar has disease resistance to Verticillium wilt, Fusarium wilt Races 1 and 2, root-knot nematodes, Alternaria stem canker and tobacco mosaic virus (Rutgers Cooperative Extension, 2013).

Tomato seeds were sown into a locally-produced organic potting mix in 50 cell flats in NY and OH (TO Plastics, Clearwater, MN) and 128 cell flats in MD, then maintained in a greenhouse with 16 h of both natural and supplemental light per day. Seedlings were moved into a cold frame for at least 24 h before transplant.

Table 1
Field setup and timeline.

	MD	NY	OH
Number of fields	1	2	3
Reps per field	6	4	4
Total reps per year	6	8	12
Total plots	30	40	60
Plot size (m)	6.4 × 12.2	2.4 × 7.6	3.1 × 6.1
Rows/plot	2	1	4
Plant distance (m)	0.9	0.6	0.6
Tilled CC 2010	4/16/2010	5/5/2010	4/14/2010
Tilled CC 2011	5/2/2011	5/14/2011	5/10/2011
Transplanted Tomatoes 2010	5/14/2010	5/27/2010	6/3/2010
Transplanted Tomatoes 2011	5/20/2011	6/10/2011	6/15/2011
Rhizosphere Collection 2010	6/14/2010	6/30/2010	7/13/2010
Rhizosphere Collection 2011	6/21/2011	7/11/2011	7/25/2011

2.2. Field design

Research was conducted in 2010 and 2011 at the University of Maryland Lower Eastern Shore Research and Education Center, Salisbury, the New York Agricultural Experimental Station, Phytophthora blight research farm in Geneva and the Ohio Agricultural Research and Development Center, Wooster. The experiment was conducted as a randomized complete-block design with five treatments and six, eight or twelve replicates, for MD, NY and OH respectively (Table 1). The five treatments of single or mixed-species cover crop combinations were different in each state based on local growing conditions and practices. The experiment included the legumes hairy vetch (*Vicia villosa* Roth), crimson clover (*Trifolium incarnatum* L.) and alfalfa (*Medicago sativa* L.). Grasses used were annual rye (*Lolium multiflorum* Lam.), winter rye (*Secale cereal* M. Bieb) and mixed-species hay which included red fescue (*Festuca rubra* L.), orchard grass (*Dactylis glomerata* L.), and timothy (*Phleum pratense* L.), as well as the legumes crimson clover and alfalfa. The brassica species used were forage radish (*Raphanus sativus* var. *longipinnatus* L.) and forage turnip (*Brassica rapa* var. *rapa* L.). MD treatments included vetch (79 kg/ha) + winter rye (79 kg/ha); vetch (25 kg/ha); vetch (42 kg/ha) + radish (42 kg/ha); mixed-species hay (125 kg/ha with composition of equal seed number); and no cover. NY treatments included vetch (34 kg/ha) + winter rye (79 kg/ha); clover (10 kg/ha) + annual rye (18 kg/ha); turnip (15 kg/ha) + winter rye (45 kg/ha); winter rye (135 kg/ha); and no cover. OH treatments included winter rye (150 kg/ha); vetch (50 kg/ha); vetch (25 kg/ha) + winter rye (75 kg/ha); radish (10 kg/ha); and mixed-species hay with 56 kg/ha in 2010 and 112 kg/ha in 2011, also with composition of equal seed number. Cover crop seed was sown in the fall and the cover crop was mowed and tilled in as a green manure the following spring three to five weeks before transplanting the tomatoes (Table 1). Fields in all states had raised beds covered with black plastic and drip irrigation. Tomatoes were grown using standard organic practices including trellising.

2.3. Tomato rhizosphere DNA extraction

DNA extraction was performed on rhizosphere samples collected at four weeks post-transplant (Table 1). Rhizosphere samples were collected from two plants per plot from each plot and each

state in 2010 and 2011. Each plant rhizosphere was processed separately to generate two DNA samples per plot. This resulted in a total of 130 plots per year, with 260 total plots for two years and 520 DNA samples total (two DNA samples per plot). The root regions of the tomato seedlings were carefully extracted from the soil with as much soil removed as possible before finely chopping all small roots less than 4 mm in diameter. DNA was extracted from 0.25 g of small root tissue from each plant using the MoBio UltraClean Soil DNA extraction Kit (MoBio, Carlsbad, CA) following the manufacture's protocol. Genomic DNA was assessed using gel electrophoresis with 1% agarose gels and visualized with UV light following ethidium bromide staining.

2.4. Macroarray analysis

Macroarray analysis was performed on one DNA sample per plot for a total of 260 arrays. The ITS region was amplified with the universal primers ITS5 and ITS4 (White et al., 1990). PCR was carried out with 1× GoTaq Green reaction buffer containing 1.5 mM $MgCl_2$ (Promega, WI), 0.2 mM dNTPs, 0.2 μM each primer, 1 unit GoTaq DNA polymerase (Promega), and 2.5 μl (15–80 ng) of genomic DNA in a 50 μl reaction. The following PCR cycling conditions were used: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min; followed by 10 min at 72 °C. PCR products were purified according to the manufacturer's protocol using the Zymo PCR clean-up kit (Zymo Research, Irvine, CA). Macroarray analysis was performed as previously described, with thirty-one total pathogen species included on the macroarray membrane (Zhang et al., 2008). Briefly, 200 ng of purified amplicon DNA was hybridized overnight using the Gene Images AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham Biosciences, NJ) at 55 °C to a nylon membrane spotted with the oligonucleotide fragments (267 μM) specific to fungal and oomycete pathogens. Hybridized amplicon DNA was visualized using Kodak Biomax Light film (Rochester, NY) exposed for 1 h. Developed films were examined visually. All macroarray analyses were performed in NY to reduce variability.

2.5. Statistical analysis of macroarray data

For macroarray data from each state, a positive detection was coded as '1′ while pathogens undetected were coded as '0'. A generalized linear model (GLM) for binomial data was used to test the effect of cover crop treatment (CCT) on the presence of individual pathogens. If the main effect of CCT was significant at $P < 0.05$, then a Tukey's Honestly Significant Differences (HSD) test was used to test for significant differences among CCTs. Analysis was performed for individual pathogens if the pathogen was detected more than four times in the state and year tested. In order to test the effect of CCT on total pathogens detected, a GLM employing a Poisson function was used. The total plots for each state per year are MD = 30, NY = 40 and OH = 60. R statistical software (R Development Core Team, 2011) and the packages lme4 (Douglas et al., 2011), multcomp (Hothorn et al., 2008), RLRsim (Scheipl et al., 2008) and agricolae (Mendiburu, 2010) were used for analyses.

2.6. T-RFLP

The T-RFLP assay was performed as described previously (Benitez et al., 2007; Benitez, 2008). Briefly, the ITS region was amplified with the universal primers ITS5 and ITS4 (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). PCR was carried out with 1× GoTaq Flexi reaction buffer containing 1.5 mM $MgCl_2$ (Promega, WI), 0.2 mM dNTPs, 0.8 μM each primer, 1.5 units GoTaq Flexi DNA polymerase (Promega, WI), 0.04 mg RNaseA and 2.5 μl of genomic DNA (15–80 ng) in a 25 μl reaction. The following PCR cycling conditions were used: 95 °C for 5 min; 33 cycles of 94 °C for 1 min, 52 °C for 45 s, and 72 °C for 2 min; followed by 8 min at 72 °C. Amplification products were purified from fluorescent primers by precipitation using 3 mM sodium acetate and ethanol. The full length PCR products were digested using Hha1 enzyme and sent to the Molecular and Cellular Imaging Center (OARDC, Wooster, OH) for imaging. There, 0.1 μl of sample was mixed with 0.2 μl 600 bp size standard (CEQ DNA size standard kit 600) and 20 μl formamide. TRF were loaded and separated on the CEQ 8800 Genetic Analysis System (Beckman Coulter, Brea, California) and individual profiles were analyzed with the CEQ fragment analysis software (CEQ 8000 Genetic Analysis System). The cut off rate for terminal restriction fragments was 1% of the highest peak in the chromatogram profile of the sample. The T-RFLP analysis utilized both DNA samples from each plot for a total of 520 T-RFLP analyses. All T-RFLP analyses were performed in OH to reduce variability.

2.7. In silico terminal restriction fragment (TRF) determination

Sequences for tomato and pathogens of interest were mined from Genbank and digested *in silico* using the Hha1 enzyme (restrictionmapper.org). The terminal restriction fragment (TRF) from the ITS5 primer end used in the T-RFLP analysis was recorded and used for further analyses. Two tomato sequences (Genbank AY552528 and JN713416) were used to generate two TRF (109 bp and 110 bp respectively). Two *Fusarium oxysporum* Schlecht. (Fo) sequences (Genbank JN859461.1 and JN253789.1) generated TRF of 317 bp and 316 bp, respectively. One *Alternaria alternata* Fr. Keissl. (Aa) sequence (Genbank JF440581.1) generated a 334 bp TRF. For Fo, a TRF value was also available from actual T-RFLP analysis from isolate cultures, where the TRF matched the *in silico* TRF value of 316 bp (Benitez, 2008).

2.8. Percent detections and comparison of macroarray and T-RFLP detections of tomato rhizosphere pathogens

All T-RFLP data from TRF correlated to specific organisms *in silico* were converted from fluorescence intensity units into a binary detection result: positive or negative. Because the T-RFLP analysis utilized both DNA samples per plot and the macroarray analysis was performed for one sample per plot, only one DNA sample was common to both tests. Therefore, in order to compare the T-RFLP and macroarray detection results within each plot, the two T-RFLP results were compiled into one detection result by coding the detection positive if at least one result was positive. For each plot, the macroarray result could then be matched directly with the T-RFLP result. For analysis of percent detection of Aa and Fo, the compiled result from the T-RFLP duplication was used in order to compare the results to the within-plot detections. However, for the percent detection of the control organism known to be present, tomato, all T-RFLP data points were considered separately in order to create a more accurate comparison of percent detection since control DNA was known to be present. Therefore, no within-plot comparison is presented for the control. The *in silico* TRFs generated were often close to those detected by T-RFLP, however, between states and years, there were often 1–3 base pair length differences. In order to do the inter-technique comparison, the most common TRF which were closest to the detected TRF sizes was chosen for the analysis. A difference of no more than 3 bp was allowed between the predicted TRF size and the detected TRF size.

In order to compare the macroarray and T-RFLP results within each plot, the two T-RFLP results for each plot had to be combined into one result. However, in order to further assess the T-RFLP efficiency, the two T-RFLP results within each plot were also compared.

Table 2
Detection of plant pathogens in tomato rhizosphere DNA collected at 4 weeks post-transplant by macroarray analysis when tomatoes are planted following different cover crops in field sites in three states over two years.

	Plots[a]					
	MD		NY		OH	
Pathogen	2010	2011	2010	2011	2010	2011
Fusarium oxysporum[b]	15	25	38	35	41	60
Alternaria alternata[b]	7	29	14	21	35	54
Fusarium solani	1	18	7	6	4	27
Phoma destructiva	3	22	10	13	19	36
Septoria lycopersici	5	7	8	2	11	27
Phytophthora capsici	0	0	1	0	0	0
Colletotrichum spp.	0	0	0	0	11	18
Pythium aphanidermatum	0	0	0	0	4	0
Pyrenochaeta lycopersici	0	0	0	0	3	7
Pythium ultimum	10	0	0	0	9	0
Pythium cryptoirregulare	1	0	0	0	0	0
Rhizoctonia solani	0	7	0	0	2	15
Pythium irregulare	3	0	0	0	0	0
Verticillium albo-atrum	2	0	0	0	0	0
Phytophthora nicotianae	0	0	0	0	0	1

[a] Total number of plots sampled each year: NY = 40, OH = 60, MD = 30. Numbers in table denote number of plots where the given pathogen was detected.
[b] *Fusarium oxysporum* and *Alternaria alternata* detection does not distinguish between pathogenic and non-pathogenic strains.

In order to do this, the percentage of pathogen positive T-RFLP plots where both results were positive was calculated.

3. Results

3.1. Macroarray analysis

Fifteen pathogens were detected using the macroarray in the three states over the two years of testing (Table 2). The most frequently detected fungi were *F. oxysporum* (Fo) and *Alternaria alternata* (Aa). *F. oxysporum*, *A. alternata*, *Phoma destructiva* Plowr., *Fusarium solani* and *Septoria lycopersici* Speg. were detected in all states and years. Although the NY fields were known to be infested with *Phytophthora capsici* Leonian, this pathogen was only detected once out of eighty plots sampled over two years. *Colletotrichum* spp. *P. aphanidermatum*, *Phytophthora nicotianae* Breda de Haan and *Pyrenochaeta lycopersici* R.W. Schneid. and Gerlach.were detected in OH only. In MD, *Pythium irregulare* Buisman, *P. cryptoirregulare* Garzón, Yánez and G.W. Moorman and *Verticillium albo-atrum* Reinke and Berthold were identified. *R. solani* and *Pythium ultimum* Trow. were detected in OH and MD (Table 2). The remaining sixteen pathogens on the array were not detected.

There were no significant affects of CCT on pathogen species detected (Table 3). In only one state/year out of six, NY 2010, and for only Aa, Tukey analysis indicated that the rye-treated plots had significantly more detections of Aa than the bare ground-treated plots (Table 4). However, Aa was consistently detected in all states and years and this outcome was not part of a larger trend.

3.2. Percent detections and comparison of macroarray and T-RFLP detections

Table 5 shows the specificity and sensitivity of macroarray and T-RFLP analysis in detecting specific organisms from the tomato rhizosphere. The control for both techniques was the detection of tomato DNA, as the ITS4–ITS5 primers used also amplify a segment of the tomato ITS gene. The quantity of DNA from this species is likely the most abundant, and therefore, should be detected consistently. The average percentage from all states and years indicates that macroarray was able to detect tomato DNA 96% of the time, with T-RFLP only detecting the control 53% of the time. While in MD in 2010, T-RFLP performed equally well as macroarray, in all other states and years, T-RFLP detected tomato DNA at a much lower rate, with the lowest success in NY 2010 with 34% detection for T-RFLP *versus* 93% detection by macroarray.

The presence of two common fungal pathogens, Aa and Fo, were also compared. Overall, the macroarray was able to detect these two organisms much more frequently than T-RFLP. The average rate of detection across states and years for Aa was 59% by macroarray, compared to 24% by T-RFLP. The results among states and years

Table 3
Results of GLM analysis testing (p-values) for significant effect of cover crop on pathogen detection by macroarray in tomato rhizosphere in three states sampled in two years. The total number of plots tested for each state is NY = 40, OH = 60 and MD = 30.

	Pathogen[a]	Treatment[b]
MD 2010	Total	0.13
	Alternaria alternata	0.02[c]
	Pythium ultimatum	1
	Septoria lycopersici	0.51
MD 2011	Total	1
	Alternaria alternata	0.26
	Fusarium solani	0.70
	Phoma destructiva	0.46
	Rhizoctonia solani	1
	Septoria lycopersici	0.41
NY 2010		
	Total	0.36
	Alternaria alternata	0.01[d]
	Fusarium solani	0.56
	Phoma destructiva	0.87
	Septoria lycopersici	0.83
NY 2011	Total	0.18
	Alternaria alternata	0.09
	Fusarium solani	0.03[c]
	Phoma destructiva	0.13
OH 2010	Total	0.30
	Alternaria alternata	0.14
	Colletotrichum sp.	0.01[c]
	Fusarium solani	0.81
	Phoma destructiva	0.65
	Pythium ultimatum	0.42
	Septoria lycopersici	0.63
OH 2011	Total	0.29
	Alternaria alternata	0.11
	Colletotrichum sp.	0.40
	Fusarium solani	0.33
	Phoma destructiva	0.32
	Pyrenochaeta lycopersici	0.06
	Rhizoctonia solani	0.05[c]
	Septoria lycopersici	0.47

[a] Test results for "Total" signify the effect of CCT on all pathogen detections in a given field. The effect of CCT on presence of individual pathogens detected in at least four plots in a field is also shown.
[b] Treatments are variable for each state. See Section 2.
[c] Although the effect of CCT was significant, there were no significant differences among CCT treatments based on Tukey analysis.
[d] See Table 4.

Table 4
Effects of CCT on *Alternaria alternata* detection, NY 2010.

Treatment	Plots detected[a]
Rye	(5)a
Clover + Rye	(2)ab
Turnip + Rye	(4)ab
Vetch + Rye	(2)ab
Bare Ground	(1)b

[a] Letters denote compact letter display from Tukey analysis. The number in parentheses is the number of detections out of eight repetitions of each treatment.

Table 5
Comparison of percent positive macroarray and T-RFLP detections of tomato, *Alternaria alternata* and *Fusarium oxysporum* DNA from tomato rhizosphere samples.

	Percent detection[a]						
	MD		NY		OH		
	2010	2011	2010	2011	2010	2011	Total[b]
Solanum lycopersicum[c]							
Macroarray	90	97	93	100	96	97	96
T-RFLP[d]	92	28	34	36	58	70	53
Alternaria alternata[e]							
Macroarray	23	97	35	53	58	90	59
T-RFLP	10	10	88	20	2	12	24
T-RFLP only[f]	7	0	63	8	2	2	14
Macroarray only[g]	20	87	10	40	58	80	49
Results same for both[h]	73	13	28	53	33	18	36
Fusarium oxysporum[e]							
Macroarray	50	83	90	88	68	100	80
T-RFLP	63	53	70	55	12	12	44
T-RFLP only[f]	27	7	5	8	0	0	8
Macroarray only[g]	13	37	25	40	57	88	43
Results same for both[h]	60	57	70	53	37	12	48

[a] DNA was extracted from two tomato rhizosphere samples per plot at four weeks post-transplant. The macroarray analysis was performed with one sample per plot, and the T-RFLP analysis was performed on both samples.
[b] 'Total' reflects the average of the percent detections over all states and years.
[c] *Solanum lycopersicum* serves as an effective positive control, since all DNA extractions used (780 samples total) were extracted from the tomato rhizosphere.
[d] For *Solanum lycopersicum*, the percentages are calculated from total detections in all samples (without combining the results of two DNA extractions per plot prior to analysis). This is because we knew control DNA to be present and did not wish to compare within-plot detections.
[e] For the comparisons of pathogen detection by macroarray and T-RFLP within each plot, one macroarray result needed to be compared to one T-RFLP result. To do this, if either result from the two T-RFLP analyses was positive, the detection was marked positive.
[f] Percentage of plots where T-RFLP detected the given organism but macroarray did not.
[g] Percentage of plots were macroarray detected the given organism but T-RFLP did not.
[h] Percentage of plots where macroarray and T-RFLP detections were either both positive or both negative.

for Aa detection were variable, with Aa detected in 97% of MD 2011 samples tested by macroarray as compared to 10% by T-RFLP, while in NY 2010, macroarray detected it 35% of the time and T-RFLP 88% of the time. The same trends were found for Fo detection, with macroarray detecting it 80% of the time overall compared to 44% for T-RFLP. In MD 2010, macroarray detected Fo less than T-RFLP with 50% *versus* 63%. However, in OH 2011, macroarray found Fo in 100% of the samples, compared to 12% for T-RFLP.

Both techniques, however, were prone to a lack of sensitivity. While macroarray overall could detect tomato DNA in 96% of samples, it did not detect the control in 10% of samples from MD in 2010. In addition, when considering the detection comparison from the state and year averages, T-RFLP detected Aa from 14% of samples and Fo from 8% of samples when macroarray did not. These differences could show a lack of sensitivity by macroarray, however, as indicated previously, these could be TRF from other organisms. The sensitivity of T-RFLP indicates low performance, as it only was able to detect tomato DNA in 53% of all samples. In addition, Aa was detected in 49% of samples and Fo in 43% of samples, by macroarray alone (not T-RFLP).

The calculation of the percentage of T-RFLP plots where both results were positive shows that few plots resulted in two positive results for the T-RFLP analysis. For MD in 2010 as well as 2011, only 3% of plots positive for Aa were positive for both T-RFLP results. For NY 2010, Aa was detected by both T-RFLP tests in 35% of positive plots, while in 2011, in only 8% of plots. For OH 2010, Aa was detected by both T-RFLP tests in only 1% of positive plots and 7% of plots in 2011. For Fo detections, the results were slightly more favorable. In MD 2010, both tests were positive in 19% of positive plots, and in 2011 it was 16%. For NY, both T-RFLP resultus were positive in 28% of plots in 2010 and 22% in 2011. For OH in both 2010 and 2011, T-RFLP results were the least consistent, with 7% of plots having both T-RFLP results positive out of the total positive plots.

4. Discussion

Effects of cover crop treatments on pathogens have been measured directly by experiments in culture, greenhouse and field trials. A variety of cover crops were found to inhibit the growth of many soilborne pathogens of potato *in vitro* and in greenhouse studies, and also demonstrated effectiveness in the field (Larkin and Griffin, 2007). Benitez et al. (2007) evaluated the effect of different field management strategies in the field and greenhouse on soilborne disease and concurrently analyzed T-RFLP profiles in order to mine for certain bacterial species associated with disease suppression (Benitez et al., 2007). Another study found that the toxins produced by the incorporation of the potential biofumigant, *Brassica juncea*, initially reduced inoculum potential of the soilborne pathogens *R. solani* and *F. oxysporum* (Friberg et al., 2009). However, Ochiai et al. (2008) found that cover crop amendment, especially larger amounts of biomass, reduced inoculum of *Verticillium dahliae*, regardless of the biofumigant potential (Ochiai et al., 2008).

In contrast to the above studies, no short term cover crop impact on pathogen detection was observed in this study. However, we do not conclude that this result is evidence that cover crops do not affect crop health or microbial communities. First, as noted earlier, general suppression may not result from a direct impact on specific pathogens. For example, in several studies, cover crops, even when associated with disease reduction, were not associated with a direct reduction in fungi. Zhou and Everts (2004) found that a vetch amendment increased the CFU of culturable fungi, as well as bacteria. This occurred in soils where Fusarium wilt was suppressed. Buyer et al. (2010) also found that cover crops increased total microbial biomass, including soil fungi, using phospholipid fatty analysis. Omirou et al. (2011) also found that cover crops impact microbial communities, but not pathogen presence, by the introduction of plant biomass. This indicates that in some pathosystems, suppression may not result from a direct impact on pathogens. Another

mechanism important in some systems is enhanced resistance of the host plant (Kumar et al., 2004). In one study on tomato, Kumar et al. (2004) demonstrated that disease suppression following a no-till hairy vetch cover crop was associated with up-regulation of specific classes of genes including defense related genes such as chitin and osmotin.

As discussed above, the complex interactions between plants, microbes and the soil environment has been measured in various ways and findings have been inconstant. Because of the variable findings of other papers, we decided to compare the results of the two PCR-based techniques we used in our study. T-RFLP has been used for years to examine microbial communities, while the macroarray was designed to be a diagnostic tool. By utilizing both macroarray and T-RFLP analyses to detect rhizosphere pathogens, the specificity and sensitivity of these two molecular techniques could be compared. Macroarray analysis is labor-intensive to design and is very specific to crop-pathogen systems as well as region. In addition, it is relatively more expensive and time-consuming than T-RFLP to run for a large-scale field study. T-RFLP also has the potential benefit of being semi-quantitative, in contrast to macroarray analysis. For these reasons, T-RFLP would be a more likely choice for similar field studies in other pathosystems. However, the macroarray is designed to detect specific organisms, and is sensitive even at very low DNA concentrations (Zhang et al., 2008). Therefore, since detection was designed to be specific to one organism only, a positive detection can be assumed accurate. In order to detect specific organisms using T-RFLP analysis, precise TRF lengths must be correlated to distinct organisms. Because there is some likelihood that more than one organism could have the same TRF length in a complex environment, false positives are possible. For both techniques in this study, an important weakness was inability to differentiate between pathogenic and non-pathogenic strains of *F. oxysporum* and *A. alternata*, as the ITS regions of these pathogens are very similar within their species (Lievens et al., 2003; Zhang et al., 2008). This is important because some *F. oxysporum* have been related to disease suppression, not pathogenicity (Larkin et al., 1996).

In order to compare T-RFLP and macroarray, we identified species detected often by macroarray, then performed *in silico* T-RFLP analysis to identify TRF that should correspond to each species. We then compared the detection results for these pathogens by calculating the percent number of detections in each plot, where 260 plots were tested over two years in three states (Table 5). In this study, the data for T-RFLP are skewed towards positive detections, because in order to compare T-RFLP and macroarray detections within each plot, two detection results from T-RFLP had to be combined into one result. In this case, one positive out of two tests resulted in a positive detection. This bias was accounted for by calculating the percent of plots where both T-RFLP results were positive out of the total number of positive plots. Nevertheless, in this study macroarray was able to detect various organisms, including the control, much more consistently than T-RFLP. In addition, though *Colletrichum* sp. and *R. solani* were commonly detected by macroarray in OH (Table 2) the TRF predicted to correlate to the presence of these organisms were not present. This suggests T-RFLP detection may be restricted to dominant and abundant species, as previously found by another study comparing detection methods (Allmér et al., 2006). This, however, cannot explain the inability of T-RFLP to detect the tomato DNA more consistently, since this DNA was likely among the most abundant in each sample. Although the PCR conditions were different for the two techniques, both T-RFLP and macroarray used the same primers, ITS4 and ITS5 (White et al., 1990), which both have a 100% identity with the tomato ITS sequence.

Previous studies have identified various problems with using T-RFLP for microbial fingerprinting. These include sources of variation due to both target gene variability in the community as well as method inconsistencies due to restriction enzyme effectiveness or PCR efficiency (Avis et al., 2006). However, it is possible that the T-RFLP methods in this experiment could have been optimized to be more effective, as studied by Osborn et al. (2000). For both T-RFLP and macroarray, one important source of variability in evaluating the effects of cover crop treatment on pathogen presence in each plot may have resulted from differences in genomic DNA quality. However, since the macroarray analyses and T-RFLP analyses utilized the same genomic DNA samples, the comparison of results for the two techniques should not be heavily influenced by variable genomic DNA quality or quantity.

Macroarray data was chosen to evaluate the cover crop treatment effects on the pathogen species we detected because the sensitivity was shown to be satisfactory and the specificity acceptable. Although no differences among treatments for pathogen detection were observed by the macroarray data in this study, this technique is not quantitative. Therefore, while it can detect the incidence of affected plants, it does not detect a reduction in pathogen levels. If a CCT decreased the population, but did not eliminate it, no difference would be observed, or would be observed only in the incidence of a pathogen. Furthermore, the pathogens that were detected may have been present at low levels that were not epidemiologically important in causing tomato disease.

Another important factor affecting which pathogens were detected was the timing of rhizosphere sampling. *P. capsici*, known to be present in the NY fields tested and causing disease later in the season, was not detected in the rhizosphere at four-weeks post-transplant when samples were collected. It is possible that rhizosphere samples collected later in the season may have shown significant effects of CCT on pathogen detection.

In conclusion, two important PCR-based molecular tools, T-RFLP and macroarray, were compared in a field study. Because of the complex nature of microbial ecology and pathogen studies, as well as the many factors involved in choosing methods with which to evaluate them, studies including and comparing more than one technique to investigate microbial communities are necessary. This continues to be important today, as more modern molecular techniques involving sequencing are also PCR-based (Anderson and Cairney, 2004). Quantitative real-time PCR (Smith and Osborn, 2009) and advances in next-generation sequencing (Schmidt et al., 2013) continue to further advance the field of microbial ecology. Our findings suggest that PCR-based techniques may need to be corroborated by concurrently using quantitative measures and checking with a diagnostic tool.

Acknowledgements

Thanks are extended to Holly Lange, Alexandra Curtis, Augusta Williams, Emily Wyckoff and Marci Day (Cornell University); Raymond Moore, Daniel Mongeon, Prasanti Babu, Ashlee Green and Wendy Mao (USDA ARS Nematology Laboratory); Matthew Hochmuth, Mason Newark, David Armentrout and Bernadette Gross (University of Maryland); and to Fulya Baysal, Martina Fraga, Seth Mauer, Elizabeth Kalikasingh, Miriam Gochnauer, Emily Nguyen, Jun Kyung Park, Seung Hawn Li, Sonia Walker, Jennifer Moyseenko and Michelle Young (The Ohio State University) for assistance in the laboratory, greenhouse and field. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Funding for this work was provided through the USDA's Organic Research and Extension Initiative (NIFA contract no. 2009-51300-05512). Funding for C.F. Summers was also

provided through an NSF pre-doctoral fellowship (DGE-1144153), and USDA-NIFA grant number 2009-55605-05184.

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