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ORIGINAL RESEARCH

Using T-RFLP to Assess the Impact on Soil Microbial Communities by Transgenic Lines of Watermelon Rootstock Resistant to Cucumber Green Mottle Mosaic Virus (CGMMV)

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Abstract To establish quantitative methods for ecological risk assessment, we assessed the impacts of transgenic watermelon rootstock (*Citrullus lanatus* (Twinser) cv. Gongdae) that was resistant to cucumber green mottle mosaic virus. The diversity of soil bacteria and fungi was monitored from May to July of 2005. Terminal restriction fragment length polymorphism (T-RFLP) was used with 16S ribosomal RNA (rRNA) coding genes for bacterial communities and with internal transcribed spacer (ITS) regions of rRNA coding genes for fungal communities. Multivariate analysis of variance (MANOVA) on the principal component analysis (PCA) scores of T-RF profiles detected no significant difference between microbial communities with transgenic or non-transgenic watermelon. Likewise, the results of our multi-response permutation procedure (MRPP) tests on non-metric multidimensional scaling (NMS) showed no significant difference between plant types. However, both MANOVA on PCA and MRPP on NMS revealed significant changes in the microbial community during the growing season. We used loading values of PCA to rank the abundances of bacterial species and found increases of some species in June and July.

Keywords Ecological risk assessment · Non-metric multidimensional scaling (NMS) · Principal component analysis (PCA) · Soil microbial community · Terminal restriction fragment length polymorphism (T-RFLP) · Transgenic watermelon

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Many transgenic crops have been developed and applied worldwide (Nap et al. 2003). Crop plants interact with soil communities to form strong links (Brimecombe et al. 2001), thereby influencing agro-ecosystems by changing gene flow and invasion and the community/food web (Dale et al. 2002). Soil microbial diversity must be adequately assessed to determine any environmental risk from such crops. We previously used terminal restriction fragment length polymorphism (T-RFLP) to investigate the impact of a transgenic watermelon resistant to cucumber green mottle mosaic virus (CGMMV) and have now established a molecular approach for elucidating the structure of that soil bacterial community (Park et al. 2005, 2006a, b; Park 2007). As a result, we have developed a procedure for principal component analysis (PCA) and multivariate analysis of variance (MANOVA) to discriminate the profiles of T-RFLP in soil bacterial communities (Park et al. 2006b).

Whereas PCA assumes linear relationships among T-RFs (Culman et al. 2008), non-metric multidimensional scaling (NMS) is an iterative method based on rank distances between sample units. It is conceptually simple (Rees et al. 2004) and useful for ecological-gradient studies because of

its general robustness and lack of assumptions about the distribution or type of data (Kruskal 1964; Mather 1976; Clarke 1993). Although its utility is important for evaluating ecological data sets, including those for microbial communities (van Hannen et al. 1999; Díez et al. 2001), it is still rarely applied as an ordination technique (Rees et al. 2004).

Our current objective was to investigate the effects of CGMMV-resistant transgenic watermelon rootstock on the diversity of soil bacteria and fungi. Two different multivariate analysis methods were compared with T-RFLP data: PCA vs NMS.

Materials and Methods

Transgenic Plants

In Korea, rootstock grafting is popular for the production of Cucurbiaceae crops because of the poor viability of their cultivar roots (Park et al. 2005). Rootstock of watermelon, *Citrullus lanatus* (Twinser) cv. Gongdae, is one of the most popular. The transgenic plant material used here had been developed to have resistance to infection by CGMMV. This virus causes mosaic symptoms, yellowish leaves, and fruit deterioration through overexpression of the CGMMV coat protein gene, *CGMMV-CP* (Park et al. 2005; Youk et al. 2009). From the 11 T_0 independent Gongdae lines created, we selected one line (12) because it had the highest degree of tolerance. This line was self-crossed to obtain the T_4 generation.

Experimental Design and Sampling

Soils were sampled from an experimental farm at Miryang (128°47′E, 35°30′N) in South Korea, a site where watermelon and rootstock are cultivated in isolated facilities. Each treatment (transgenic or non-transgenic) comprised three replication plots (3×5 m each) on which ten watermelon plugs were planted per plot. Their growth rate in 2005 was 1–2 m per month, so we used a small corer (to 5 cm deep) to collect 12 soil samples on May 12, 9 on June 16, and 3 on July 25. These were obtained from a layer approximately 10 cm deep around three plugs per plot. All soil samples and root material from the experimental plots were immediately placed on dry ice. Upon return to our laboratory, they were frozen at −80°C.

DNA Extraction of Soil Microbial Organisms

Total DNA for the microbial community was acquired from soil samples, using a FastDNA SPIN Kit (Qbiogene, USA) that was designed to extract PCR-ready genomic DNA. This total DNA was used for the T-RFLP analyses (Marsh 1999).

16S rDNA Region Primer and PCR for Bacteria

PCR for the bacteria was conducted using extracted DNA (final concentration: 50 ng per 50 μl) as a template to amplify the 16S rRNA gene. Primers included fluorescence dye (FAM)-labeled 8F-FAM (5′-AGAGTTTGATCCTGGCTCAG-3′; LaMontagne et al. 2002) and unlabeled 1492R (5′-TACGGTTACCTTGTTACGACTT-3′; Martin-Laurent et al. 2001). The reactions were performed with 50-μl (final volume) mixtures containing 10× Taq buffer, 1 μl of each deoxyribonucleotide trisphosphate at a concentration of 0.25 mM (Promega, USA), 1 μl of each primer at a concentration of 10 pmol, and 2 U of Taq DNA polymerase (SolGent Inc., Korea). Conditions included an initial denaturation step for 3 min at 94°C; then 25 amplification cycles of denaturation (45 s at 94°C), annealing (45 s at 55°C), and elongation (2 min at 70°C); followed by final extension for 7 min at 72°C (Ritchie et al. 2000). PCR products from T-RFLP were purified with a QIAquick PCR purification kit (QIAgen, USA) and detected by 1% agarose gel electrophoresis.

ITS Region Primers and PCR for Fungi

PCR for the fungal community was conducted using extracted DNA (final concentration: 100 ng per 50 μl) as a template to amplify an approximate 650-bp fragment of the internal transcribed spacer ITS1-5.8S-ITS2 (ITS) rRNA gene. Primers included FAM-labeled EF3RCNL (5′-CAAACTTGGTCATTTAGAGGA-3′) and unlabeled ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; Lord et al. 2002). The reactions were performed with 50-μl (final volume) mixtures containing 10× Taq buffer, 7 μl of each deoxyribonucleotide trisphosphate at a concentration of 0.25 mM, 5 μl of each primer at a concentration of 10 pmol, and 2 U of Taq DNA polymerase. PCR conditions included initial denaturation for 2 min at 94°C; then 30 amplification cycles of denaturation (1 min at 94°C), annealing (1 min at 56°C), and elongation (2 min at 72°C); followed by a final extension step for 10 min at 72°C (Ritchie et al. 2000).

PCR Product Digest and Analysis

To enhance T-RFLP performance, we combined products from six PCR runs (total volume: 300 μl), followed by purification with a QIAquick PCR purification kit (QIAGEN, Germany). Purified products (1 μg) were digested with the restriction endonucleases *Hae*III (Promega, USA), *Hha*I (Promega, USA), and *Hinf*I (Promega, USA) at 37°C for 2 h. Those enzymes were chosen because they showed the highest polymorphism of cleavage sites at the extremities of the amplified DNA fragment. Reactions were conducted using 20-μl (final volume) mixtures containing

2 μl of the 10× buffer, 2 μl of the 10× bovine serum albumin acetylated (Promega, USA), and 1 μl of restriction endonuclease (10 U). Digests (1–2 μl) were mixed with 12 μl of formamide and 0.5 μl of size standard (GeneScan-1000 ROX; Applied Biosystems). The samples were denatured at 96°C for 4 min, then placed on ice; lengths of the restricted fragments were determined on an automated ABI DNA sequencer (Model 3100, Applied Biosystems) for 1 h 32 min and 10 s. The fluorescently labeled 5′-T-RFs were detected and analyzed by a GeneScan 3.7 test (Applied Biosystems), with size markers ranging from 29 to 677 (Lukow et al. 2000). We used a web-based analysis tool (PAT+) provided by MiCA3 (http://mica.ibest.uidaho.edu/) to identify bacterial species for important T-RF peaks, based on the RDP Release 9.60 16S rRNA gene database (Shyu et al. 2007; Nakano et al. 2008).

PCA

Those T-RF peaks identified from individual T-RFLP profiles were compiled and manually aligned (tolerance limit: ±0.5 bp) to produce large data matrices. Centered T-RFLP profile data were standardized to relative abundance prior to PCA. We assigned a value of '0' if there was no matching peak. PCAs were applied using covariance data matrices to reduce their dimensionality. MANOVA was conducted to discriminate PCA scores from different groups (Park et al. 2006b). Using the PCA scores, we performed MANOVA on two plant types (transgenic vs non-transgenic) and three seasonal factors (May vs June vs July). Variances for each PC were examined with Rule N (Overland and Preisendorfer 1982; Termonia 2001), and several PCs were selected for MANOVA. Normality of the data sets was checked with a Kolmogorov–Smirnov test. PCA and MANOVA were performed with S-Plus 6 for Windows (Insightful Corp., USA).

NMS and Multi-Response Permutation Procedures

Non-metric multidimensional scaling was done using PC-ORD version 4.28 (McCune and Mefford 1999; McCune and Grace 2002). The pooled main matrices for each data had high beta diversity, moderate to extreme row and column skewness, and a high coefficient of variation (CV) among the sums of the columns (species) in the matrix. Thus, rare species that occurred in less than 5% of the samples were deleted, and the data were then transformed by taking logarithms. Relativization by column (species) maxima was then done to equalize the weights between abundant and less abundant species. The Sørensen distance measure was used for all analyses.

Our simple repeated-measure designs, which are common in ecology, required multi-response permutation procedures (MRPP; McCune and Grace 2002). This MRPP is useful for analyzing ecological data because assumptions of normality and constant variance are not necessary (Biondini et al. 1988; McCune and Mefford 1999). Because our research data fitted well, we used MRPP. That particular analysis is a non-parametric procedure for testing a hypothesis of no difference between two or more groups of entities; it randomly reassigns the observed values to different treatments.

Results

T-RFLP Profiles

We obtained 138 T-RFLP profiles out of metagenomes extracted from soils in which virus-resistant watermelon (69 profiles) and a non-transgenic parental line (69 profiles) were grown over 3 months (66 in May, 54 in June, and 18 in July). *Hae*III, *Hha*I, and *Hinf*I endonucleases were used here (46 profiles per endonuclease). In July, most watermelons were harvested, and only one plot per treatment remained. Most T-RFs ranged in size from 50 to 650 bp. After manual alignment of T-RFs, with a tolerance limit of ±0.5 bp, we identified 199 (*Hae*III), 242 (*Hha*I), and 171 (*Hinf*I) different T-RF occurrences for our bacteria data set and 374 (*Hae*III), 386 (*Hha*I), and 397 (*Hinf*I) T-RFs for the fungi data set. Generally, the fungi T-RFLP had many more occurrences than the bacteria T-RFLP, regardless of the endonuclease.

Principal Component Analyses of T-RFLP Profiles and MANOVA on PCA Scores

PCA calculated scores for the T-RFLP profiles of bacteria and fungi with *Hae*III, *Hha*I, and *Hinf*I (Fig. 1). For the bacteria, PCA explained 40% (*Hae*III), 37% (*Hha*I), and 44% (*Hinf*I) of total variability with two principal component axes. For the fungi, PCA explained 29% (*Hae*III), 27% (*Hha*I), and 26% (*Hinf*I) of all variability with the first two principal component axes. Although PCA plots showed no distinct separation between PCA scores from transgenic and non-transgenic treatments, seasonal differences were evident. Bacterial communities in May were grouped against those in June and July on the second principle component axis (PC2), while fungal communities in May were grouped against those in June and July on the first principle component axis (PC1).

MANOVA on the PCA scores of T-RFLP profiles revealed that microbial communities in soils containing transgenic or non-transgenic watermelon did not differ at the 95% significance level for any data sets (Table 1). However, those results did clearly show that microbial

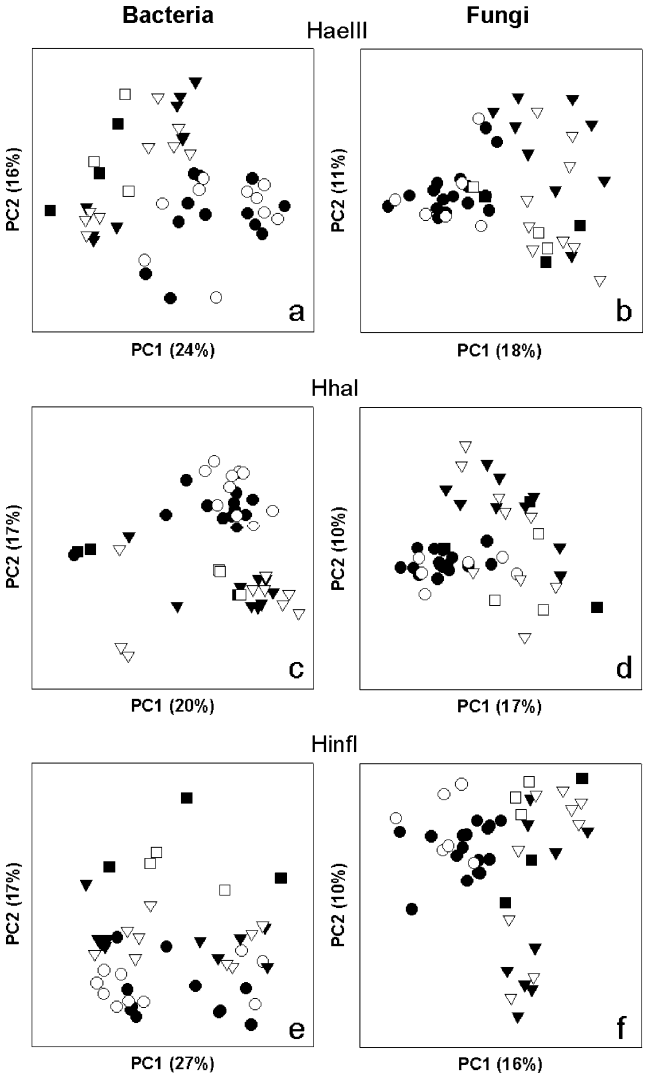

Fig. 1 PCA scores for T-RFLP profiles of bacterial and fungi communities from soils containing non-transgenic (*closed symbols*) and transgenic (*open symbols*) watermelon plants in May (*circles*), June (*triangles*), and July (*rectangles*)

communities in May were significantly different from those in June and July ($p<0.001$). Changes in those communities from June to July were also significant, having much higher p values ($p<0.05$) except for the bacterial data set with *Hinf*I ($p<0.001$).

Non-Dimensional Scaling and Multi-Response Permutation Procedures on T-RFLP Profiles

Patterns for soil microbes from the NMS analysis were evaluated for our three restriction enzymes (Fig. 2). For bacterial communities, Axis 1 and Axis 2 of the NMS ordination accounted for 32% and 54% of the total variation for *Hae*III, 27% and 37% for *Hha*I, and 29% and 39% for *Hinf*I, respectively. For fungal communities, Axes 1 and 2 accounted for 32% and 31% of the total

Table 1 MANOVA results for PCA scores

Group	Bacteria						Fungi					
	HaeIII		Hha1		HinfI		HaeIII		Hha1		HinfI	
	T^2	p	T^2	p	T^2	p	T^2	p	T^2	p	T^2	p
NT-T	0.023	0.977	0.644	0.530	0.057	0.945	1.016	0.371	1.113	0.338	1.766	0.183
May–June	58.002	<0.001***	69.290	<0.001***	19.727	<0.001***	51.285	<0.001***	30.831	<0.001***	61.988	<0.001***
May–July	52.438	<0.001***	19.900	<0.001***	66.518	<0.001***	13.737	<0.001***	12.265	<0.001***	16.532	<0.001***
June–July	3.839	0.029*	3.680	0.037*	24.794	<0.001***	4.444	0.018*	3.531	0.038*	4.251	0.021*

Comparisons were made between microbial communities with transgenic (T) and non-transgenic (NT) watermelon plants, and among microbial communities in different months. T^2 indicates Hotelling's T^2 and p, its probability

*$p<0.05$
**$p<0.01$
***$p<0.0$

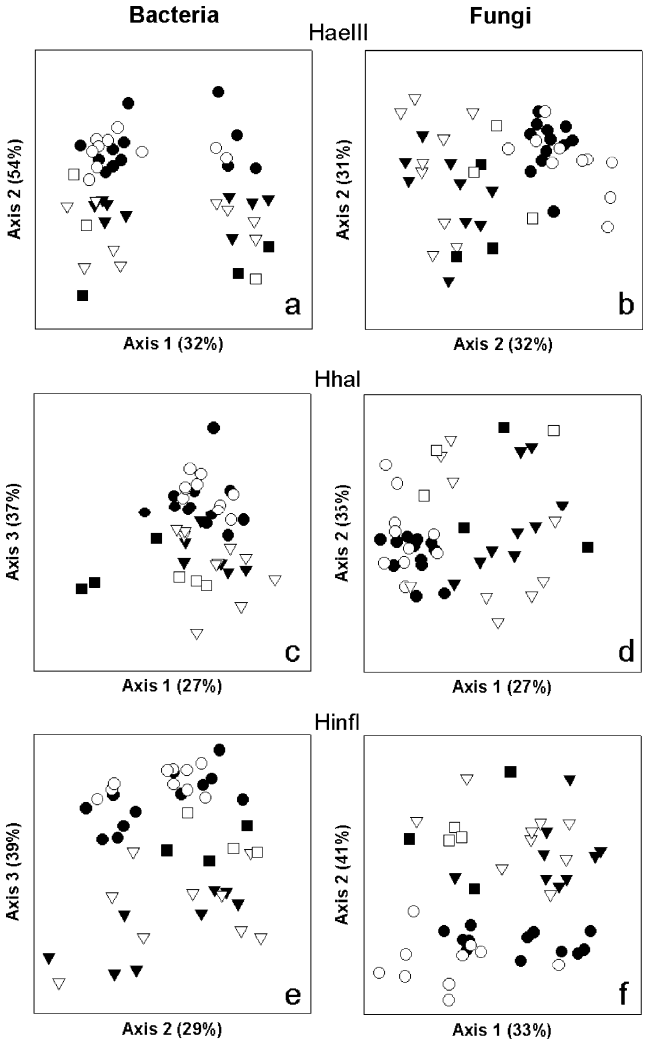

Fig. 2 NMS ordination for evaluating soil collected from sites with non-transgenic (*closed symbols*) and transgenic (*open symbols*) watermelon plants in May (*circles*), June (*triangles*), and July (*rectangles*). Final instability, 0.00116; final stress, 19%; iteration, 35

variation for *Hae*III, 27% and 35% for *Hha*I, and 33% and 41% for *Hinf*I, respectively. Each NMS result for bacteria and fungi with the three enzymes showed significant differences among soils by time of sampling but not by plant type.

We applied an MRPP test to identify any statistically explicit difference between plant type or among months, basing this on the graphical results from our NMS ordination (Table 2). Here, plant type was not associated with any statistically significant difference in the composition of a microbial community ($p>0.05$). In contrast, the seasonal factor led to statistically significant differences in the structures of both bacterial and fungal communities ($p<0.0001$).

Identification of Bacterial Species Using PCA Loading Values

Because the bacterial communities in May were very different from those in June/July, we attempted to list those species that most contributed to such seasonal changes in structure. PC2 had proven important for these patterns, so we investigated T-RF peaks with the 12 highest positive loadings (>0.10) for the *Hae*III and *Hinf*I data set and the 12 lowest negative loadings (<−0.08) for the *Hha*I data set. Using PAT+ in MiCA, we searched 12 peaks for our three restriction enzymes (36 peaks total) and selected bacteria identities within the 12th loading rank for all three data sets (Table 3). We then ordered those identities based on their averages and standard deviations of loading ranks.

Discussion

PCA and NMS, with subsequent statistical analyses, suggested that overall, bacterial and fungal community

Table 2 MRPP results from NMS analysis

Source	Endonuclease					
	HaeIII		HhaI		HinfI	
	Type	Season	Type	Season	Type	Season
Bacteria						
T	0.3940	−7.1944	−10.9756	−0.7545	0.1054	−15.2165
A	−0.0028	0.0753	0.0906	0.0004	−0.0006	0.1362
p	0.5476	<0.0001***	0.3928	<0.0001***	0.4592	0.0001***
Fungi						
T	−0.9535	−17.0502	−2.1284	−14.9762	−2.9719	−16.0724
A	0.0040	0.1041	0.0093	0.0953	0.0131	0.1037
p	0.1540	<0.0001***	0.0399*	<0.0001***	0.0148	<0.0001***

Comparisons were made between communities with non-transgenic and transgenic watermelon plants (type) and among communities in different months (season)

*$p<0.05$

**$p<0.01$

***$p<0.001$

Table 3 Bacterial species identified by MiCA, based on the RDP 9.60 16S rRNA gene database

Species suggested by MiCA	T-RF length (bp)			Rank		
	HaeIII	Hha1	HinfI	HaeIII	Hha1	HinfI
*Burkholderia*sp. JB1	196	371	326	5	6	6
*Burkholderia xenovorans*TCo-26	219	98	329	5	3	7
Burkholderiaceae bacterium KVD-1894-09	219	60	329	2	1	7
*Dechloromonas*sp. CL	219	203	321	1	8	5
*Dechloromonas*sp. HZ	219	203	321	1	8	5
Kinetoplastibacteriumcrithidii	219	203	321	1	8	5
Uncultured beta Proteobacterium UCT N112	219	206	323	4	10	3
Uncultured beta Proteobacterium UCT N161	219	206	323	4	10	3
Uncultured beta Proteobacterium	219	206	323	4	10	3
Uncultured soil bacterium UC8	219	206	323	4	10	3
*Variovorax paradoxus*1-94	219	206	323	4	10	3
*Variovorax*sp. KS2D-23	219	206	323	4	10	3
*Pseudoalteromonas*sp. 8039	216	206	323	4	10	3
Uncultured beta Proteobacterium UCT N032	222	206	323	4	10	3
*Acidovorax*sp. JS42	216	206	323	4	10	3
*Acidovorax*sp. PD-10	216	206	323	4	10	3
Beta Proteobacterium NOS8	216	206	323	4	10	3
Chitinimonas taiwanensis(T) cf.	216	206	323	4	10	3
*Comamonas*sp. PHD-10	216	206	323	4	10	3
Diaphorobacter nitroreducens(T) NA10B	216	206	323	4	10	3
*Diaphorobacter nitroreducens*KSP3	216	206	323	4	10	3
*Diaphorobacter nitroreducens*KSP4	216	206	323	4	10	3
*Diaphorobacter*sp. NA5	216	206	323	4	10	3
*Diaphorobacter*sp. PD-12	216	206	323	4	10	3
*Mitsuaria chitosanitabida*12	216	206	323	4	10	3
*Bifidobacterium breve*BGM6	255	206	323	4	10	3
*Bifidobacterium breve*BR2	255	206	323	4	10	3
*Bifidobacterium longum*BG	255	206	323	4	10	3
Burkholderiaceae bacterium KVD-1894-01	219	203	326	4	8	6
Burkholderiaceae bacterium KVD-1894-02	219	203	112	3	8	8
Burkholderiaceae bacterium KVD-1894-03	219	203	112	3	8	8
Burkholderiaceae bacterium KVD-1894-04	219	203	112	3	8	8
Burkholderiaceae bacterium KVD-1894-05	219	203	112	3	8	8
Burkholderiaceae bacterium KVD-1894-06	219	203	112	3	8	8
*Sphingomonas*sp. A1	219	206	323	6	10	3
*Burkholderia*sp. AK-5	219	371	326	9	6	6
*Burkholderia*sp. PYX3	219	371	326	9	6	6
*Burkholderia xenovorans*B2-5	219	371	326	9	6	6
*Corynebacterium glutamicum*TCCC27018	232	203	112	1	8	8
*Corynebacterium glutamicum*TCCC27021	232	203	112	1	8	8
*Corynebacterium glutamicum*TCCC27030	232	89	343	10	4	1
Burkholderiaceae bacterium KVD-1894-07	219	97	329	12	12	7
Burkholderiaceae bacterium KVD-1894-10	219	371	329	12	6	7
Burkholderiaceae bacterium KVD-unk-47	219	371	329	12	6	7
Burkholderiaceae bacterium KVD-unk-48	219	371	329	12	6	7
Candidatus*Burkholderia verschuerenii*19750204	219	371	329	12	6	7
*Corynebacterium glutamicum*TCCC27034	232	97	326	1	12	6

These candidates were selected from T-RF peaks with high loading values on their second principal components (PC2). T-RF lengths and their ranks on PC2 are presented for each restriction enzyme in the order of lower sum of ranks and standard deviation of ranks

compositions in soils with transgenic watermelons were not significantly different from those with non-transgenic plants. However, both PCA and NMS were able to detect significant compositional fluctuations among communities over time.

Although PCA and NMS are entirely different ordination methods, both provided congruent results here (Tables 1, 2). This was of interest to us because it is generally thought that the former is less appropriate for T-RFLP community analysis due to its linearity assumption (Rees et al. 2004; Culman et al. 2008). Nevertheless, if T-RF data are centered, PCA is one of the most robust ordination procedures for analyzing T-RFLP data (Culman et al. 2008). Our results support the conclusion that many ordination methods produce comparable patterns when using soil T-RF data sets that are usually low in sample heterogeneity (Culman et al. 2008).

In terms of their representative variances, NMS showed higher r^2 values for two axes than did the ratios of eigenvalues to total variance from PCA. However, the distance-based r^2 from NMS is an "after-the-fact" means for reducing data quality, whereas PCA uses a "built-in" method for variance (McCune and Grace 2002). Therefore, we think that these values for variability as represented by NMS and PCA are not directly comparable.

Our results suggested that ecological risk assessments of transgenic crops on soil microbes require a thorough examination of temporal (seasonal) and spatial variabilities if researchers are to provide baselines for such detection (see also Lynch et al. 2004, and Ikeda et al. 2006). Here, the presence of transgenic watermelon appeared to have little impact on bacterial and fungal communities. However, microbial communities underwent significant changes in their species composition as the watermelon crop continued to develop into the Summer. This is in accordance with many other observations of strong shifts in soil compositions during plant growth (Smalla et al. 2001; Heuer et al. 2002; Gomes et al. 2003).

To compare PCA scores from different treatments, we used MANOVA with scores from PCA (Park et al. 2006b). Researchers must provide a quantitative means for deciding whether structures of soil microbial communities are different when associated with transgenic crops. Such assessments are as important as general studies of those communities (Rees et al. 2004; Widmer 2007). MANOVA can test if scores from different groups are significantly different regardless of the number of axes used (Kourtev et al. 2002; Park et al. 2006b). However, MANOVA on PCA scores is not equivalent to a combination of ANOVAs for each PC axis. Even though MANOVA can reveal significant differences in scores between or among groups, ANOVAs on each axis may not discriminate groups with any statistical significance (Cuthill et al. 1999). Therefore, conducting MANOVA on PCA scores simultaneously would improve statistical power to discriminate scores from different groups compared with ANOVAs on each PC axis. When MANOVA is able to discriminate scores from different groups, e.g., seasonal patterns, it is possible to identify important T-RF peaks by using the loading values of PCA (Esperschütz et al. 2007; Nam et al. 2008). Indeed, we conducted a loading analysis to learn which bacterial species contributed most to those seasonal changes (Table 3). Here, that loading approach provided in-depth information about important T-RF peaks and their identification.

In conclusion, we have assessed the impacts of transgenic watermelon on the bacterial and fungal community structure of soils in which it is growing. Neither PCA nor NMS could detect any significant difference in microbial compositions, although significant community changes were observed over time. We also determined the major bacterial identities associated with those seasonal patterns. To evaluate the ecological risks of transgenic crops on soil communities, we recommend that researchers take quantitative approaches, e.g., MANOVA on scores from ordination methods. PCA loadings can provide further information on important T-RF peaks.

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