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Influence of soil fumigation by methyl bromide and methyl iodide on rhizosphere and phyllosphere microbial community structure

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Rhizosphere and phyllosphere microbial communities were evaluated on roots and leaves of growth chamber-grown lettuce (*Lactuca sativa* (*L.*) cv. Green Forest) plants by culture-dependent and -independent methods after soil fumigation. Denaturing gradient gel electrophoresis (DGGE) with 16S rRNA primers followed by cloning and sequencing was used to identify major rRNA bands from the rhizosphere and phyllosphere. Three weeks after fumigation, there were no differences ($P = 0.16$) in rhizosphere microbial communities between the fumigated treatments and the control. The same effect was observed during week seven after fumigation ($P = 0.49$). Also, no significant differences ($P = 0.49$) were found in the phyllosphere microbial communities between the fumigated treatments and the control during the growth period of the plant. A majority of the bands in the rhizosphere were related to known bacterial sequences with a 96 to 100 % sequence similarity. Some of the derived sequences were related to *Pseudomonas syringae* pv. tomato DC300 and *Bradyrhizobium japonicum* USDA 110. A total of 23 isolates were identified from leaf surface by both culture-dependent and independent methods, and only *Photorhabdus luminescens* was found on leaf surface using both techniques. All the Biolog isolates from phyllosphere were from the *Proteobacteria* phylum compared to the culture-independent bands from the leaves that were from different bacterial phyla. Based on our data, methyl bromide (MeBr) and methyl iodide (MeI) did not have any significant negative effects on rhizosphere and phyllosphere microbial communities throughout the growing period of lettuce.

Keywords: Denaturing gradient gel electrophoresis; rhizosphere, phyllosphere; microbial communities; methyl bromide methyl iodide.

Introduction

Methyl bromide (bromomethane, MeBr) has been used widely since the 1940s as an effective pre-plant soil fumigant for controlling nematodes, plant pathogens, weeds and insects.[1] Fumigant use is vital for the economic viability of many crops, including strawberries, tomatoes, peppers, eggplants, tobacco, ornamentals, nursery stocks, vines and turves.[2,3] Its success as a fumigant is largely due to its wide spectrum of activity against pests at many stages of life, its ability to penetrate the fumigated zones, and the ease of application.[4] MeBr was phase-out in the United States in 2005[5] because of its stratospheric ozone depletion potential. Methyl iodide (MeI, iodomethane) is another fumigant that is in the registration process. Methyl iodide is often referred to as the "drop-in replacement" because its fate, transport characteristics and effectiveness as a biocide are similar to those properties of MeBr.[6] Methyl iodide has been identified as a stand-alone alternative to MeBr.[6] MeI has efficacy equal to or better than MeBr against fungi, nematodes, and weeds on equimolar basis.[6] The generally accepted mechanism of MeBr and MeI biological activity is through a bimolecular, nucleophilic displacement (S_N2) reaction with functional groups, such as NH_2 and SH, in various amino acids and peptides of the target organisms.[7] In soil, MeBr and MeI degrade through nucleophilic substitution reactions with water and nucleophilic sites on soil organic matter.[8]

Preliminary work with strawberry rhizosphere colonizers in fumigated compared to native soils suggests that there may be differences in deleterious and beneficial rhizosphere colonizers following soil fumigation.[9]A basic understanding of the soil and rhizosphere microbiology can simplify the identification of specific microorganisms that can be used directly for disease management, enhancement of plant growth or altered crop management practices to enhance their populations. One excellent example is the identification of specific bacterial rhizosphere

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colonizers that are capable of protecting apple roots from pathogens associated with apple replant disease and enhancing their soil populations by cropping specific cultivars of wheat.[10] Soil fumigation generally increases root health, growth, and fruit yields in strawberries even when major pathogens are not present in soil.[11,−13] Soil fumigation has been shown to reduce the incidence of *Pythium*, *Cylindrocarpon* and binucleate *Rhizoctonia spp* damaging to strawberry roots.[14,15] The reduction of pathogens is the major benefit of fumigation. The process does not result in soil sterilization but in some cases it results in changes in the microbial community structure. *Pseudomonas* species have been shown to survive fumigated soil and recolonize strawberry rhizospheres rapidly and in high numbers after fumigation.[16] The increase in numbers of different *Pseudomonas sp* in the strawberry rhizospheres after fumigation correlated to a significant increase in the growth of the strawberry plants in field and greenhouse experiments.[16]

The next hypothesis that was tested in this study was whether fumigation alters leaf surface microbial community. The rhizosphere is a nutrient-rich environment, while the phyllosphere is very limited in nutrient composition. The leaf surface topography and the nutrients present on the leaf surface are generally recognized as important regulators of phyllosphere microbial communities, but no research has been done at the whole community level after soil fumigation. Nothing has been done on the microbial community structure of fresh produce grown in different soils after fumigation with MeBr and MeI. In this study we used DGGE[17] in an attempt to obtain an overview of the structural diversity of microbial communities in the rhizosphere and phyllosphere after soil fumigation and Biolog assay to assess functional microbial communities in the phyllosphere. The main advantage is the presentation of microbial development dynamics with plant maturity in a way that the culture-dependent method lacks. The aim of this study was to increase our understanding of the distribution, diversity, and composition of microbial communities associated with developing lettuce plants grown in different soil types after fumigation.

Materials and methods

Soils and plant conditions

Clay soil (Willows silty clay, saline-alkaline) and sandy soil (Dello loamy sand) were collected from Mystic Lake dry bed and the Santa Ana River bed, respectively.[18,19] Seeds of green romaine lettuce (*Lactuca sativa* (*L.*) cv. Green Forest) were purchased from Johnny's Selected Seed Co. (Albion, ME). The plants were grown at 20° C, 70 % humidity and a photoperiod consisting of 16 h of light and 8 h of darkness in a walk in growth chamber.

Growth chamber experiment and fumigant application

Plastic trays (58.2 × 43.2 × 18.5 cm) were filled with approximately 40 kg of soil, and irrigated with approximately 2.2×10^8 *E. coli* O157:H7. Bacteria were inoculated into the irrigation lines as described.[18] Fumigant application rates were selected according to the recommendations for each chemical. Methyl iodide (>99 % purity) was purchased from Chem Service (West Chester, PA) and methyl bromide (>99 % purity) was obtained from Great Lakes Chemical company (West Lafayette, IN). Application rates for MeBr and MeI were approximately 48 Kg ha^{-1} and 40 Kg ha^{-1}, respectively. These rates were designated 1X to indicate approximate field application rates. Methyl bromide and MeI were also applied at half this rate (0.5X) to examine the effect of lower fumigant concentrations on the soil microbial diversity, and non-fumigated controls (0X) were included.

To avoid the emission of fumigants to the growth chamber, syringes were used to inject MeBr (gas) and MeI (liquid) into the soil through the tarp, and the punctured hole was covered immediately with duct tape. Soil trays were left in the growth chamber for 10 d. After 10 d, trays were moved outside and the plastic film was removed. Trays remained outside in an enclosed area, open and aerated a for 2 d before they were moved back to the growth chamber for the continuation of the experiment. At this point, a total of 14 d had elapsed since fumigant application. Soil samples were collected for the second time for community analysis, *E. coli* O157:H7(pGFP) concentration and heterotrophic plate counts.

Plant growth and sampling

Lettuce seedlings were grown in 50 % Hoagland's solution[20] and transplanted into the soils in two growth chambers. The experiment was a completely randomized design. The growth chambers were kept under the same environmental conditions at 20°C and 70 % relative humidity. Plants in both soils were irrigated with distilled water daily and received filtered sterilized nutrient solution weekly. Rhizosphere, bulk soil, and leaf surface samples were collected weekly for 5 weeks for *E. coli* O157:H7 and heterotrophic plate counts. The samples were collected in separate sterile petri dishes or collection bags. Leaf samples were collected above the soil surface with a sterile blade, placed in a stomacher bag and weighed. The rhizosphere samples were collected also and placed in stomacher bag and weighed. Non-rhizosphere soil (0–10 cm) was collected 30 cm from the plant stem using a 2 cm diameter sterile stainless steel soil probe. These samples were transferred to plastic bags and 10 g of sample was used for serial dilution. Total bacterial community DNA and *E. coli* O157:H7 was extracted from rhizosphere and leaf surfaces by homogenization with 100 mL of phosphate buffer saline (PBS) for 2 min at 260 rpm in a Seward stomacher 400 Circulator (Seward Ltd., London, UK). The homogenate was centrifuged

at 3000 × g for 10 min and the pellet was resuspended in 2 mL of 10 mM phosphate buffered saline, [PBS] (Fisher Scientific, Pittsburgh, PA), pH 7 and used for community DNA extraction. The *E. coli* O157:H7 data has previously been published.[21]

DNA extraction, PCR amplification, and DGGE analysis from rhizosphere and phyllosphere

Community DNA was extracted from rhizosphere and phyllosphere samples with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Solana Beach, CA) and stored at – 20° C after further cleanup steps. A 236-bp DNA fragment in the V3 region of the small subunit ribosomal RNA genes of eubacteria was amplified by using primer set PRBA338f and PRUN518r.[22] Ready-To- Go polymerase chain reaction (PCR) beads (GE Healthcare, Piscataway, NJ) and 5 pmol of primers in a total volume of 25 mL were used in the PCR reaction. PCR amplifications were done under the following conditions: 92°C for 2 min; 30 cycles of 92°C for 1 min, 55°C for 30 s, 72°C for 1 min followed by a final extension at 72°C for 6 min. DGGE was performed with 8 % (wt/vol) acrylamide gels containing a linear chemical gradient ranging from 30 to 70 % denaturant with 100 % defined as 7 M urea and 40 % formamide.[18]

Characterization of phyllosphere bacterial communities in BIOLOG EcoPlates by DGGE

Microbial community analysis using Biolog EcoPlates (Hayward, CA) was used to relate culturable bacteria to specific substrates. In order to obtain substrate utilization patterns of phyllosphere microbial communities, cell suspensions were prepared by extracting total bacterial DNA from the phyllosphere with PBS. Cell suspension was serially diluted and 150 μL suspension was added into the Biolog plates with an 8-channel repeating pipette and incubated at 25° C for 72 h. One mL of the microbial suspension from each leaf sample was also used to extract total DNA for microbial community assay using DGGE. DNA was extracted as stated above.

Statistical analysis of DGGE bands

DNA fingerprints obtained from the 16S rRNA banding patterns on DGGE gels were photographed and digitized using Image Master Labscan (GE Healthcare, Piscataway, NJ). The gel images were straightened and aligned using Image Master 1D Elite 3.01 (GE Healthcare, Piscataway, NJ) and analyzed to give a densitometric curve for each gel.[23] Band positions were converted to Rf values between 0 and 1, and profile similarity was calculated by Pearson similarity coefficients. Data were integrated and analyzed using ImageMaster 1D database 2.01 (GE Healthcare, Piscataway, NJ). Data obtained were used for the construction of a library to determine the best-fit profile and to integrate the area under each peak for every gel and for the construction of a dendrogram between treatments.

The Shannon index of diversity (H) was compared using a one-way analysis of variance,and Tukey HSD test for post hoc analysis.[24] Diversitywas calculated by comparing changes in diversity of microbial communities within all treatments at each time[25] by using the following function:

$$H = -\Sigma P_i \log P_i$$

when $P_i = n_i/N$, n_i is the height of peak, and N is the sum of all peak heights in the curve.

Results

Changes in rhizosphere soil microbial community structure following fumigation

Microbial community structure on the rhizosphere of lettuce from the two soils was examined to determine microbes that were loosely attached to the roots (rhizosphere) during plant growth. To validate changes in rhizosphere microbial composition with plant age after soil fumigation, DGGE banding patterns of rhizosphere microbial communities from week three, four, five, and seven were analyzed. Three weeks after fumigation (Fig. 1a; 1 wk after planting), there were no differences ($P = 0.16$) in rhizosphere microbial communities between the fumigated treatments with and without *E. coli* O157:H7 and the control (Table 1). The same effect was observed during week seven after fumigation (Fig. 1b; $P = 0.49$). However, there were major differences in banding patterns between week three and week seven as the numbers of dominant bands increased from a maximum of about 20 in week three to about 50 bands in week seven (Fig. 1 a&b). Pearson coefficient was used to compare DGGE patterns from different days for comparisons of all profiles, and unweighted pair group method with mathematical averages (UPGMA) was used to create a dendrogram describing pattern similarities (Fig. 1 c, sandy soil). Data from sandy soil rhizosphere revealed bacterial communities from week seven were 80 % different from microbial community from week three (Fig. 1 c). The same level of differences was observed in clay soil (data not shown). Microbial community structure on the phyllosphere of lettuce using DGGE analysis of microbes released from whole leaves by sonication, revealed distinct 16S rRNA banding patterns from week three to week seven (Fig. 2a&b). Banding patterns were more complexed, but with stable pattern during week seven than week three. There were no differences ($P = 0.49$) in phyllosphere microbial communities between the fumigated treatments and the control (Table 1) during week three. The same effect was observed during week seven after fumigation ($P = 0.60$) and inoculation with *E. coli* O157:H7 did not have any effect on microbial community. However, there were major increases in the Shannon index of diversity from week three

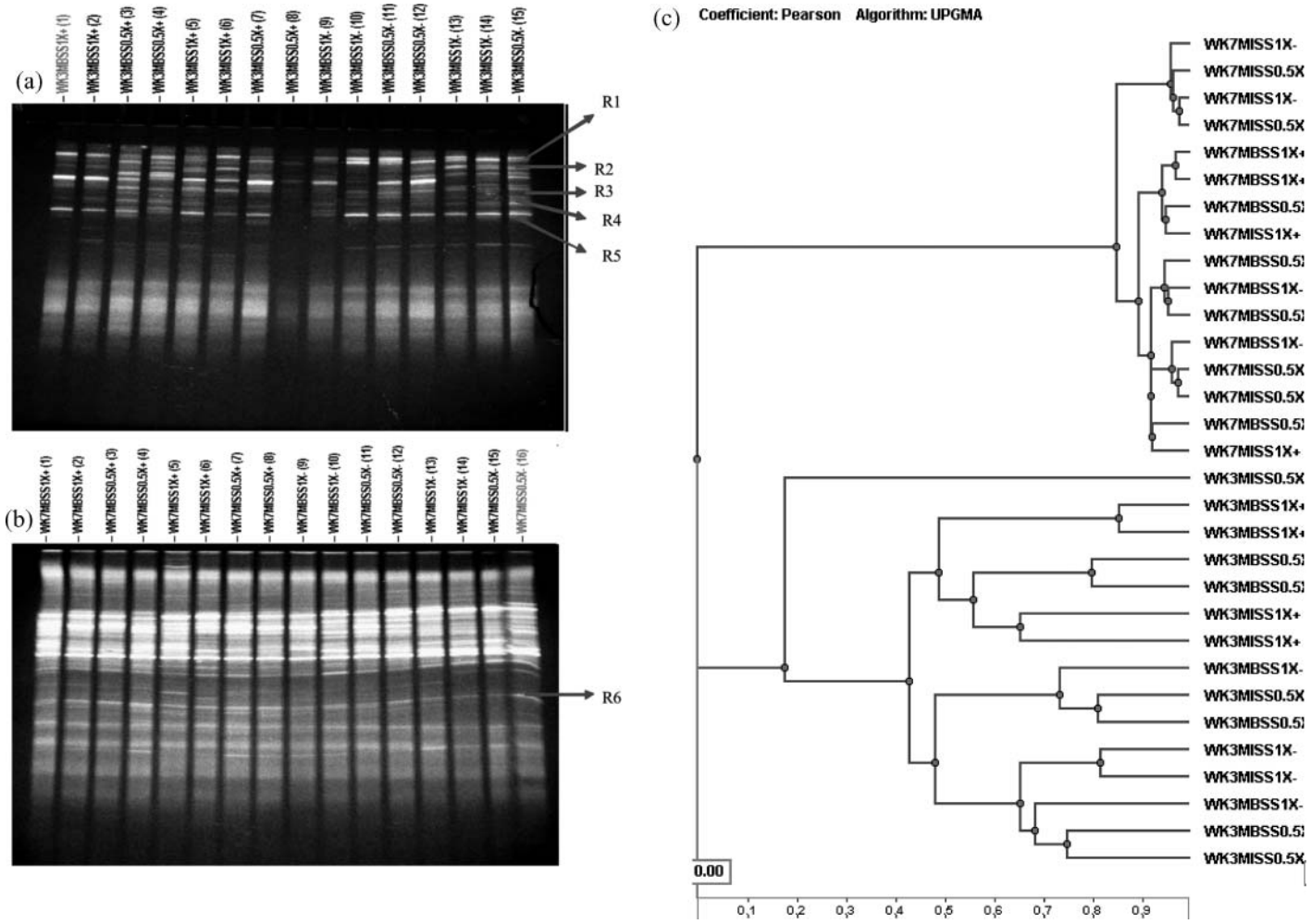

Fig. 1. Denaturing gradient gel electrophoresis(DGGE) analysis of 16S rRNA fragments of total bacterial population from rhizosphere samples a grown in a growth chamber containing sandy soil (a) three weeks after fumigation or one week after planting and (b) seven weeks after fumigation or five weeks after planting. Gel image from rhizosphere samples collected on week three and seven from plants grown on sandy soil fumigated with methyl bromide (WK3MBSS and WK7MBSS) and methyl iodide (WK3MISS and WK7MISS). The numbers 1X and 0.5X represent normal fumigant application rate and half the normal application rate. The sign '+' and '-' at the end indicate treatments with and without contamination with *E. coli* O157:H7. The numbers in parenthesis represent gel lanes. (C) Cluster analysis of microbial communities generated by the analysis of DGGE 16S rRNA PCR patterns. Symbols are as shown in Figs. 1 a and b. Cluster analysis for clay soil showed the same separation pattern (data not shown).

to week seven (Table 1). The banding profiles were analyzed using cluster analysis to examine the relative similarities of bacterial communities on the phyllosphere during week three and week seven. The phyllosphere community profiles from week three clustered as their own group but differ by 70 % from the communities from week seven (Fig 2c).

Phyllosphere microbial community by Biolog

When Biolog substrate utilization patterns from epiphytic phyllosphere microbial communities from samples collected one week after planting were examined after 72 h incubation, eleven out of 31 substrates showed redox dye color changes (Fig. 3a). These were L-arginine (A4), pyruvic acid methyl ester (B1), D-galacturonic acid (B3), L-asparagine (B4), Tween 40 (C1), Tween 80 (D1), D-mannitol (D2), 4-hydroxy benzoic acid (D3), L-serine (D4), N-acetyl-D-glucosamine (E2), and putrescine (H4). Substrate utilization increased from eleven during week one to fifteen in week two, and to twenty six in week three and five (Fig. 3 a–d). Lactose (H1) was utilized during week three and five after planting and this substrate did not show any unique pattern compared to other substrates. Lactose has been used previously as indicator of coliform activity.[26] The increase in substrate utilization also resulted in greater diversity of microbes that were utilizing a particular carbon source. After three weeks of plant growth, cultured dependent epiphytic microbial communities as determined by Biolog assay had stabilized. DNA from these Biolog wells (Fig. 3; week1–week 5 after planting) was

Table 1. Numerical analysis of denaturing gradient gel electrophoresis(DGGE) bands from growth chamber samples (Fig.1) with Shannon index of diversity (*H*).

Treatment	*Week 3 (R)*	*Week 7 (R)*	*Week 3 (P)*	*Week 7 (P)*
CB 0.5X+E*	1.18 a	1.60 a	0.71 a	1.43 a
CB 0.5X−E	1.12 a	1.62 a	0.87 a	1.22 a
CB 1.0X+E	0.94 a	0.76 a	1.03 a	0.72 a
CB 1.0X−E	0.80 a	1.57 a	0.99 a	1.32 a
CI 0.5X +E	1.24 a	0.83 a	0.97 a	0.76 a
CI 0.5X−E	1.24 a	0.77 a	0.85 a	0.79 a
CI 1.0X+E	0.59 a	1.57 a	0.85 a	1.49 a
CI 1.0X−E	1.03 a	1.55 a	0.73 a	1.28 a
CO 0+E	1.07 a	1.66 a	0.35 a	1.55 a
SB 0.5X+E	1.30 a	1.58 a	0.87 a	1.44 a
SB 0.5X−E	1.30 a	1.56 a	0.80 a	1.47 a
SB 1.0X+E	1.07 a	1.55 a	0.69 a	1.40 a
SB 1.0X−E	1.13 a	1.55 a	1.40 a	1.49 a
SI 0.5X +E	1.05 a	1.58 a	0.86 a	1.62 a
SI 0.5X−E	1.25 a	1.48 a	0.66 a	1.43 a
SI 1.0X+E	1.22 a	1.60 a	0.74 a	1.48 a
SI 1.0X−E	1.25 a	1.45 a	0.84 a	1.45 a
SO 0+E	1.30 a	1.51 a	1.12 a	1.40 a
SO 0−E	1.13 a	1.60 a	0.59 a	1.51 a

Means with different letters within each column are significantly different at $P \leq 0.05$ using Tukey's Studentized Range Test.
R= rhizosphere; P= phyllosphere.
C = clay soil; B = methyl bromide, I = methyl Iodide; S = sandy soil; 0.5X, 1X and 5.0X represents half agricultural application rate, recommended agricultural application rate, and five times the recommended application rate, respectively.
+ E = E .coli O157:H7 applied to the samples and – E not applied to the sample.

extracted and analyzed on DGGE gels, and distinct 16S rRNA banding profiles were observed from Biolog wells with the same carbon source but from different sampling days.

To examine the relationships among the different sampling dates, cluster analysis was performed with the utilized substrates. The cluster clearly separated carbon sources utilized during week one from week two and five except N-acetyl-D-glucosamine (E2), putrescine (H4), glycyl-L-glutamic acid (F4), and D-malic acid (H3) (Data not shown). Further analyses were done to compare phyllosphere bacterial communities as determined by culture-independent analysis with the Biolog method that examined only cultured dependent phyllosphere microbial communities. Cluster analysis showed distinct pattern separations between epiphytic microorganisms directly extracted from the leaf surface as compared with those that grew in Biolog wells during week seven after fumigation or week five after planting (Fig. 3C). This confirmed what has been previously reported in citrus plants that epiphytic microbial communities are vastly different between cultured and uncultured populations and that phyllosphere

Table 2. Sequence analysis of bands excised from DGGE gels derived from bacterial 16S rRNA extracted from rhizosphere.

Bands	*Related bacterial sequences*	*Sequence similarity*	*Accession no.*
R1	*Peudomonas syringae* pv. Tomato str. DC3000	96	AE016858
R2	*Bradyrhizobium japonicum* USDA 110	100	AP005960
R3	Uncultured bacterium clone AIZ/4	99	AY465166
R4	*Sporosarcina sp*. 2216	100	AB094469
R5	*Photorhabdus luminescens*	100	AY444555
R6	*Rickettsia* endosymbiont	96	AB113215

populations are more complex that previously realized.[27] Therefore, microbial community composition is vastly different between culture dependent and culture independent population in lettuce leaf surfaces.

Identification of dominant bacterial communities

The analysis of predominant bacterial species was carried out with rhizosphere, phyllosphere, and Biolog samples from different sampling dates. Bands selected for analysis are shown in Figures 1, 2, and 3 for rhizosphere, phyllosphere, and Biolog, respectively. A summary of the prominent bands recovered from the DGGE gel analysis are presented in Table 2 for rhizosphere samples and Table 3 for phyllosphere and biolog samples. A majority of the bands in the rhizosphere were related to known bacterial sequences with a 96 to 100 % sequence similarity. The derived sequences from these bands confirmed R1 to be 96 % similar to *Pseudomonas syringae* pv. tomato DC3000 and R2 had 100 % similarity to *Bradyrhizobium japonicum* USDA 110.

For detailed understanding between the culture-independent and Biolog analysis of epiphytic bacteria, dominant 16S rRNA DGGE bands directly obtained from lettuce leaves (culture independent) or from Biolog wells (culture-dependend) were isolated, cloned, and sequenced (Table 3). A total of 15 bands from the leaves and eight from the Biolog plates were analyzed. Only *Photorhabdus luminescens* with accession number AY444555 was present in both culture independent and culture-dependend samples. All the Biolog isolates were from the *Proteobacteria* phylum compared to the bands from the leaves that were from different bacterial phyla. The dominance of the *Proteobacteria* phylum from the Biolog isolates indicates the culturability of some of the members of this group. The microorganisms that grew in Biolog wells produced dominant 16S rRNA DGGE banding patterns from eleven major carbon sources (L-arginine, pyruvic acid methyl ester, D-galacturonic acid, L-asparagine, Tween 40, Tween 80, D-mannitol, 4-hydroxy benzoic acid, L-serine, N-acetyl-D-glucosamine, and putrescine) that were recovered for sequence analysis during

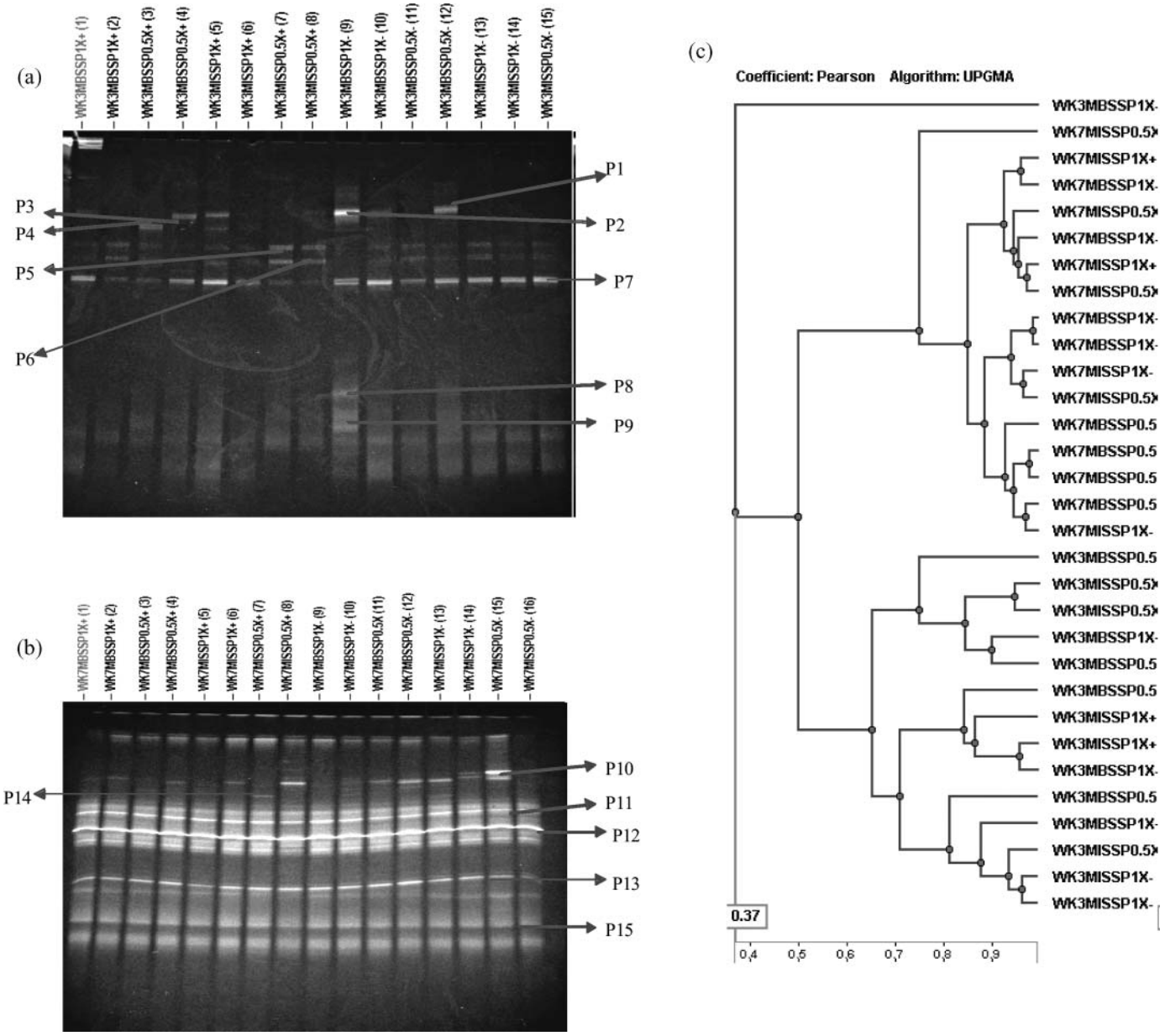

Fig. 2. Denaturing gradient gel electrophoresis(DGGE) analysis of 16S rRNA fragments of total bacterial population from phyllosphere samples grown in growth chamber containing sandy soil (a) three weeks after fumigation or one week after planting and (B) seven weeks after fumigation or five weeks after planting. Gel image from phyllosphere samples collected on week three and seven from phyllosphere samples grown on soil fumigated with methyl bromide (WK3MBSS and WK7MBSS) and methyl iodide (WK3MISS and WK7MISS). The numbers 1X and 0.5X represent normal fumigant application rate and half the normal application rate. The sign '+' and '-' at the end indicate treatments with and without contamination with *E. coli* O157:H7. The numbers in parenthesis represent gel lanes. (c) Cluster analysis of microbial communities generated by the analysis of DGGE 16S rRNA PCR patterns. Symbols are as shown in Figs. 3 a & b. Cluster analysis for clay soil showed the same separation pattern (data not shown).

the first week and increased to more than 25 during week five after planting.

Discussion

There were no significant differences observed in rhizosphere microbial structure as a result of the application of the two fumigants. The reason may be that 14 d had passed before samples were taken from the rhizosphere and the fumigants may not have any impact on rhizosphere bacteria. At this point the influence of plant exudates may be stronger that the residual effects of fumigants. Soil fumigation does not result in soil sterilization but in some cases may result in changes in the microbial community

Table 3. Bacterial isolates identified via predominant 16s rRNA DGGE bands from phyllosphere samples of lettuce leaves incubated for 72 h in BIOLOG EcoPlates containing different carbon sources, and from community DNA from lettuce epiphytic bacteria.

Bands	*Related bacterial sequences*	*Sequence similarity*	*Carbon source*	*Accession no*
b1	*Pseudomonas sp*. TS1138	100	A4, B1, B3, D1, D2, D3, D4, E2, H4	AY536741
b2	*Pseudomonas fluorescens*	98	B4, C4, E2	AY472116
b3	*Pseudomonas sp.*	94	B4, C4, E2	AY191342
b4	*Stenotrophomonas maltophilia*	100	A4, B1, B4	AY748889
b5	*Pseudomonas sp.* B65	100	A2, A3, B1	AF332541
b6	*Photorhabdus luminescens*	100	B2, B3, C1, C2	AY444555
b7	*Photorhabdus luminescens*	98	B1, E2, F2,	AY444555
b8	*Pseudomonas syringae*	96	C2, C4, D3	AE016858
P1	Uncultured bacterium	95	NA	AY256614
P2	Uncultured bacterium	100	NA	AY853674
P3	Uncultured bacterium	100	NA	AY345564
P4	*Photorhabdus luminescens*	100	NA	AY444555
P5	*Gamma proteobacterium* MS-1	100	NA	AF005656
P6	*Bacillus sp. Eint 1b*	100	NA	AM062716
P7	Uncultured bacterium	99	NA	AJ232875
P8	*Paenibacillus lactis*	99	NA	AY257868
P9	*Leptolyngbya angustata* UTCC 473	100	NA	AF218372
P10	*Pseudomonas fluorescens* PfO-1	98	NA	CP000094
P11	*Erwinia pyrifoliae*	96	NA	DQ180962
P12	*Pseudomonas brenneri*	98	NA	AM086254
P13	*Pseudomonas fluorescens* PfO-1	97	NA	CP000094
P14	Uncultured bacterium	99	NA	AY842561
P15	*Sporosarcina sp*. 3061	100	NA	AM111010

structure. However, the changes may not affect rhizosphere microbial composition as it was observed in this study because plants are grown on soil at least two weeks after fumigation. The major bands from the rhizosphere were dominated by *Proteobacteria* such as *Pseudomonas syringae*. *Pseudomonas sp.* has been shown to survive fumigated soil and recolonize strawberry rhizospheres rapidly and in high numbers after fumigation.[16] The increase in numbers of different *Pseudomonas sp* in the strawberry rhizospheres after fumigation correlated to a significant increase in the growth of the strawberry plants in the field and greenhouse experiments.[16] Other studies with strawberry rhizosphere colonizers in fumigated compared to native soils suggest that there may be differences in deleterious and beneficial rhizosphere colonizers following soil fumigation.[9] A basic understanding of the soil and rhizosphere microbiology can simplify the identification of specific microorganisms that can be used directly for disease management, enhancement of plant growth or altered crop management practices to enhance their populations and improve soil quality.[10]

There were no significant effects of fumigants on bacterial composition on the leaf surface. Our main interest was to determine whether *E. coli* O157:H7 that was inoculated into the soil before fumigation had survived and become the major component of leaf surface microbial community. To confirm our hypothesis, fifteen bands were excised from phyllosphere DGGE gels and the bands were cloned, sequenced, and identified. A biolog assay was also conducted to test lactose utilization by phyllosphere microbial communities. The two assays confirmed that *E. coli* O157:H7 was not part of the dominant microbial composition of the phyllosphere, since none of the bands from phyllosphere and Biolog samples were identified as *E. coli* O157:H7. Lactose utilization was only observed during the last few weeks of the study with the Biolog plates. Therefore, *E. coli* O157:H7 was not a major member of the phyllosphere microbial community, either because the fumigants had killed a large number of pathogens, or the DGGE technique was unable to detect them because the pathogen had become a minor component of the community. The two explanations are quite possible since DGGE only detects the major bacterial components of the community.[17] However, Ibekwe et al.[21] indicated that this pathogen was detected on leaf surfaces by real-time PCR and immunomagnetic separation throughout this study, but only detected by plate count in the fumigated samples during the first week.

Identification of the major bands (Table 3) showed that all bands from the biolog plate were identified as *Gamma Proteobacteria*, while bands from the phyllosphere obtained by cultured independent approach were mostly uncultured bacterium (P1, P2, P3, P7, and P14), *Firmicutes* (P6, P8, and P15), *Cyanobacteria* (P9), and *Gamma Proteobacteria* (P4, P5, P10, P11, P12, and P13). From all

bands excised from DGGE gel, three clones were sequenced from each band. This was done to check for the inherent problems associated with DGGE limitations and to some extend PCR bias with complex microbial communities. In this study, three bands (P1, P2, and P3) migrated to almost the same electrophoretic position and these bands were identified as uncultured bacterium with different accession numbers (Table 3). Two other bands (B6 and B7)

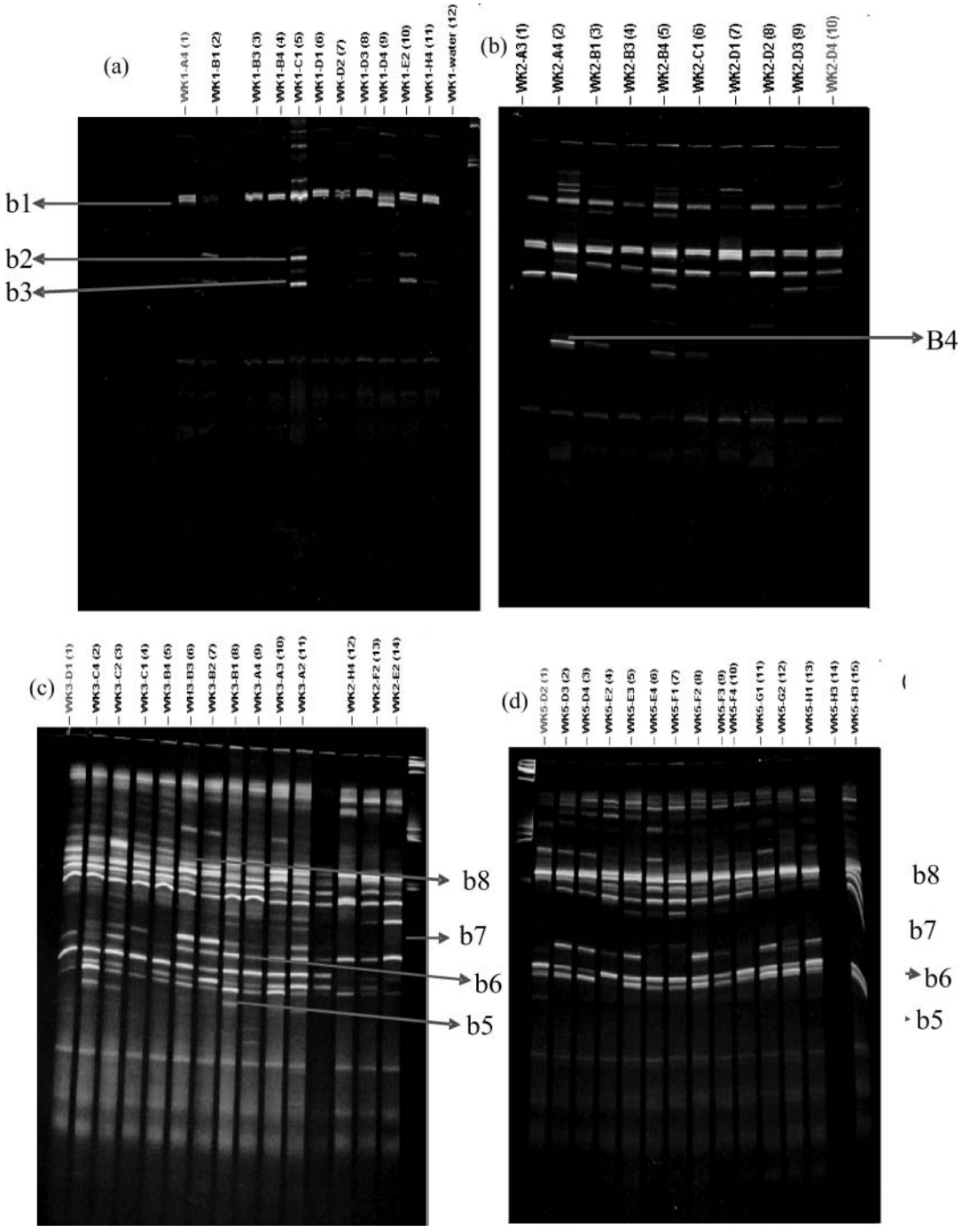

Fig. 3. Bands from polymerase chain reaction (PCR) denaturing gradient gel electrophoresis(DGGE) 16S rRNA banding profiles of epiphytic bacteria on lettuce extracted after 72 h growth in different wells of Biolog plates at 25°C with different carbon sources. Gels a, b, c, and d are Biolog plates from wk 1, 2, 3, and 5 after fumigation. Letters followed by numbers after wk 1, 2, 3, and 5 are substrates utilized. There were a total of 26 substrates utilized. DNA was extracted from some wells for the identification of dominant bacteria utilizing the substrate. (e) Cluster analysis of 16S rRNA banding profiles for epiphytic bacteria from the phyllosphere of lettuce grown on Biolog plate after 72 h incubation during weeks 5 after planting. Note that the comparisons between culture independent epiphytic microbial compositions are noted as WK7 and culture dependent (Biolog) microbial composition during week 5 of plant growth. (*Continued*)

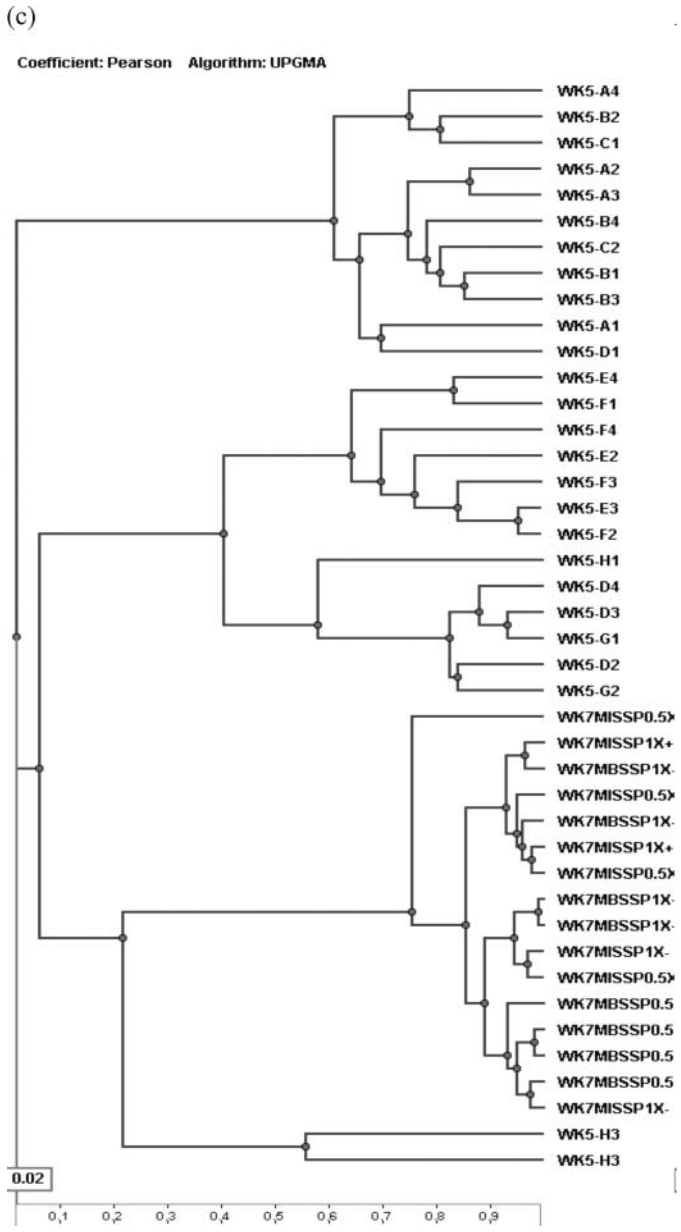

Fig. 3. (Continued)

from the Biolog plate that were a few percent apart from each other were identified as the same bacterial species with the same accession number, but different percent sequence similarity. Therefore, detail analysis of DGGE bands continue to show some of the limitations of the technique despite its huge advantages in explaining changes in complex microbial community structure subjected to different treatments.

Our data clearly showed greater complexity of microbial communities obtained from the phyllosphere based on a culture independent approach compared to the distinct and simpler community structures obtained from the Biolog assay. It should be noted that the carbon metabolizing profiles in Biolog plates are often used as a reflection of the catabolic potential of a community,[28,−30] and these culture conditions clearly do not reflect the epiphytic microbial community *in sit*u. The reasons for the differences include different nutritional requirements, generation times, and antagonistic/synergistic interactions among phyllobacteria community. Our DGGE results showed that different subsets of phyllosphere communities became dominant in the Biolog wells through enrichment, such that fewer bacteria were present in each dominant DGGE band sequenced. This study is in agreement with Yang et al.[27] that showed 10 dominant DGGE bands from eight major carbon sources on which bacteria grew. The dominant bacteria found in Biolog plates in our study were common phyllosphere bacteria.[19,31] The Biolog assay skewed the observed microorganisms to the *Proteobacteria* phylum relative to the culture-independent method because it is likely that *Proteobacteria* grew faster than others in the biolog culture.

In conclusion, MeBr and MeI may not have any significant negative effects on rhizosphere and phyllosphere microbial communities. This may be due to the reduction in population of plant pathogens such as nematodes, soilborne diseases, and weeds thus enhancing bacteria to rebound after the initial decline in population and use the available carbon sources for growth.

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