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Bacterial diversity in cucumber (*Cucumis sativus*) rhizosphere in response to salinity, soil pH, and boron

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ABSTRACT

Soil salinity is a major factor relating microbial communities to environmental stress in the microbial selection process as stress can reduce bacterial diversity. In the San Joaquin Valley (SJV) of California, the problem of increasing salinity and consequently, decreasing crop productivity, due to reuse of saline drainage water are major concerns. An experiment was conducted in a closed, recirculating volumetric lysimeter system (VLS) consisting of 24 experimental plant growth units to determine the interactive effects of salinity, boron and pH on rhizosphere and non-rhizosphere microbial composition of cucumber (*Cucumis sativus* L. cv. Seminis Turbo hybrid). Plants in the VLS were irrigated from individual reservoirs containing a modified half-strength Hoagland's nutrient solution combined with salinity, boron (B), and pH treatments. The results indicated that salinity and pH were the most influential factors affecting the growth of plants and the effect of boron on the plant was more severe under slightly acidic conditions. Total bacterial DNA was extracted from rhizosphere and non-rhizosphere samples, and a 236-bp DNA fragment in the V3 region of the small subunit ribosomal RNA genes of eubacteria was amplified. The 16S rRNA and the products were subjected to denaturing gradient gel electrophoresis (DGGE) and sequencing. Analyses of bacterial diversity showed that the effects of salinity, boron, and pH were more severe on the rhizosphere bacterial population during the first week of growing cucumber, with decreasing impacts with plant growth. However, there was no salinity–B–pH interaction effects on plant biomass, but the effects were seen in the number of heterotrophic bacteria in the rhizosphere and on species richness and diversity during week seven of the study. These suggest that the effects of salinity–B–pH interactions may influence microorganisms first before plants and may pose long term effects on soil quality.

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1. Introduction

Reuse of saline drainage water is a necessary management practice for reducing the volume of drainage produced in the Westside of California in USA. Increasing soil salinity due to the reuse of saline drainage water is a major concern for sustainable agriculture in the San Joaquin Valley (SJV) in California. Another concern with this practice is the extent to which B, a naturally occurring element in drainage water, will affect crop growth and yields. Numerous studies have demonstrated the effects of either salinity or B on growth and yield responses on crops (Eaton, 1944; Ehret and Ho, 1986; Maas and Grattan, 1999). Other studies have addressed the effects of both with mixed conclusions (Holloway and Alston, 1992; El-Motaium et al., 1994; Grattan et al., 1996; Grieve and Poss, 2000; Alpaslan and Gunes, 2001; Ferguson et al., 2002; Wimmer et al., 2003). Soil solution pH is known to affect B availability in soils, B-ion reactions and ion interactions with other trace elements such as As, Se, etc in the drainage water. The pH conditions recorded in salinity and B experiments (Sternberg et al., 2001; Ben-Gal and Shani, 2002,) were either slightly acidic (pH 6.5) or alkaline (pH 7.5–8.5) but pH was not used as an experimental variable. It has also been reported that trace elements can move up the aquatic food chain and become more concentrated, resulting in high levels of exposure for those animals near the top of the food chain (Fan et al., 1988). In some habitats, reproductive failures and deformities were observed in waterfowl (Tanji et al., 1986; Letey et al., 2002). Currently, there is no information available about the microbial communities as affected by the interaction among salinity, pH, and B content in the SJV soils. This information is needed as microorganisms maintain the whole ecosystem health and functioning.

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Salinity is a major factor in controlling microbial abundance, diversity, composition and functions (Borneman et al., 1996). Salinity has been shown to have significant effects on microorganisms (Polonenko et al., 1981; Omar et al., 1994; Matsuguchi and Sakai, 1995). Environmental stress in soil gain importance, especially in saline agricultural soils, where high salinity results from irrigation practices and application of chemical fertilizer. This effect is always more pronounced in the rhizosphere pursuant to increased water uptake by the plants due to transpiration. The simple explanation for this is that life in high salt concentrations is bioenergetically taxing because microorganisms must maintain an osmotic balance between their cytoplasm and the surrounding medium while excluding sodium ions from the cell interior, and as a result, sufficient energy is required for osmoadaptation (Oren et al., 2002; Jiang et al., 2007). Other factors such as soil composition, organic matter, pH, heavy metals, water and oxygen availability, along with the host plant, also play a major role in the selection of the natural flora in the soil during salinity (Ross et al., 2000).

It has been shown that the accumulation of B may be more of a limiting factor to plant growth than the total salt concentration (Ayars et al., 1993). Boron is an essential micronutrient for plants, but it is toxic to many plants at higher concentrations. The optimum concentration range of plant-available B is very narrow for most crops (Grattan and Grieve, 1999). The B tolerance of crops is species dependent and can vary widely among cultivars within a given species from <6 to 10 g m^{-3} (Benlloch et al., 1991). In arid and semiarid irrigated areas, high B concentrations in soils are often associated with high salt concentrations (Grieve and Poss, 2000). This observation was further shown by a linear relationship ($r^2 = 0.81$) between soil B content and soil salinity in a three year study in the San Joaquin Valley (Shouse et al., 2006). The authors concluded that the correlation between salinity and B in the field probably exists because they share a common origin, namely the alluvium derived from sedimentary marine deposits of the Coast Range Mountains on the western side of the San Joaquin Valley (Letey et al., 2002). The effect of B on microorganisms is largely unknown; however, studies with pure cultures have shown than B can inhibit growth of bacteria (Bringmann and Kuhn, 1980; Butterwick et al., 1989) at high concentrations.

Recently, Nelson and Mele (2007) studied the subtle changes in rhizosphere microbial community structure in response to increased B and sodium chloride concentrations. They concluded that B and sodium chloride are more likely to affect rhizosphere microbial community structure indirectly through root exudates quantity and/or quality than directly through microbial toxicity, and that plant health is a major determinant in rhizosphere microbial community structure and normal N cycle. However, this study did not include pH as a variable. San Joaquin Valley soils that have the combination of high salinity and B are alkaline in nature (pH range of 7.5 to nearly 9.0). Very little is known about how pH influence salinity–B interactions in this soil, and how they affect plant growth. To the best of our knowledge, nothing has been reported about how these variables affect soil microbial composition and functions in the rhizosphere of vegetable crops in this region. Incorporation of pH into the factorial design of the experiment may provide insights into salinity–B interactions and the modified tolerances of B in the presence of salinity observed in many other studies. The objectives of this study were study the effects of soil salinity, B, and pH on rhizosphere and non-rhizosphere microbial composition by using both culture dependent and independent approaches. To achieve these goals polymerase chain reaction combined with denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing of 16S rRNA genes were used to explore how the incorporation of pH into the experimental variables may provide insights into how salinity–B interactions affect rhizosphere microbial population.

2. Materials and methods

2.1. Plant growth

The experiment was conducted in a closed recirculating volumetric lysimeter system (VLS) (Poss et al., 2004) consisting of 24 experimental plant growth units (81.5 cm wide $\times$ 202.5 cm long $\times$ 85 cm deep) at the U.S. Salinity Laboratory in Riverside, CA to determine the interactive effects of salinity, B and pH on the rhizosphere and non-rhizosphere microbial composition of cucumber (*Cucumis sativus* L. cv. Seminis Turbo hybrid). The lysimeters were constructed in 1995. Between 1995 and 2004, many types of plants had been grown. Crops grown in the model soil whose physical properties were found to be comparable to field soils by Wang (2002) have included many different type of crops such as Poplar trees, Paspalum, Bermudagrass, Saltgrass, Pistachio rootstocks, leafy vegetables, and two years of alfalfa and tall wheatgrass. The year prior to the current cucumber study, a floral crop, was grown. Each crop was irrigated with solutions having a minimum electrical conductivity of 2 dS m^{-1}. The lysimeters were filled with sand (particle size distribution ranging from 0.09 mm to 4 mm) that resulted in a medium with volumetric water content of 0.1–0.3 $cm^3\ cm^{-3}$ and similar thermal conductivities and heat capacities as field soil (Wang, 2002). This medium had a high saturated soil hydraulic conductivity (400 cm day^{-1}) and provided limited exchange of soil water inorganic constituents with the solid phase, thus simplifying control of soil water chemistry. Twenty-four sand tanks, arranged in a randomized complete block design, were irrigated from individual reservoirs containing a modified half-strength Hoagland's nutrient solution combined with various salinity, B, and pH treatments. Each tank was plumbed with 5.1 cm PVC pipes, one for irrigation to the sand tank, and one for return flow to a 1740-L reservoir in the basement below. The pH adjustments on the reservoirs were performed most week days and salinity and B concentrations were periodically monitored. Crop water use was determined volumetrically by reservoir water depletion. The rhizosphere samples were collected after shaking loosely held soil on the roots into the stomacher bags and weigh and 10 g of non-rhizosphere samples were collected at least 10 cm away from plants. All samples were collected weekly for five weeks (weeks 1, 3, 4, 5, and 7). Samples were also collected for heterotrophic plate counts and analyzed by serial dilution as well as samples for total community DNA extraction.

2.2. Treatments

Treatments included a concentration of two salinity levels, 3 and 8 dS m^{-1}; three B concentrations of 0.7, 5, and 8 mg L^{-1}; and two pH levels where solutions were frequently adjusted to 6.5 and 8. Treatments were replicated twice. The concentration of B was selected based on the B concentrations in drainage waters in SJV (Letey et al., 2002). Plants were routinely observed for foliar injury and fruit development. Data collected included fresh and dry weights of plants, leaves, stems, and roots as well as cucumber fruit weight and quantity. Collected tissues were analyzed for various ions to determine their distributions within the plant and ion interactions.

2.3. DNA extraction, PCR-DGGE, and phylogenetic analysis

Community DNA was extracted from rhizosphere and non-rhizosphere samples with the Ultra Clean Soil DNA Kit (MoBio Laboratories, Solana Beach, CA) and stored at $-20\ ^\circ C$ 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 (Øverås et al., 1997). Ready-To-Go 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. Gels were run for 3.5 h at 200 V with the Dcode™ Universal Mutation System (Bio-Rad Laboratories, Hercules, CA). DNA was visualized after ethidium bromide staining by UV transillumination and photographed with a Polaroid camera. Major bands were excised for identification of bacterial species. Bands were placed into sterilized vials with 20 ml of sterilized, distilled water and stored overnight at 4 °C to allow the DNA to passively diffuse out of the gel strips. Ten milliliter of eluted DNA was used as the DNA template with the bacteria primers above but without the GC-clamp. DNA was cloned into the TOPO TA cloning kit (Invitrogen, Carlbad, CA). Isolation of plasmids from *Escherichia coli* was performed using the Qiagen plasmid mini kit (Valencia, CA). Four plasmids from each band were sequenced to check for purity of clones. The purified plasmids were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, CA) with forward and reversed primer M13.

Amplification of 16S rRNA genes for phylogenetic analysis from soil-extracted DNA using was done using Ready-To-Go PCR bead (Amersham Pharmacia GE Healthcare Bio-Sciences Corp., Piscataway, NJ), 2 μl of forward primer 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). Amplification of the 16S rRNA was done using the following steps: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension step of 72 °C for 7 min. The 1500 bp PCR products were excised from gel, purified and cloned directly into the TOPO TA cloning kit (Invitrogen, Carlbad, CA) as described above. Isolation of plasmids from *E. coli* was performed using standard protocols from the Qiagen plasmid mini kit (Valencia, CA) as stated above. Colonies were screened by *Eco*RI restriction endonuclease digestions for inserts and digested for 3 h at 37 °C. Digestion products were visualized on a 1.5% agarose gel to ensure the presence of inserts of the expected size. DNA plasmids were commercially sequenced with an Applied Biosystems Prism 377 DNA sequencer using universal M13 forward and reverse primers. DNA sequences were edited manually to correct falsely identified bases and trimmed at both the 5′ and 3′ ends using the Chromas software (Technelysium Pty. Ltd, Helensvale, Australia). Only sequences with unambiguous reads of about 600 bp were used for further analysis. For phylogenetic analysis, clone sequences were BLAST analyzed and the predicted 16S rRNA sequences from this study were compared with 16S rRNA sequences from the BLAST gene bank (ftp://ftp.ncbi/nih.gov/blast/executables/LATEST) and a FASTA-formatted file containing the predicted 16S rRNA sequences. Matrices of evolutionary distances were computed using the Phylip program with the Jukes–Cantor model (Jukes and Cantor, 1969). Phylogenetic trees were constructed and checked by bootstrap analysis (1000 data sets) using the program SEQBOOT. Bootstrap values represented the frequency of resampling that supported a specific branching pattern. The computer program DOTUR (Schloss and Handelsman, 2005, 2006) was used to calculate species richness estimates and diversity indices from sequence data. Another program, LIBSHUFF (Singleton et al., 2001), was used to compare the similarities of bacterial clone libraries. The distance matrices for both programs were obtained using an algorithm located at the Greengenes website (DeSantis et al., 2006a,b). For dendrogram construction, partial 16S rRNA sequences representing the most prevalent OTUs from the dominant phylum were aligned using CLUSTALX version 1·8 for Windows (Thompson et al., 1997).

2.4. Statistical analysis of DGGE bands

DNA fingerprint obtained from the 16S rRNA banding patterns on DGGE gel were photographed and digitized using Image Master Labscan (GE Healthcare, Piscataway, NJ). The lanes were normalized to contain the same amount of total signal after background subtraction, which was carried out using the rolling disc mechanism. 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 (Ibekwe et al., 2001). Band positions were converted to Rf values between 0 and 1, and profile similarity was calculated by Dice's similarity coefficients. Data were integrated and analyzed using Image Master 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 richness, evenness, and diversity indexes were compared using a one-way analysis of variance, and Tukey HSD test for post hoc analysis (SAS, 2005). Richness (S) refers to the number of bands detected in a given soil sample. The DGGE evenness (E), a measure of how evenly DGGE bands were distributed in a given soil sample, was calculated as $E = H/\ln(S)$. Diversity was calculated by using the Shannon index of diversity (H) to compare changes in diversity of microbial communities within all treatments at each time (Shannon and Weaver, 1963) by using the following function:

$$H = -\sum P_i \log P_i$$

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. Simpson's diversity index (D) for infinite population = 1 − sum ($P_i \times P_i$) where P_i = importance probability in element i (element i relativized by row total).

3. Results

3.1. Cucumber growth under salinity–B–pH interaction

Salinity and pH were the most significant ($P < 0.01$) factors affecting the growth of cucumber (Table 1). Both increased salinity and increased pH reduced yield and plant biomass. Increased B also reduced total biomass, water usage, and fresh fruit weights ($P < 0.05$). Most interactions among the variables, however, were not significant. The only exception was a significant B–pH interaction affecting water usage ($P < 0.05$). These data indicate that under slightly acidic conditions, increased B had a much more dramatic reduction in plant biomass and yield than did the same increase under slightly alkaline conditions.

3.2. Diversity analysis of DGGE banding patterns

Rhizosphere and non-rhizosphere soils were treated with two salinity levels, three B concentrations and two pH levels and analyzed over time to determine the changes in microbial community structure (Fig. 1A and B). To compare DGGE patterns, Pearson's indices were determined for comparisons of all profiles, and unweighted pair group method with mathematical averages (UPGMA) was used to create a dendrogram describing pattern similarities in the rhizosphere (Fig. 1C). The analysis clearly distinguished between bacterial community structure in week one and seven.

Table 1
Probability values of parameters measured at harvest.

Source	Total fresh biomass	Fresh vine weight	Fresh fruit weight	Water usage	Number of fruit per plant	HPC-R* Log_{10} CFU g^{-1}	HPC-S Log_{10} CFU g^{-1}
Salinity	<0.001	<0.0001	0.0003	<0.0001	0.0003	0.7971	0.3393
Boron	0.0445	0.0818	0.0610	0.0222	0.0407	0.3632	0.4499
Salinity–Boron	0.4440	0.8569	0.3290	0.0834	0.1825	0.2764	0.3994
pH	<0.0001	<0.0001	0.0004	<0.0001	0.0003	0.0968	0.5813
Salinity–pH	0.5071	0.1924	0.8576	0.976	1.000	0.6269	0.2831
Boron–pH	0.1075	0.1241	0.1613	0.0328	0.3368	0.6146	0.2164
Salinity–Boron–pH	0.5853	0.5217	0.6791	0.6212	0.7235	0.0041	0.1779

*R and S indicate significant level ($P = 0.05$) bacterial population by heterotrophic plate counts in the rhizosphere (R) and non-rhizosphere soil (S).

Analysis of variance to determine time (weeks) effects on the population structure when time was included in the model with salinity, B, pH and the interaction of these variables showed that time was a significant factor affecting richness, Shannon, and the Simpson indices in both rhizosphere and non-rhizosphere soils (data not shown). Due to these findings, data were analyzed to see the weekly effects in both rhizosphere and non-rhizosphere soils (Tables 2 and 3). There were no significant effects of these variables on bacterial diversity indices during week 0 (Table 2). However, during week one, there was a significant diversity reduction due to pH effect on the non-rhizosphere soil. During weeks three, four, five, and seven, there were significant decreases of bacterial diversity indices (except B week 3) due to salinity and B effects. The pH also significantly decreased these indices during the same period, except for week 5 and 7. There were significant salinity–pH and B–pH interaction effects on all bacterial diversity indices during week 5 and 7 as well as salinity–B–pH interaction effects on all bacterial diversity indices during week 7. The salinity–B–pH interaction effects on bacterial diversity in bulk soil during week 7 correlated with the rhizosphere data during the same period, resulting in significant bacterial diversity reduction (Tables 2 and 3).

In the rhizosphere, several indices of microbial community structure were significantly affected by salinity, B, and pH during week one of our studies (Table 3). Salinity, B, and pH significantly reduced species' richness and the Shannon index of diversity, but not affected the Simpson diversity index during the first week of our study (except pH significantly decreased Simpson's diversity richness). The combined effects of salinity, B and pH also resulted in a significant decrease in species richness but not the Shannon and the Simpson indices. During week 4, only pH had significant effects on the diversity indices (Shannon–Weaver and Simpson), and also salinity–B and B–pH interactions had significant effects on bacteria diversity indices. During week 7 salinity was the only variable with a significant effect on bacterial diversity indices. However, there were significant B–pH and salinity–B–pH interaction effects on bacterial diversity indices. Our results indicate that the effects of salinity, B, and pH caused severe decrease in the rhizosphere bacterial population during the first week of growing cucumber, with decreasing impacts with plant growth.

3.3. DGGE clone sequence analysis

To gain insight into the identities of major bacterial populations, prominent DGGE bands derived from rhizosphere soil were excised and used for nucleotide sequence analysis (Supplementary material Table 1). Four clones from each band were sequenced for species identification and test for RNA heterogeneity. DGGE bands in a single gel that appeared to be identical based on mobility produce identical nucleotide sequences (data not shown). The majority of the DGGE bands showed the highest levels of identity to clones recovered from soil or sequences obtained from strains isolated from soils or rhizosphere environments. Bands excised from the rhizosphere samples were correctly identified with 93–100% identity to closely related database sequences (Supplementary material Table 1). Most of the sequences were uncultured bacteria, *Proteobacteria*, and *Bacteroides*.

3.4. Phylogenetic analysis

Cloning and sequencing revealed clear differences in the community composition and diversity of control and salinity induced soil in pre-plant, non-rhizosphere, and rhizosphere soils. The rarefaction curves showed that all clone libraries were far from saturation with coverage between 51% and 64% (Fig. 2 and Table 4). A total of about 264 sequences were obtained and were distributed among 9 different major bacterial phyla and 186 operational taxonomic units (OTUs). The clone libraries of the three soils showed clear differences in the phylogeny and percentage distribution (Fig. 3 and Supplementary material Fig. 1). The majority of the clones had $\geq$ 91% percent similarity with 16S rRNA sequences of cultivated bacteria available in the GenBank nucleotide data library. Uncultured bacteria have the highest percent composition in the three soils followed by *Cyanobacteria* in pre-plant soil (Fig. 3A) and non-rhizosphere soil (Fig. 3B), and *Gammaproteobacteria* (Fig. 3C).

In the *Cyanobacteria* group, their sequences became more associated with higher salinity than the control soil in the pre-plant treatment, and 39% of the cyanobacterial clones fell next to sequences of *Phormidium lumbricale*, *Oscillatoria* spp. and *Stanieria cyanospaera* whereas 61% were related to uncultured *Cyanobacteria*. In the non-rhizosphere samples, most sequences were affiliated to unicellular thermophilic cyanobacteria, *Oscillatoria spearlei*, *S. cyanospaera*, *Phormidium terebriformis*, and uncultures *Cyanobacteria* in the high pH (SJV) samples. In the control samples, *Cyanobacteria* was dominated by *Pleurocapsa* sp., *Oscillatoria* sp., and uncultured *Cyanobacteria*.

Pre-plant, non-rhizosphere, and rhizosphere soils exhibited different distribution of *Proteobacteria*. In the pre-plant soil, *Alpha-* and *Gammaproteobacteria* were detected only in control soil (low salinity), and *Deltaproteobacteria* were detected in salinity induced soil, and these were mainly *Myxococcus macrosporus* and *Corallococcus coralloides* (Supplementary material Fig. 1A). In the non-rhizosphere soil, *Alpha*, *Beta-* and *Gammaproteobacteria* were detected (Supplementary material Fig. 1B). As seen in Fig. 3B *Alphaproteobacteria* was slightly higher than other members of the *Proteobacteria* phyla in non-rhizosphere soil and *Deltaproteobacteria* was not detected. In the rhizosphere, *Alpha*, *Beta*, *Delta-* and *Gammaproteobacteria* were detected with *Gammaproteobacteria* having the highest composition in this group (Fig. 3 and Supplementary material 1C). The *Bacteroides* group constituted 6% of the total clones in pre-plant soil, 9% in non-rhizosphere soil, and 7% in

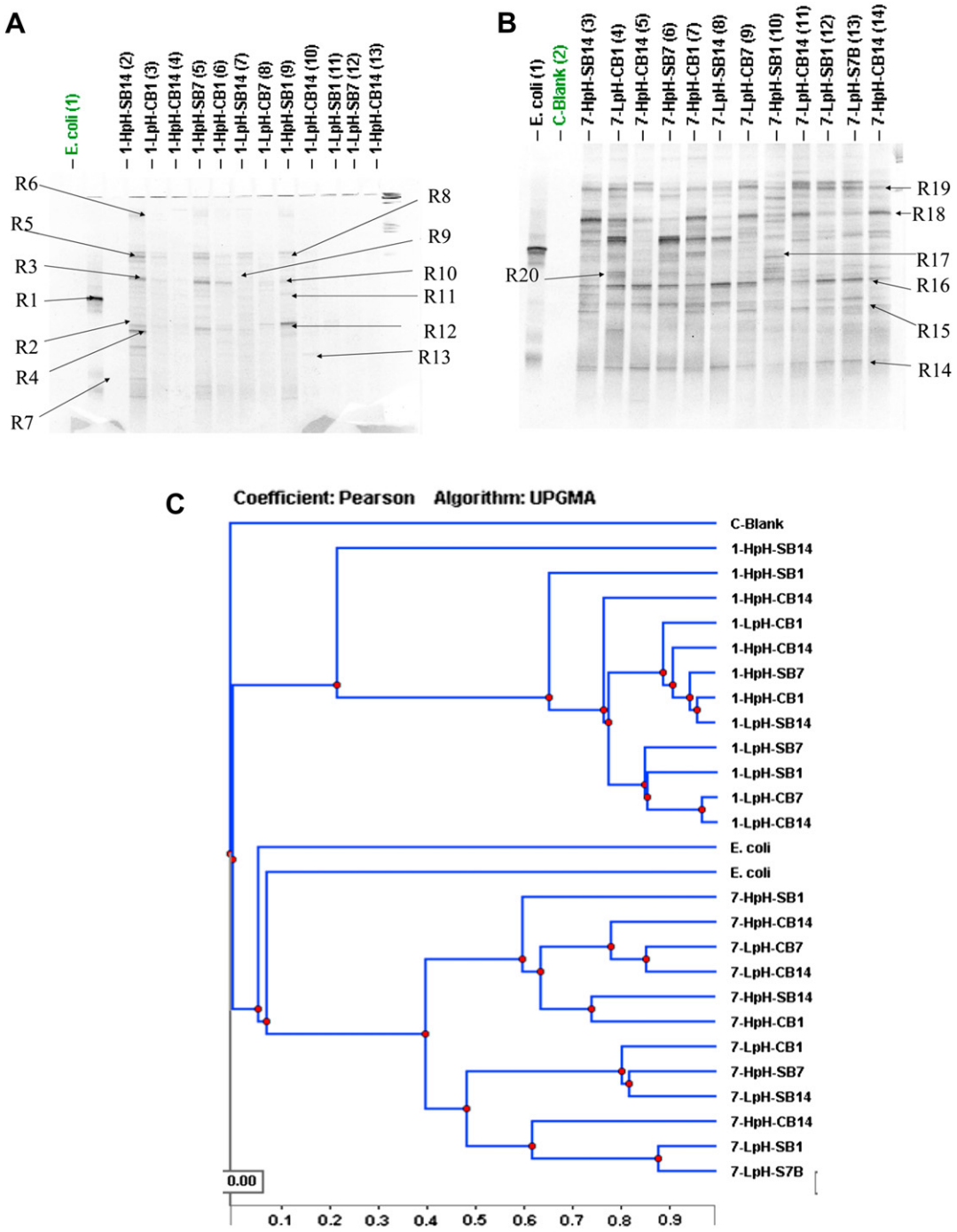

Fig. 1. DGGE analysis of 16S rRNA fragments of rhizosphere samples, at different times after planting of cucumber, collected from duplicate lysimeters treated with different concentrations of boron, salinity, and pH. Amplified products were separated on a gradient gel of 30–70% denaturant. All labeled bands were excised from the gel, reamplified, and subjected to sequence analysis. These reamplification products were cloned and screened as described in the text. (A) Community structures at week 1, 7 days after planting. (B) Community structures after 7 weeks of planting. (C) UPGMA tree with Pearson coefficient, representing the genetic similarity of the microbial community profiles obtained by PCR-DGGE. Numbers 1 through 14 in parentheses refer to the lane numbers in the DGGE gels. Numbers 1 and 7 at the beginning indicate week 1 and 7. HpH and LpH indicate high pH (8.0) and low pH (6.5). S and C indicate high salinity (8 dS m^{-1}) and low salinity or control (3 dS m^{-1}). B 1, 7 and 14 indicate boron at 0.7, 5, and 8 mg L^{-1}, respectively.

the rhizosphere soil (Fig. 3). Uncultured *Bacteroides* were detected in the pre-plant soil in comparison to the detection of additional species like *Algoriphagus terrigena*, *Flexibacteraceae bacterium*, *Sphingobacteria bacterium* in non-rhizosphere, and *Flavobacteria*, and *Dyadobacter* sp. PF-B in rhizosphere soil. The remaining clones were related to *Firmicutes*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, and *Gemmatimonas* groups.

4. Discussion

4.1. Plant growth and microbial diversity with salinity–B–pH interaction

One critical question that was asked at the beginning of the study was whether there may be a salinity–B–pH interaction effect

Table 2
Bacterial diversity colonizing cucumber soil by DGGE.

Source	Bacterial diversity indices	Week 0	Week 1	Week 3	Week 4	Week 5	Week 7
Salinity	*S*	0.3839	0.0164	0.0771	0.0539	0.0004	0.0357
	H	0.4803	0.0589	0.0512	0.0239	0.0055	0.0191
	D	0.6578	0.1811	0.0259	0.0107	0.0518	0.0141
B	*S*	0.1508	0.9410	0.0002	0.0030	0.1415	0.0062
	H	0.1359	0.8542	0.0001	0.0019	0.2507	0.0029
	D	0.1287	0.6851	<0.0001	0.0011	0.3386	0.0023
Salinity–B	*S*	0.6719	0.0074	0.0038	0.0295	0.9641	0.1856
	H	0.5824	0.1602	0.0299	0.0688	0.5084	0.1027
	D	0.5252	0.7571	0.0379	0.0510	0.3036	0.0814
pH	*S*	0.3839	0.0004	0.0771	0.0018	0.0633	0.2599
	H	0.4777	0.0060	0.0742	0.0024	0.4910	0.1556
	D	0.5635	0.0448	0.0359	0.0028	0.8284	0.1040
Salinity–pH	*S*	0.4487	1.0000	0.1175	0.1648	0.0008	<0.0001
	H	0.4062	0.6859	0.0279	0.0661	0.0055	<0.0001
	D	0.3662	0.5559	0.0058	0.0217	0.0364	<0.0001
B–pH	*S*	0.4551	0.0422	0.5101	0.8571	0.0036	0.0149
	H	0.4842	0.1369	0.2148	0.7652	0.0120	0.0226
	D	0.5740	0.3664	0.0357	0.2122	0.0518	0.0739
Salinity–B–pH	*S*	0.1281	0.8488	0.0098	0.0556	0.2075	0.0072
	H	0.0927	0.7828	0.0080	0.1065	0.6325	0.0081
	D	0.0833	0.5559	0.0014	0.0553	0.6150	0.0132

S = Richness = number of non-zero elements in row, *H* = Diversity = −sum (P_i*ln(P_i)) = Shannon's diversity index and *D* = Simpson's diversity index for infinite population.

on bacterial diversity since this has never been done. This effect was not observed during the first five weeks of the study, but during week seven these three variables had significant interaction effects on the bacterial diversity, indicating that salinity–B–pH interactions may result in decrease in bacterial diversity thus posing long term effects on soil quality. These results are very similar to previous findings by Nelson and Mele (2007) which showed a significant decrease in diversity and species richness in high B rhizosphere soil and suggested that both B and NaCl affected utilization of several individual substrates as an indication of plant stress. However, they characterized these changes as subtle. In our study, pH was added as an additional variable because of the alkaline nature of Westside SJV soils and its known influence on B uptake by plants. Analysis of variance indicated that increased salinity and pH significantly reduced total biomass, vine fresh weight, fruit yield, fruit number per plant and cumulative water use of cucumber. Significant interactions were also found between B and pH, but not between salinity and pH or between salinity and B. These data indicate that under slightly acidic conditions, increased B caused a much more dramatic reduction in plant biomass and yield than did the same increase under slightly alkaline conditions. The alkaline conditions are characteristic of soils on the Westside of the SJV. Similar results on the effects of B and NaCl in wheat emergence have been reported previously (Gupta et al., 1976; Bingham and Strong, 1987) and seedlings in water culture (Badenhorst and Burgers, 1973). It has previously been suggested

Table 3
Bacterial diversity colonizing cucumber rhizosphere by DGGE.

Source	Bacterial diversity indices	Week 1	Week 4	Week 7
Salinity	*S*	<0.0001	0.2629	0.0054
	H	0.0042	0.3293	0.0049
	D	0.6252	0.4298	0.0042
B	*S*	0.0092	0.5512	0.1738
	H	0.0793	0.5291	0.1381
	D	0.3592	0.5124	0.1052
Salinity–B	*S*	0.0015	0.0043	0.4020
	H	0.0077	0.0031	0.3394
	D	0.1170	0.0024	0.2697
pH	*S*	<0.0001	0.0930	0.3905
	H	<0.0001	0.0536	0.4887
	D	0.0065	0.0306	0.6077
Salinity–pH	*S*	<0.0001	0.9873	0.7278
	H	0.0002	0.9321	0.6475
	D	0.0246	0.8467	0.5655
Boron–pH	*S*	0.0685	0.0398	0.0196
	H	0.0006	0.0474	0.0170
	D	0.0082	0.0612	0.0143
Salinity–B–pH	*S*	0.0092	0.1177	0.0116
	H	0.1386	0.0944	0.0081
	D	0.7756	0.0624	0.0054

S = Richness = number of non-zero elements in row, *H* = Diversity = −sum (P_i × ln (P_i)) = Shannon's diversity index and D = Simpson's diversity index for infinite population.

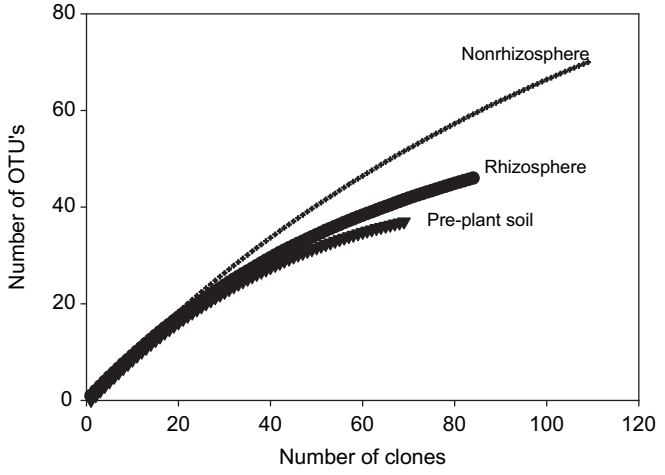

Fig. 2. Rarefaction curves depicting the effect of 3% dissimilarity on the number of OUT's identified from pre-plant soil, rhizosphere, and non-rhizosphere soils.

Table 4
Diversity-richness measures of control and salinity induced soil during pre-plant, non-rhizosphere and rhizosphere soils by cloning.

	Pre-plant	Non-rhizosphere	Rhizosphere
No of clone	67	89	109
No of total OTUs	55	65	66
No of phyla	5	7	6
Coverage (%)	53	51	64
Species richness	25	32	32
Species evenness	1	1	1.2
Shannon–Weaver index	3.5	3.6	3.9

PP-SJA = Pre-plant soil with salinity concentrations commonly found IN drainage water of San Joaquin Valley (SJV) of central California; PP-cont = control with normal salinity of 3.5 dS m^{-1}. NR = non-rhizosphere; R = rhizosphere.

that the addition of NaCl and B may alter the physical characteristics of the rhizosphere due to osmotic pressure. It may also impair the functions of plants in the high NaCl soils resulting in lower utilization of applied water (Nelson and Mele, 2007). However, there was no salinity–B–pH effects on cucumber biomass, vine weight, fruit weight, water usage, and the number of fruits per plant, but the effects were seen in the number of heterotrophic bacteria in the rhizosphere and on species richness and diversity during week seven of the study. These suggest that salinity–B–pH stress may affect microorganisms first before plants. Further explanation of this conclusion may indicate a general trend towards poor soil quality as bacterial species and diversity are affected first due to salinity–B–pH interaction.

4.2. Diversity of bacterial community with salinity as main effect

The degree of salinity effect on soil microbial composition on the studied soils was clearly dependent on the salinity range they experienced. Each of the studied soils apparently developed its own microbial communities in correlation with distinctly different salinity regimes. However, the abundance and diversity of *Cyanobacteria* were higher in pre-plant soil with higher salinity (9 OTUs) but dropped significantly to six OTUs in the control soil (low salinity), probably in favor of species that are better adapted to lower salinities and salinity fluctuations. In our study, the high salinity soil was dominated by *Phormidium*-related *Cyanobacteria* at salinity of 8.0 dS m^{-1} (Casamatta et al., 2005) while *S. cyanospaera* dominated salinity at 3.5 dS m^{-1} (Ishida et al., 2001). The trend in our study was that the longer the soil was exposed to high salinity gradient the greater the reduction in cyanobacterial abundance and

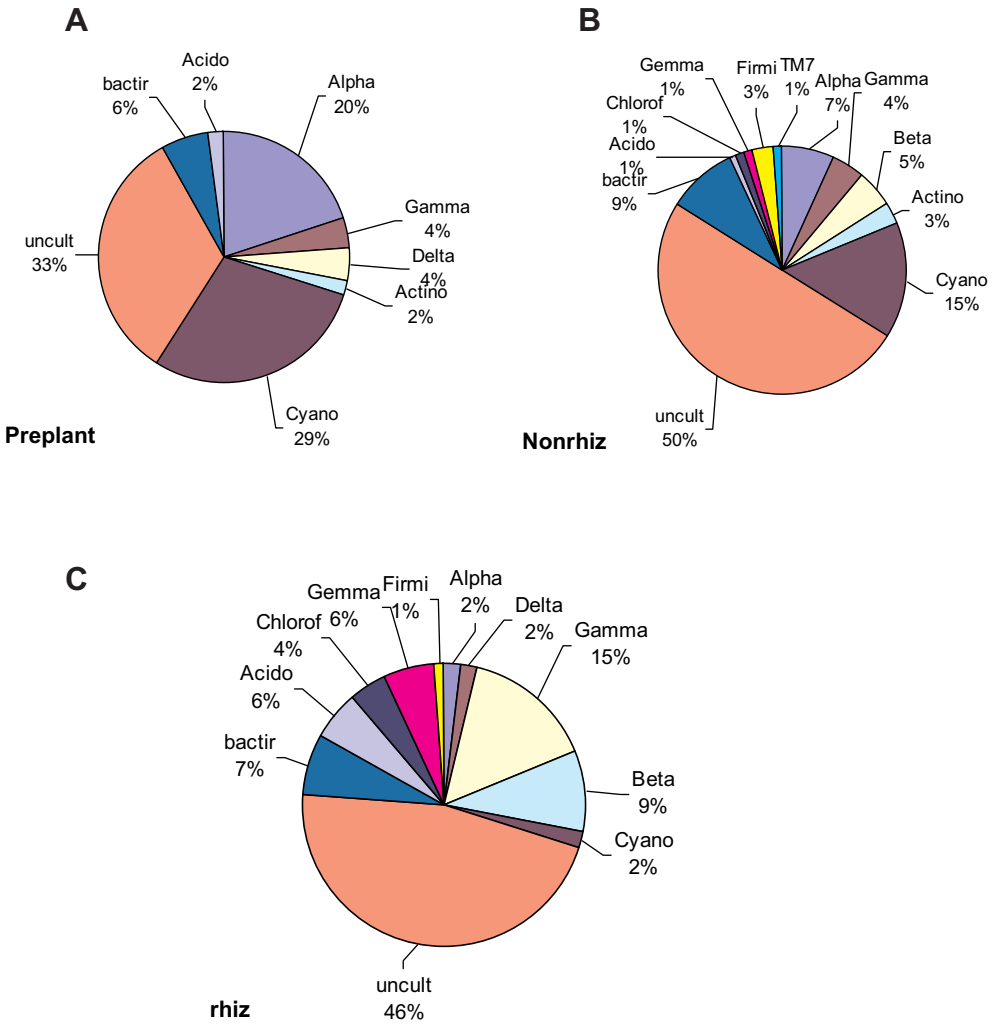

Fig. 3. Comparison of the bacterial community composition in three soil, collected pre-plant soil (A), non-rhizosphere soil (B) and rhizosphere soil (C) as revealed by 16S rRNA cloning. Shown fractions indicate the relative percentage to the total number of clones. Alph, *Alphaproteobacteria*; Gamma, *Gammaproteobacteria*; Beta, *Betproteobacteria*; Delta, *Deltaproteobacteria*; bactir, *Bacteroides*; chlorof; *Chloroflexus*, Cyano, Cyanobacteria; Actino, *Actinobacteria*; *Acido*, *Acidobacteria*; *Firmi*, *Firmicutes*; Gemma, *Gemmatimonas*; uncult, uncultured bacteria.

diversity. The same trend was shown in the *Bacteroidetes* group, which was more abundant and diverse in the control soil with low salinity. It was also interesting to note that higher abundance of *Deltaproteobacteria* were more predominant in soil with higher salinity, whereas *Alpha-* and *Gammaproteobacteria* were more predominant in control soil with low salinity (Fig. 3). The *Deltaproteobacteria* was dominated by *C. coralloides* (Li et al., unpublished) and *M. macrosporus* (Sproer et al., 1999). *C. coralloides* is a strain that was previously isolated by the above authors from marine mud and has been reported as having salt-resistant properties.

5. Conclusion

Addition of an assessment of microbial composition and diversity in soils treated with saline water drainage in the SJV region holds potential to provide important information of its effects in ecosystem health and functioning. Our results indicate that the effects of salinity, B, and pH were more severe on bacterial population during the first week of growing cucumber, with decreasing impacts with plant growth. Our study also showed significant B–pH and salinity–B–pH interaction effects on bacterial diversity indices; which were not previously available. Since salinity–B–pH interaction effects were more apparent during week seven, these three variables had significant interaction effects on the bacterial diversity indices compared to the insignificant effect on cucumber during the same period. This suggests that changes in microbial diversity may be the first indicator of stress in salinity–B–pH affected soils. Therefore, if stress can be detected early enough in salinity–B–pH affected soils, then some remedial action may be possible to improve soil quality and crop performance.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.soilbio.2009.11.033.

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