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Research Article

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Effect of Non-indigenous Bacterial Introductions on Rhizosphere Microbial Community

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Abstract

BACKGROUND: Towards achievement of sustainable agriculture, using microbial inoculants may present promising alternatives without adverse environmental effects; however, there are challenging issues that should be addressed in terms of effectiveness and ecology. Viability and stability of the bacterial inoculants would be one of the major issues in effectiveness of microbial pesticide uses, and the changes within the indigenous microbial communities by the inoculants would be an important factor influencing soil ecology. Here we investigated the stability of the introduced bacterial strains in the soils planted with barley and its effect on the diversity shifts of the rhizosphere soil bacteria.
METHODS AND RESULTS: Two different types of bacterial strains of *Bacillus thuringiensis* and *Shewanella oneidensis* MR-1 were inoculated to the soils planted with barley. To monitor the stability of the inoculated bacterial strains, genes specific to the strains (XRE and *mtr*A) were quantified by qPCR. In addition, bacterial community analyses were performed using v3-v4 regions of 16S rRNA gene sequences from the barley rhizosphere soils, which were analyzed using Illumina MiSeq system and Mothur. Alpha- and beta-diversity analyses indicated that the inoculated rhizosphere soils were grouped apart from the uninoculated soil, and plant growth also may have affected the soil bacterial diversity.
CONCLUSION: Regardless of the survival of the introduced non-native microbes, non-indigenous bacteria may influence the soil microbial community and diversity.

Key words: *Bacillus thuringiensis*, *Shewanella oneidensis MR-1*, Rhizosphere Bacterial Diversity

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Introduction

Microorganisms are ubiquitously found in the four spheres of the earth. In the soil environment especially for soils utilized for agricultural activities, its presence is vitally important as it directly affects the soil health [1]. These microbes affect soil health as it carries out recycling of nutrients such as carbon, nitrogen, phosphorus, and sulfur, that are known to be essential for the growth of plants [1, 2]. Over the years as population grows higher, there has been a higher demand for food production. Thus, during the Green Revolution several technologies were developed to meet this demand through the enhancement of plant breeds coupled with the application of inorganic fertilizers and pesticides. However, the latter has brought about undesirable consequences especially to the environment as these chemicals were found to be consistently persistent that later became environmental contaminants [3-5]. This

realization has then shifted the goal of sustainable agriculture to what considers mitigating climate change and preserving agroecosystem [5, 6]. Different remediation approaches have been utilized to different environmental contaminants. Apparently, among those methods, cost effectiveness is one of the primary factors of consideration for application, which could be provided by various microorganisms in the process known as bioremediation [7-10]. Furthermore, utilization of these microorganisms, such as plant growth-promoting rhizobacteria (PGPR), also encourages reduction of reliance to chemical fertilizers and pesticides by improving the acquisition of nutrients from soil, releasing of plant hormone regulators, protecting the roots from pathogens, and improving plant stress tolerance [11, 12].

Several strains of microorganisms have already been studied and found to have plant growth promotion effects and potential degrading capabilities of organic and inorganic pollutants [3, 13]. The use of microbial inoculants currently presents promising alternatives towards a sustainable agriculture without adverse environmental effects; however, there are some challenges that should be addressed up-front. Some of these are the viability and stability of the bacterial inoculants as well as the changes within the indigenous microbial communities at which the inoculant is introduced as the soil itself contains humongous number of microorganisms [13-15].

This study investigated the stability of the added bacterial inoculants to the soils planted with barley and its effect onto the composition of resident soil microorganisms. The bacterial inoculants using *Bacillus thuringiensis* KCTC 3452 (hereafter represented by Bt) and *Shewanella oneidensis* MR-1 were monitored by estimating gene copy numbers of XRB gene of *B. thuringiensis* KCTC 3452 and *mtr*A gene from *S. oneidensis* MR-1 after the inoculations. The XRE gene encoding transcriptional regulator has been known to be specific to Bt srain [16], while the *mtr*A gene that encodes a periplasmic decaheme cytochrome has been known to be specific to dissimilatory metal-reducing bacteria such as *S. oneidensis* MR-1 [17]. In addition, a comparison of the composition of the resident soil microorganism prior and after addition of the bacterial inoculants was also done with 16S rRNA gene sequences acquired using the Illumina MiSeq sequencing.

Materials and Methods

Plant–soil microcosm setup

The topsoil at about 15 cm depth was collected from the Jeonbuk National University campus farm. The soils were immediately transported to the laboratory and used to fill pots with approximately 1 kg of the soil. To each pot, 10 surface-sterilized barley (*Hordeum vulgare*. L) seeds were placed onto the soil surface. The barley seeds were surface-sterilized with 10% commercial bleach solution and then washed with sterile water.

For inocula, the strains Bt and MR-1 were grown in tryptic soy broth (TSB) separately at 30°C and 28°C, respectively, for 18 h. Cells were collected by centrifugation at 5,000 xg for 10 min and washed twice with 0.1X PBS by centrifugation. The bacterial pellets were resuspended in 0.1X PBS and adjusted to an OD of 0.1 ($1.0x10^8$ CFU/mL). The bacterial suspensions (5mL per each pot) were applied onto the soil surfaces after a week of seedling emergence and mixed with a sterile stainless spatula. For the control set up, only 0.1X PBS without the cells was used at the same volume. The two inoculated and the control planted pots were placed under an LED light source with 15 h of light and 9 h of dark condition at room temperature (25°C). Three plant-soil microcosm setups were designated as the control, Bt, and MR-1 (Fig. 1).

From each set up, 0.25 g of non-rhizosphere (pot) soils were collected with three replicates at 10 days, 20 days, and 50 days of post inoculations for the gene copy number analysis, and the rhizosphere soils were collected at time 0 and 30 days for the rhizosphere bacterial community analysis. The barley plants were harvested at 30 days. The soils adhered loosely to the roots were removed by shaking the plants, and approximately 0.25 g of firmly adhered soils were collected, which was designated as rhizosphere soil.

Total DNA extraction and quantification of XRB and *mtr*A genes

DNAs from soil samples were extracted using the DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturer's instructions. Monitoring of the Bt and MR-1 strains was performed by quantification of XRE gene and *mtr*A gene, respectively. Primer set for XRE gene (XRE Fwd - AAG ATA TTG CAA GCG GTA AGA T, XRE Rev - GTT TTG TTT CAG CAT TCC AGT AA; product size – 246 bp) was referred from the previous article [16], and the one for *mtr*A gene (MtrA Fwd - CTA AAG GCG CCG ATT CTT GC; MtrA Rev - TTG CGA CGT GTA CTT GGT GA; product size – 327 bp) was designed based on the whole genome sequence of *S. oneidensis* MR-1 using the NCBI Primer-BLAST [18].

The quantitative PCR (qPCR) amplification and detection

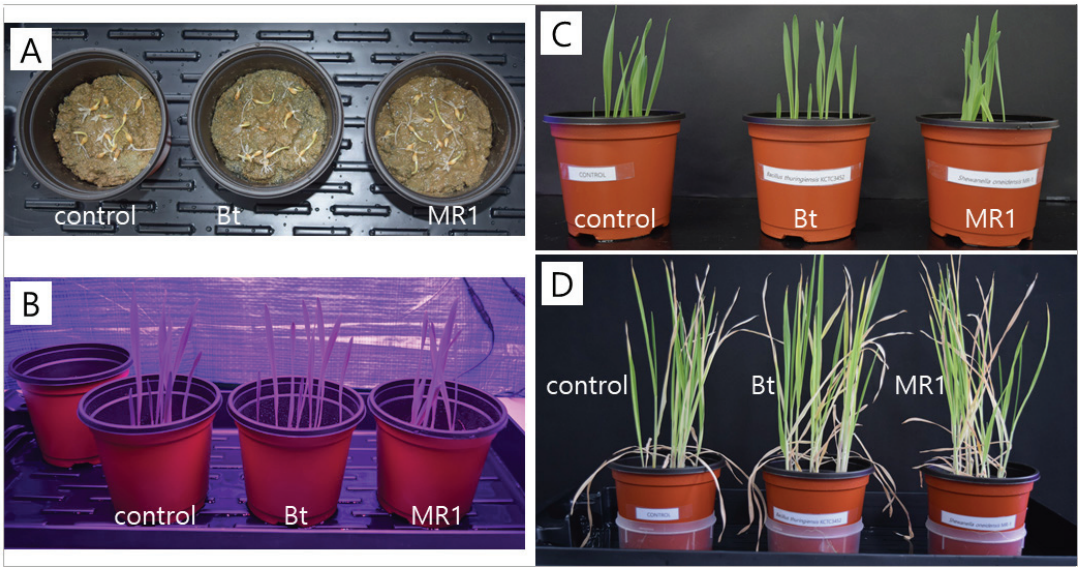

Fig. 1. The pot experiment showing the setup of the control pot and inoculated pots with Bt and MR-1 strains. (A) Barley seed pot preparation, (B) Experimental pots under LED culture, (C) Barley growth after 1 week, (D) Barley growth after 30 days.

were performed in 20-μL reaction solution. Amplification was performed in triplicates with a negative control. Each reaction tube was prepared using 10 μL AccuPower 2X GreenStar qPCR Master mix (Bioneer, Daejeon, Korea), 0.2 μM of forward and reverse primers, 1 μL DNA template, and deionized water. The qPCR was performed using CFX Connect™ Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA, USA) and the conditions were programmed as follows: initial denaturation at 95°C for 10 min, followed by 39 cycles of denaturation at 95°C for 15 sec and annealing at 59°C for 1 min. The melt curve analysis was set to raise from 65°C to 95°C at 0.3°C increments for 5 sec to test the specificity of the primer. One standard curve was prepared for both XRE and *mtr*A genes since a small difference in their expected amplicon base pair length. A standard curve was established following the procedures indicated by Nogrado *et al.* [19] and absolute gene copy number was calculated directly from the extracted plasmid [20].

Microbial community analysis by MiSeq sequencing and Mothur processing

The concentration of extracted DNA was first measured using Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The hypervariable v3-v4 region of the 16S rRNA gene was amplified using the primer set 341F (5′-CCTACGGGNBGCASCAG-3′) and 805R (5′-GACTACNVGGGTATCTAATCC-3′). The library was prepared by adding Illumina sequencing adapters and dual-index barcodes to the amplicon target and sequencing was performed using paired 300-bp reads on the Illumina MiSeq platform. The obtained sequences were submitted to the NCBI (National Center for Biotechnology Information) Sequence Read Archive (https://www/ncbi.nlm.nih.gov/sra) with accession numbers SRR13445520, SRR13444976, SRR13445002, and SRR13445519 under project accession number PRJNA692299.

The raw data of fastq was analyzed using Mothur package (v.1.44.3) [21]. Quality filtering of sequences was done briefly by generating contigs, removing nucleotide sequence errors, removing chimeric nucleotide sequences using VSEARCH, and removing nonbacterial nucleotide sequences, referring the standard operating procedure [22]. Nucleotide sequences were aligned against SILVA database v.132 [23]. Operational taxonomic units (OTUs) were classified at the species level with 3% of dissimilarity, and then classified using RDP database [24] which resulted in a total of 49,945 reads in all 4 samples and a total of 5,432 OTUs.

Results and Discussion

Stability of non-indigenous bacteria by detection of specific genes

The absolute copy number of *mtr*A gene decreased more than 10 folds from 10 days to 20 days (5.67×10^5 to 5.29×10^4), and approximately 2 folds from 20 days to 50 days (2.43×10^4) (Fig. 2). In case of XRE gene, the decrease rate was higher from 1.06×10^3 at 10 days to non-detectable at 50 days, than that of *mtr*A (Fig. 2).

Although XRE gene was not detected at 50 days, with still a high copy number of *mtr*A gene (approximately four orders of magnitude), looking at the data at 20 days, the addition of the Bt and MR-1 strains in the microcosms demonstrated relative stability of added bacterial strains in soils. An inference to the higher copy number of *mtr*A than XRE gene could be due to paralogy of *mtr*A in *S. oneidensis* MR-1 genome, which has over 50% gene similarity [25, 26].

Diversity indices by plant growth and non-indigenous bacteria

The effect of adding bacterial inoculants on the soil resident microorganisms was also determined. Based on the normalized read numbers using the minimum read count of 7,605 from the sample T0, the soil sample at the start of the experiment (T0) showed the highest number of 1,956 OTUs which decreased after 30 days (T30-Ctrl; 1,770 OTUs), but slight less decreases of OTU counts were observed in the rhizosphere soils inoculated with Bt (T30-Bt; 1,822 OTUs) and MR-1 (T30-MR1; 1,799 OTUs) (Table 1). Chao1 index, representing estimated species richness indicated the similar trend about the non-inoculated controls, with the highest value at T0 (4,030 OTUs) which later decreased after 30 days (T30-Ctrl; 3,860 OTUs). Similarly, the Shannon and inverse Simpson indices, representing both species richness and evenness showed higher values at the start of the experiment (T0), and also indicated decreases in both the control and the inoculated pots after 30 days, which may suggest that plants may have affected the diversity loss possibly due to heterotrophic bacterial growths by plant-derived organic materials. Interestingly, Shannon and inverse Simpson indices showed less decrease in the inoculated pots than those in the control (Table 1), suggesting a potential of non-native bacterial introduction effect on native microbial community.

Bacterial community by plant growth and non-indigenous bacteria

Composition of the bacterial communities was determined and classified into 21 phyla. There was dramatic increases of phylum *Cyanobacteria* in all three samples after 30 days (Fig. 3). *Cyanobacteria* has been known to promote plant growth by supplying nutrients through soil organic carbon amendment and nitrogen fixation [27]. Although it is speculative for the opposite

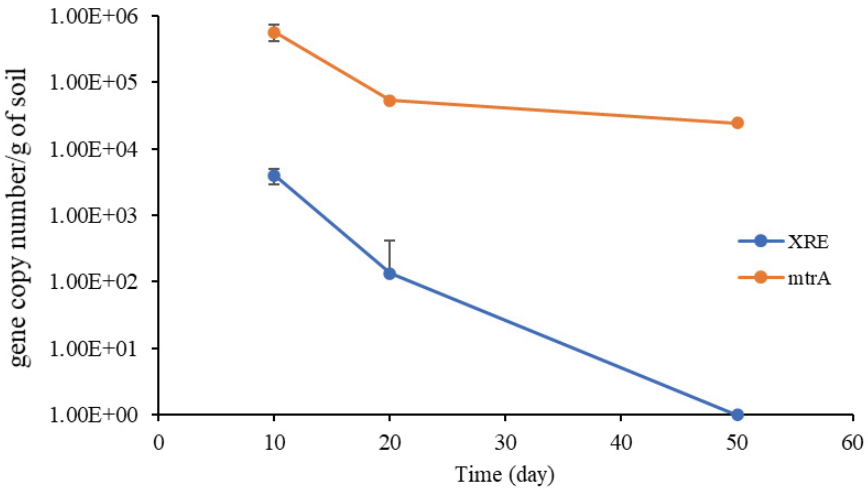

Fig. 2. The copy numbers of XRE and *mtr*A genes per gram of soil, analyzed by absolute quantification of qPCR.

Table 1. Microbial community diversity indices of soil samples from the pot experiment

Sample	Normalized no. of sequence	Good's coverage	observed species (OTU)	Chao1	Shannon	inverse Simpson
T0	7605	0.8533	1956.0	4029.9	6.568	256.26
T30-Ctrl	7605	0.8612±0.0024	1770.0±15.6	3859.7±144.6	5.944±0.014	59.61±1.36
T30-Bt	7605	0.8688±0.0030	1822.5±20.2	3631.2±155.8	6.485±0.016	240.24±6.34
T30-MR1	7605	0.8664±0.0030	1798.7±18.8	3615.2±148.2	6.328±0.015	173.07±4.51

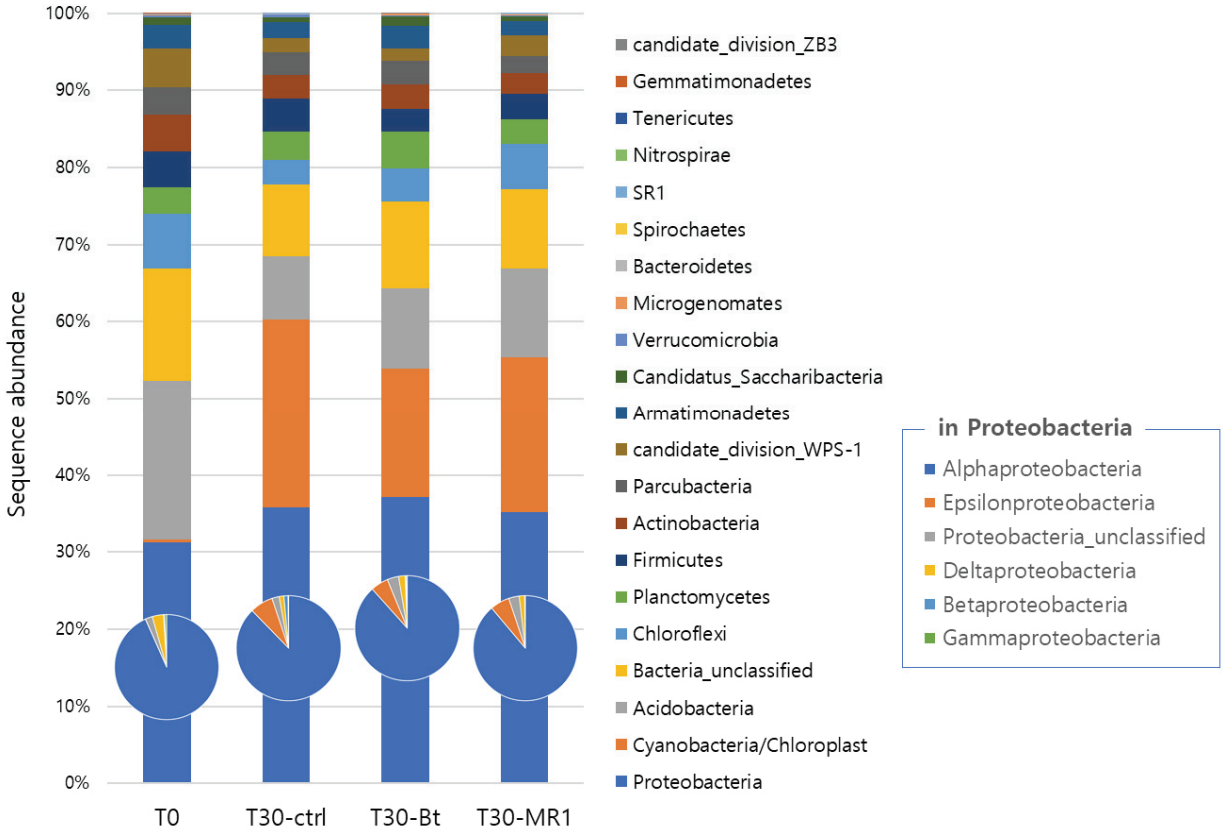

Fig. 3. Relative sequence abundance at phylum level. Sub pie charts for classes of *Proteobacteria* and legend shown in the box.

notion, there may be a potential that plant growth has increased the *Cyanobacteria* in the soils and rhizosphere. *Proteobacteria* have increased in the incubated soils with the barley plants, while *Acidobacteria* was observed to have decreased. Both *Proteobacteria* and *Acidobacteria* has been known to be dominant in forest soils [28], and substantial portions of members of *Proteobacteria* are heterotrophs, suggesting possible addition of organic carbon to the soils from the plants.

Heatmap representing sequence abundance at genus level indicated that only several genera were dominant with most of genera in minor ratios, by visualizing the top 40 genera among the total 307 genera (Fig. 4). The dominant bacterial genera also appeared to be correlated somewhat with each other, indicated by the vertical dendrogram. The horizontal dendrogram correlating the samples based on Bray-Curtis dissimilarity showed the similar results with those from diversity indices (Table 1) and community distribution at phylum level (Fig. 3). The initial soil (T0) was most different from the planted soils, regardless of non-indigenous inoculations, after 30 days of incubation. Also, the inoculated soils (T30-MR1, T30-Bt) were differentiated from the uninoculated soil (T30-ctrl), which was suggestive that addition of external bacteria had an effect on the composition of soil microbial community in the rhizosphere at least in this study under controlled laboratory conditions.

Beta-diversity analyses

Dissimilarity distances among the samples calculated by Yue & Clayton theta distance [29] were visualized by principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) (Fig. 5). The rhizosphere soil bacterial communities from the inoculated pots (T30-Bt, T30-MR1) formed a group, apart from the initial soil (T0) and the uninoculated rhizosphere soil (T30-ctrl) in both PCoA and NMDS. This might help explain differentiation of non-native bacteria introduction to soils from uninoculated rhizosphere soil. Based on the UniFrac analysis that compares similarity of the samples by incorporating phylogenetic distances of the microorganisms [30], the communities from the inoculated rhizosphere soils (T30-Bt, T30-MR1) were separated from the initial (T0) and uninoculated (T30-ctrl) soils (Fig. 6), which was similar with the results from

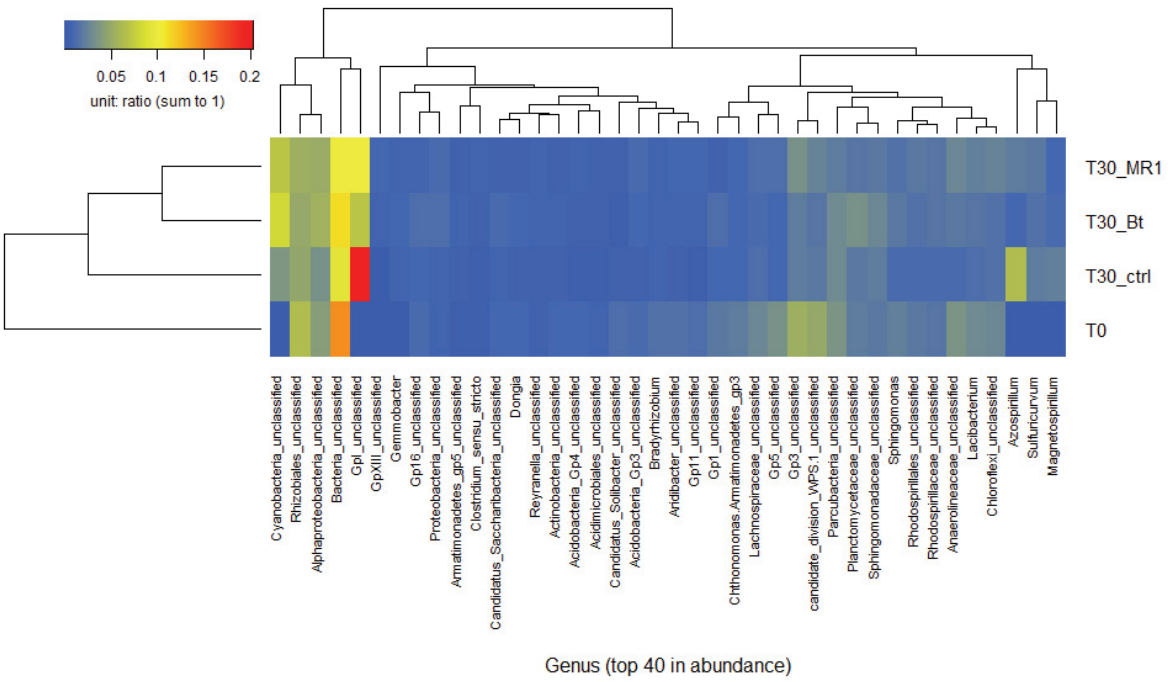

Fig. 4. Heatmap representing sequence abundance of top 40 among the total 307 genera at genus level. Vertical and horizontal dendrograms based on Bray-Curtis dissimilarity distance.

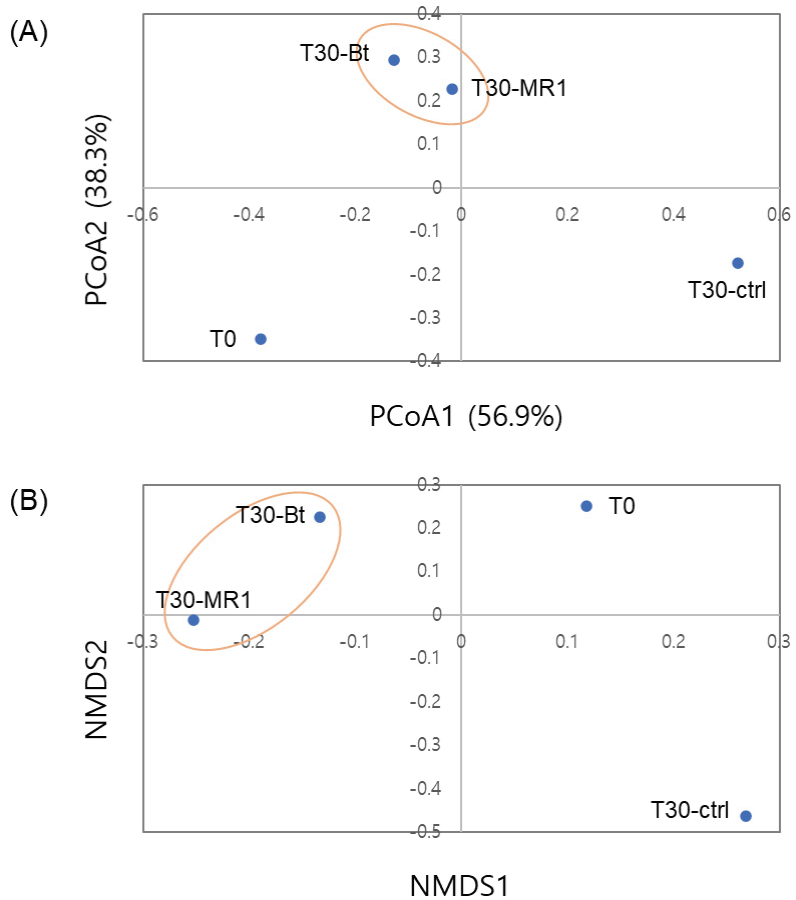

Fig. 5. Ordination analyses using Yue & Clayton dissimilarity theta distance. (A) Principal coordinates analysis, (B) Non-metric multidimensional scaling.

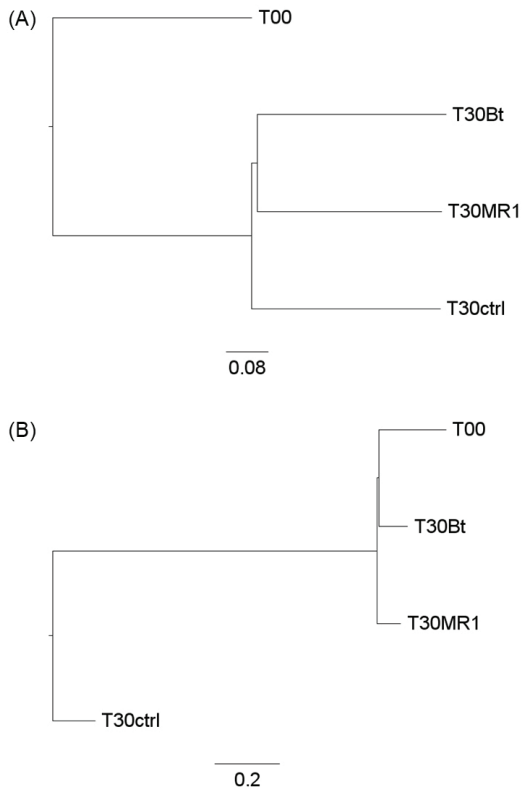

Fig. 6. Unweighted (A) and weighted (B) UniFrac analyses.

community distribution (Fig. 3, 4) and ordination analyses (Fig. 5). This is also suggestive of non-native bacterial introduction effect on the rhizosphere soils.

Effect of non-indigenous bacteria introduction

It is important to note that the sequences of the inoculated *Bacillus* and *Shewanella* were not detected. This suggests that bacterial inoculants were unsuccessful to colonize the rhizosphere. Also, the specific genes, XRE and *mtr*A of the inoculated *Bacillus* and *Shewanella*, decreased in detected copy numbers over time. Nonetheless there were bacterial diversity separations between the inoculated and uninoculated soils, identified by heatmap, ordination, and UniFrac analyses. This might suggest that introduction of non-native microbes may have influences on the soil microbial community and diversity, even though the introduced microorganisms do not survive long or colonize dominantly.

Viability of the introduced bacteria in the soil environment would be one of the major issues for certain purposes, such as microbial pesticides. Colonization of the bacterial inoculants on the rhizosphere would be a challenge, particularly if the bacterial strain is added for plant-growth promoting activities. Other factors such as appropriate carrier of the bacterial inoculum as well as method of application should be taken into consideration, which will aid the survival of the bacterial strain in the soil environment [11].

Note

The authors declare no conflict of interest

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