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ARTICLE

Effect of genetically modified rice producing resveratrol on the soil microbial communities

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Abstract Cultivation of genetically modified (GM) crops has rapidly increased in the global agricultural area. However, cultivation of GM crops in the field evoked the concern of the possibility of unintentional consequences from transgenic plant into environment. In our present study, we have assessed the effects of RS526, GM rice producing resveratrol on the surrounding soil microbial community. The effects of RS526 on the soil microbial community in its field of growth were assessed using a conventional culture technique and culture-independent molecular methods. Three replicate field plots were planted with single GM rice and a non-GM counterpart, Dongjin. The soil microbial communities around these plants were compared using colony counting, denaturing gradient gel electrophoresis (DGGE), pyrosequencing analysis, and community-level physiological profiling during the growing periods. The bacterial, fungal, and actinomycetes population densities of the RS526 soils were found to be within the range of those of the non-GM rice cultivar. The DGGE banding patterns of the GM and non-GM soils were also similar, suggesting that the bacterial community structures were stable within a given month and were unaffected by the presence of a GM plant. The data obtained from pyrosequencing analysis showed that the bacterial community distribution at the phylum level were highly similar to DGGE patterns between two tested groups. The substrate utilization pattern of RS526 and Dongjin rice soil was quite similar between each sampling time. These results indicate that soil microbial communities are not significantly affected by the cultivation of RS526 within the experimental time frame.

Keywords Genetically modified rice · Resveratrol · Soil microbial community

Introduction

Soil contains the most diverse organisms within the natural environment and soil organisms control numerous integral processes that are not only important for production, but maintaining the healthiness of the ecosystem (Brussard et al. 1997). Microorganism activity plays an important role in the stability of the soil's cohesiveness. Most soil organisms are concentrated toward the plant's rhizosphere soil (Gupta et al. 2000). Thus, any changes that affect the rhizosphere have the potential to alter the soil microbial community and activity which may be either beneficial or harmful. It has been reported that different plant species tend to prefer specific microorganism communities. Plants have different composition of root exudates which can affect the relative abundance of specific microorganisms that are present in the rhizosphere (Somers et al. 2004). For example, the rhizosphere of a primitive variety of wheat is dominated by a rhizobacteria with a diverse phylogenetic,

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whereas the rhizosphere of a modern variety of wheat was found to be dominated by fast-growing *Proteobacteria* (Graner et al. 2003). Although the soil exudates are an important determining factor of soil microbial flora, understanding the role of mono-compounds associated with soil exudates in mediating the interaction between microorganisms is still at its early stages (Bais et al. 2006; Haichar et al. 2008). Genetically modified (GM) crops were known to alter root exudate composition through the introduction of a functional gene to plant. Thus, both the short-term and long-term effects of releasing such GM crop on the soil ecosystem must be investigated for monitoring of their safety to agricultural environment (Gupta et al. 2000).

The effect of GM crop on soil microbial community has been studied for various crops such as potato, corn, cotton, canola, and rice. Some examples of the traits incorporated into the GM crop include disease resistance (Ahrenholtz et al. 2000; Sessitsch et al. 2003; Brusetti et al. 2004; Baumgarte and Tebbe 2005; Rui et al. 2005; Shen et al. 2006; Fang et al. 2012; Wei et al. 2012), herbicide resistance (Becker et al. 2001; Schmalenberger and Tebbe 2002; Dunfield and Germida 2003; Sessitsch et al. 2004), and changed starch composition (Milling et al. 2004). It has been reported that the effects of these GM crops on the soil microbial community are either negligible, have temporal or spatial differences and comparable to natural factors (Becker et al. 2008). Important changes to the microbial community were reported to be from unintentional changes in trait of GM crop, especially the change in number of root exudates (Sessitsch et al. 2003; Rui et al. 2005; Icoz and Stotzky 2008). When the bird's foot trefoil was modified to synthesize opine, for example, it resulted in a change of the microbial community due to strongly stimulated bacteria degrading the metabolite (Oger et al. 1997, 2000).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a non-flavonoid polyphenol-type compound found in peanuts and grapes (Creasy and Coffee 1988; Jeandel et al. 1991). It is produced by the plant as a defensive response under the attack from a pathogen (Langcake et al. 1979). Properties of resveratrol include antioxidant through antagonism similar to quercetin (Mikstacka et al. 2010), extended life span through increased expression of SIR2 enzyme (Guarente and Picard 2005; Markus and Morris 2008), repressing the expression of NF-κB and it has also been reported to be effective against pancreatitis through anti-inflammatory effects (Meng et al. 2005). Moreover, it has been shown to repress the production of both fat and non-fat fatty livers in animals (Bujanda et al. 2008).

In gramineae crops such as rice, resveratrol is not synthesized because they are lack of resveratrol synthase. Baek et al. (2013) introduced the *AhSTS1* (*RS3*, GenBank accession no. DQ124938) into the commercial rice variety Dongjin and developed resveratrol transgenic rice line RS526, which contained a single copy transgene and had good performance of agronomic traits. RS526 was found to significantly reduce body weight, blood glucose, triglycerides, total cholesterol, and low-density lipoprotein-cholesterol in the diet experiment with mice (Qin et al. 2013). Therefore, commercialization of RS526 could contribute to the human health. This study is a preliminary step toward the commercialization of RS526, which compares how the transgenic rice affects the soil microbial community in comparison to its parental wild-type, Dongjin rice. To achieve this, the following studies were carried out on the soils from the two varieties of rice: (1) soil chemical composition analysis; (2) soil microbial density by culture-based analysis; (3) Denaturing gradient gel electrophoresis (DGGE) analysis to investigate the effect of resveratrol rice on the total soil microbial community; (4) Pyrosequencing analysis of soil bacterial 16S rRNA gene to compare the microbial composition between the two soils; and (5) community-level physiological profiling (CLPP) analysis to investigate the metabolic capability and functional diversity of the two soil samples.

Materials and methods

Site and sampling

The experimental plot for RS526 and Dongjin rice to investigate their effect on soil microbial community was set up in an isolated GMO experimental field at the National Academy of Agricultural Science (NAAS) located in Korea. RS526 and Dongjin were each plotted three times on the experimental field with each plot being 4 × 4 m in size. The rice seeds were sown on a seedling box then transplanted after 3 weeks in June 2013. Soil samples were collected in ends of June (seedling stage), August (tillering stage), and October (maturity stage). The rhizosphere soil samples were collected in three repeats by uprooting the rice and removing the surrounding soil in rice roots.

Soil chemical analyses

Rhizosphere soil samples of RS526 and Dongjin rice were collected, dried, and then passed through a 2 mm sieve for further study. Chemical analysis of the soil was done following the NAAS soil and plant analysis method (NIAST 2000), pH was measured using a pH meter with soil and distilled water at a 1:5 ratio, respectively, total nitrogen and carbon composition was obtained via the Elemental analyzer (Vario Max CN, Elementar, Germany), Available phosphate was measured by Lancaster method, using calorimetry assay. Exchangeable cations such as calcium,

potassium, magnesium, and sodium were diffused by 1N ammonium acetate (pH 7.0) and then analyzed using ICP (GBC Integra XL, Australia).

Microbial community density analysis

The density of soil microbes was assessed by enumeration of cultured total bacteria and fungi after inoculating soil samples in each selective media. Ten gram of the collected sample was immersed in 90 mL of sterilized 0.85 % NaCl solution then suspended for 30 min using a shaking incubator (Vision Co., Korea) with 200 rpm. A series of dilutions were made using the suspension and smeared onto a R2A agar (Difco, USA) containing cycloheximide (0.05 g/L) for bacterial culture and on a R2A agar containing chloramphenicol (0.02 %) for fungal culture. The inoculated media to grow bacteria and fungi were incubated at 28 °C for 2 and 4 days, respectively, prior to counting the number of colonies. The number of microorganisms for each sample was calculated by counting the number of colonies in each of the three petri dishes then using the average value as the colony forming unit ($CFUg^{-1}$ dry soil).

DNA extraction and DGGE analysis

Metagenomic DNA was extracted using FastDNA Spin Kit (Qbiogen, USA) from the soil according to manufacturer's manual. The 16S rRNA was amplified using polymerase chain reaction (PCR) reaction for DGGE analysis. The primers used for PCR were the F352T-519r incorporating a eubacteria's V3 region (Ahn et al. 2006), the primer sequence being F352T: 5′-CGCCCGCCGCGCGCGGGCG GGGCGGGGGCACGGGGGGACTCCTACGGGTGGC-3′, 519r: 5′-ACCGCGGCTGCTGGCAC-3′. Volume of the PCR reaction was 50 μL, consisting of 5 μL of 10× PCR buffer, 10 ng of template DNA, 25 pmol of each primers, 200 μM of each dNTP, and 2.5 U of f-*Taq*-DNA polymerase (Solgent, Korea). The PCR condition involved denaturation at 95 °C for 5 min, 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and was carried out for 30 cycles. The last step involved a 7 min reaction at 72 °C. The PCR product was electrophoresed in an 8 % acrylamide gel with a formamide concentration gradient of 40–70 % using Dcode Universal Mutation Detection System (Bio-Rad, USA). The unfolded DNA was dyed with SYBR Green I (Cambrex BioScience, USA) and EtBr and was observed using Gel Doc 2000 (Bio-Rad) under UV transilluminator.

Pyrosequencing analysis

After DGGE analysis for preliminary sample comparisons of microbial community from soil samples of RS526 and Dongjin rice, we obtained detailed information of bacterial community composition using high-throughput pyrosequencing. The extracted metagenomic DNA was used for pyrosequencing analysis as in previous reports by Hur et al. (2011). PCR amplifications were performed using a C1000 Touch thermal cycler (Bio-Rad, USA) and barcoded fusion primers (http://www.ezbiocloud.net/resource/M1001). A total of 100 ng template DNA was added to the PCR reaction (total of 50 μL), which contained Ex Taq buffer, 0.2 mM each dNTP, 0.5 μM each primer, and two units Ex Taq (Takara, Japan). After initial denaturation (94 °C, 5 min), the PCR reaction was carried out using the Touch-down program to undergo ten cycles of denaturation (94 °C, 30 s), annealing (60 °C, 45 s), extension (72 °C, 90 s) where the annealing temperature was decreased by 0.5 °C for each subsequent cycle. Further 20 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 45 s), extension (72 °C, 90 s) were carried out. The amplified products were confirmed by 2 % agarose gel electrophoresis and visualized using the Gel Doc system (Bio-Rad). Amplicons were purified using a QIAquick PCR purification kit (Qiagen, USA) and quantified using a PicoGreen dsDNA Assay kit (Invitrogen, USA). Equimolar concentrations of each amplicon from different samples were pooled and purified using an AMPure bead kit (Agencourt Bioscience, USA) and then amplified on sequencing beads by emulsion PCR. Sequencing reactions were performed using a Roche GS FLX Titanium system at ChunLab, Inc (Korea) according to the manufacturer's instructions.

Data analysis of pyrosequences

The obtained sequences were compared and classified with EzTaxon database (http://www.ezbiocloud.net). A reaction curve that shows the increase in ratio of operational taxonomic unit (OTU)s to analyzed sequence number to other OTUs was made based on CD-HIT and Mothur package (Schloss et al. 2009). The number of OTU was calculated from a sequence group that shows a 97 % sequence homology based on TBC de novo clustering algorithm (Lee et al. 2012). To compare OTUs between the samples, shared OTUs were obtained with taxon exclusive or (XOR) analysis using CLcommunity program (ChunLab, Korea) according to Khodakovskaya et al. (2013). For this analysis, the number of reads for all soil samples was normalized using the program prior to carrying out XOR analysis at phylum, class, order, family, genus, and species levels. The similarity between each pair of communities was estimated using the Fast UniFrac web interface (Hamedy et al. 2010) and visualized using principal coordinate analysis (PCoA).

Community-level physiological profiling (CLPP) analysis

The potential of the microbial community functionality was investigated by inoculating soil samples into each well

of the EcoPlate (Biolog, USA). For this experiment, soil samples were diluted at a 1:10 (w/w) ratio with sterile water prior to stirring at 200 rpm for 10 min. Then, 150 μL of the supernatant was taken to fill each Eco plate wells. Next it was incubated at 20 °C and each of the well's change in color was measured and analyzed at 595 nm wavelength on 24 h intervals using Multiskan Ascent (Thermo Labsystems, Finland). Each EcoPlate was divided into three identical zones, serving as replicates, and the absorbance value of each carbon source was corrected by subtracting the absorbance value of the control well without any carbon substrate; negative values were set to zero. Average well color development (AWCD) was calculated for each replicate and for each sampling time as the mean of the 31 corrected values (Garland and Mills 1991; Wei et al. 2012).

Results and discussion

Chemical characteristics of soil samples

The chemical composition of rhizosphere soil collected from RS526 and Dongjin rice was investigated. Because a difference in the soil chemical composition can affect the soil microbial community, the soil pH, available phosphate, electrical conductivity, cations, total nitrogen, and organic matter were analyzed to compare the difference in rhizosphere soil's chemical composition between RS526 and Dongjin rice (Table S1). There was no significant difference in the soil pH between RS526 (pH 5.9–6.3) and Dongjin rice (pH 6.2–6.5) for the periods studied. The available phosphate was shown to be 52.8–71.3 mg kg^{-1} for RS526 and 55.4–71.1 mg kg^{-1} for Dongjin rice, showing that there is no significant difference between the two soils. The soil electrical conductivity for both RS526 and Dongjin rice was found to be 0.3 dS m^{-1} and the total nitrogen content was also found to be similar. Moreover, there was no significant difference in either organic matters or cations such as K, Ca, Mg, or Na between RS526 and Dongjin rice soil. It has been reported that cation composition is related to organic matter composition suggesting that RS526 and Dongjin rice had similar soil chemical characteristics (Clark et al. 1998).

Soil microbial community density analysis

To investigate how RS526 cultivation affects the soil microorganism community density, the bacteria and fungi community densities of rice rhizosphere soil were observed at seedling (June), tillering (August), and maturity stage (October). Differences in the bacterial population density were found according to the growth stage of rice except for fungi which did not show any significant differences. The difference in the bacterial population density according to growth stage is in agreement with previous reports of the diversity and activity of bacterial communities changing during plant growth (Houlden et al. 2008). The reason behind no significant difference being found in fungi is likely due to the *fungistatic* phenomenon, which is known to repress fungal growth and fungal spore germination in most soil types (Lockwood and Filonow 1981). In conclusion, there were differences found in the microorganism density between growth stages, but no significant differences were found between GM and non-GM crop (Table S2).

Comparative analysis of the soil microbial community using DGGE analysis

In order to compare the major changes in total rhizosphere soil microorganisms at different time periods between RS526 and Dongjin rice, a DGGE analysis was performed. The V3 region of 16S rRNA was amplified using PCR and the PCR product was confirmed the expected size of 323 bp (data not shown). Differences in the bands of DGGE profile of RS526 and Dongjin rice rhizosphere soils were observed for different time periods, but nearly no significant differences were observed between the RS526 and Dongjin rice rhizosphere soils (Fig. S1), suggesting that the major bacterial phylotypes of two samples are highly similar.

Bacterial community structure analysis using pyrosequencing

Throughout the research periods, the range of the reads of pyrosequence analyzed in Dongjin rice soils was from 4484 to 5313 and the number of OTUs was 1827–2713, while the range of the reads in RS526 rice soils was 4249–5283 and OTUs were 2138–2657 (Table 1). A rarefaction curve is similar for all samples studied (data not shown). Analyzing the bacterial distribution at a phylum level showed RS526 soil microbial community to consist in the order of *Proteobacteria* (27 %), *Cloroflexi* (24 %), *Firmicutes* (13 %), *Acidobacteria* (8 %), *Actinobacteria* (7 %), *Bacteriodetes* (5 %) and the Dongjin rice soil as *Proteobacteria* (31 %), *Cloroflexi* (28 %), *Firmicutes* (16 %), *Acidobacteria* (10 %), *Actinobacteria* (8 %), *Nitrospirae* (5 %) in seedling stage soil (Fig. 1A). We obtained 4249 and 4484 of pyrosequences from tillering stage soil samples of RS526 and Dongjin rice, respectively. Analyzing the microbial distribution at a phylum level showed RS526 soil microbial community to consist in the order of *Proteobacteria* (25 %), *Cloroflexi* (24 %), *Firmicutes* (13 %), *Actinobacteria* (10 %), *Acidobacteria* (9 %), *Nitrospirae*

Table 1 Bacterial diversity indices in Dongjin and RS526 rice rhizosphere soils

Growth stage	Sample	Total no. of reads	Observed phylotypes (OTUs)	Average length (bp)	Ace	Chao1	Shannon index	Coverage (%)
Seedling stage	DJ	5368	1968	453.7	3520.4	3439.0	7.0	80.6
	RS526	5283	2657	464.8	9878.3	6112.2	7.4	66.7
Tillering stage	DJ	4484	1827	452.8	3431.4	3229.2	7.0	77.4
	RS526	4249	2138	468.2	7490.8	4851.2	7.2	67.0
Maturity stage	DJ	5313	2713	468.1	11,482.0	6902.6	7.4	64.8
	RS526	4716	2501	467.6	10,237.5	6272.5	7.3	63.0

Estimates of shannon index were obtained based on 3 % differences in DNA sequence alignments

(4 %) and the Dongjin rice soil as *Proteobacteria* (25 %), *Cloroflexi* (24 %), *Firmicutes* (15 %), *Acidobacteria* (8 %), *Actinobacteria* (7 %), *Nitrospirae* (5 %) (Fig. 3B). We also obtained 4716 and 5313 pyrosequences from maturity stage soil samples of RS526 and Dongjin rice, respectively. Analyzing the microorganism distribution rate at a phylum level showed RS526 soil microorganism to consist in the order of *Cloroflexi* (30 %), *Proteobacteria* (29 %), *Firmicutes* (11 %), *Acidobacteria* (7 %), *Actinobacteria* (7 %), *Nitrospirae* (4 %) and the Dongjin rice soil as *Cloroflexi* (33 %), *Proteobacteria* (25 %), *Firmicute* (11 %), *Acidobacteria* (8 %), *Actinobacteria* (7 %), *Nitrospirae* (5 %) (Fig. 1C). The dominant microbial community distribution at the phylum level was very similar between RS526 and Dongjin rice soil which suggests RS526 rice cultivation does not have a significant different effect on soil microbial community structure. The result of the bacterial 16S rRNA sequencing of the California field-grown M103 rhizosphere soil sample showed the presence of 10 phyla including *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Plactomycetes*, *Proteobacteria*, and *Verrucomicrobia.* Studying the 2008 and 2009 samples showed that *Proteobacteria* composed 44 and 50 %, respectively, and *Acidobacteria* composed 24 and 30 %, respectively, of the total bacterial community. The phyla which composed less than 4 % of the bacterial community were *Actinobacteria, Bacteroidetes,* and *Firmicutes* (Spence et al. 2014). This study carried out a 16S rRNA sequence analysis of the rice soil which showed the presence of phyla including *Proteobacteria, Cloroflexi, Firmicutes, Acidobacteria, Bacteroidetes, Actinobacteria, Nitrospirae, Planctomycetes, Clorobi, Bacillariophyta,* and *Gemmatimonadetes*. The distribution of these bacteria showed minor differences depending on the growth stage and was found to be around 24–29 % for *Proteobacteria,* 24–33 % for *Cloroflexi*, 11–16 % for *Firmicutes*, 7–10 % for *Acidobacteria*, 7–10 % for *Actinobacteria* and the other phyla including *Bacteroidetes, Nitrospirae, Planctomycetes, Clorobi* and *Gemmatimonadetes* were found to be below 4 %. This result was different to the microorganism distribution of the M104 cultivation soil and is likely to be due to the environment where the rice was cultivated, rather than the cultivar of rice that were cultivated. XOR analysis showed that there were differences of phylotypes between RS526 and Dongjin rice soil (Table 2). The unique phylotypes in two soils could be due to the heterogeneity of the soil rather than the cultivation of RS526, since there were also differences of phylotypes between two non-GM rice soils (data not shown).

A PCoA analysis based on Fast UniFrac on all samples for different time periods and soil type was carried out to make a UPGMA dendrogram which showed that soils collected at seedling and tillering are not grouped by time period or soil type. It was found that the soils from seedling and tillering stage are not grouped separately by growth stage or soil sample (Fig. 2). However, it was found that the soil from RS526 or Dongjin rice does not generally group together. These results suggest the cultivation of RS526 does not have a significant effect on the soil ecosystem. It has been reported that a study on GM poplar showed that in the early stages of GM poplar release, there was a difference in the microbial community structure between GM poplar and non-GM poplar. However, after a period of 3 years, the difference stabilized and became negligible between GM and non-GM (Hur et al. 2011). Thus, it is suggested that analyzing the effect of cultivating GM crop on soil microbial community should be carried out on a case by case basis and both short-term and long-term experiments should be included.

Pyrosequencing, a next generation sequencing technique, was used to investigate the effect of GM crop cultivation on soil microbial community using transgenic *Bt* maize crop transformed with *Cry1Ab* gene where the study showed that over a cultivation period of 4 years, there was no difference in the microbial community structure between *Bt* and non-*Bt* maize. It was concluded that difference in rhizobacterial community structure is highly likely to be a result of climatic factors and not GM (Barriuso et al. 2012). However, it was reported that Cry

proteins are rapidly absorbed to clay minerals, which render the proteins resistant to biodegradation in soil, thus facilitating a potential longer exposure of non-target organisms to the toxin (Icoz and Stotzky 2008; Koskella and Stotzky 1997; Stotzky 2004). It also has been reported that infective fungal mycorrhizae could colonize *Bt* maize roots more efficiently than non-*Bt* ones and the persistence of Cry proteins in soil may also be related to the decrease of some microbial activity (Icoz and Stotzky 2008).

The difference between the DGGE analysis and pyrosequencing is that DGGE analysis is to compare the total

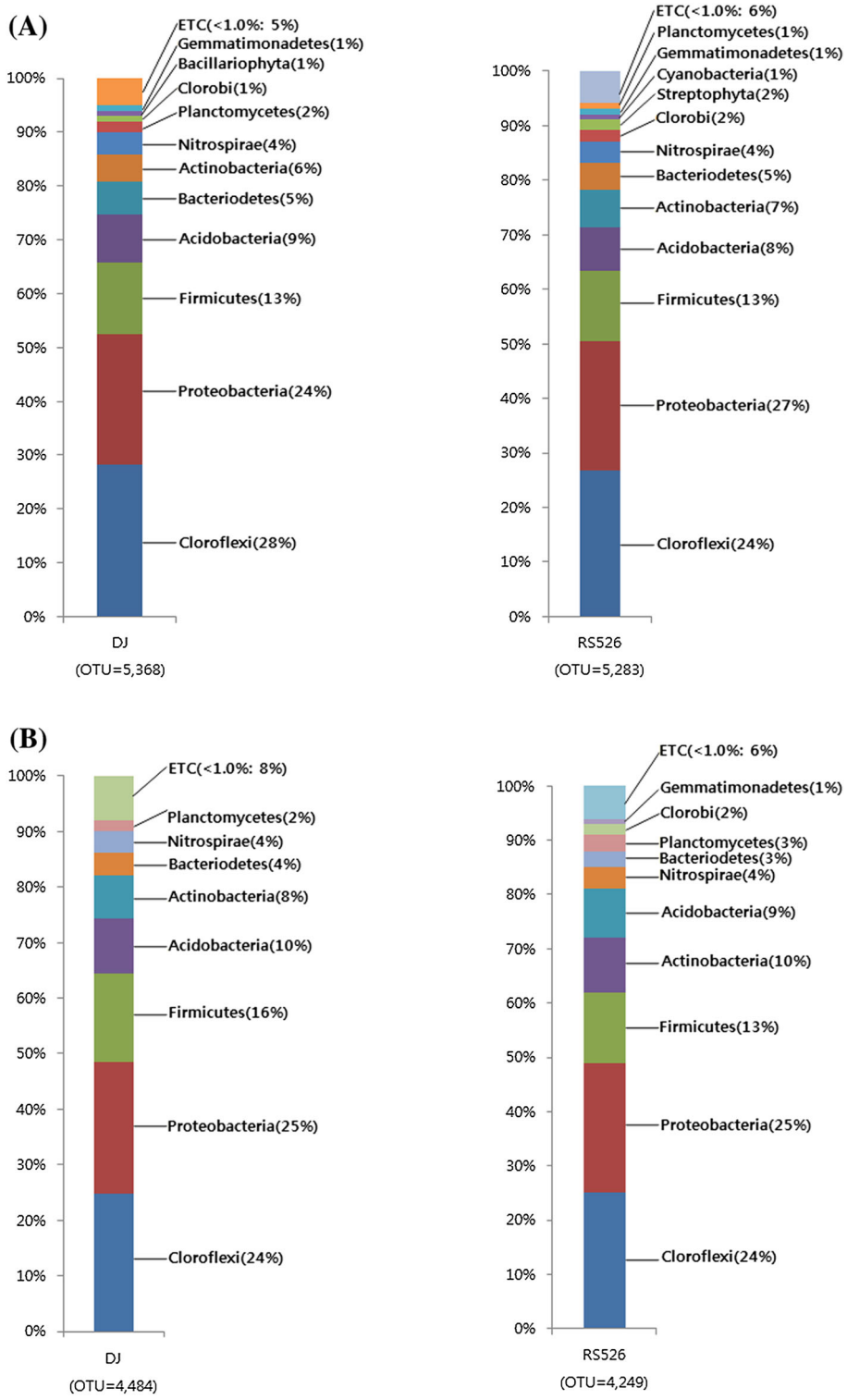

Fig. 1 Comparison of bacterial composition in Dongjin and RS526 rhizosphere soils. (**A**) Seedling stage, (**B**) tillering stage and (**C**) maturity stage. *DJ* Dongjin, *RS526* resveratrol rice 526, *OTU* operational taxonomic unit

Fig. 1 continued

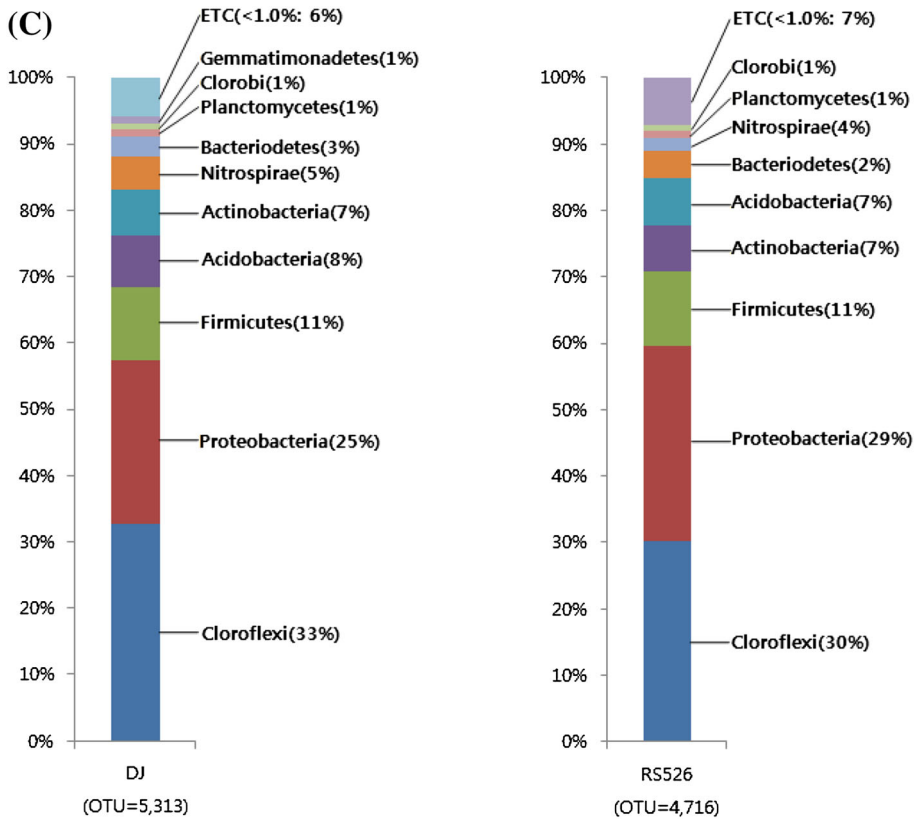

Table 2 Taxon XOR analysis between Dongjin and RS526 rhizosphere soil bacterial communities

Rank	Seedling stage		Tillering stage		Maturity stage	
	DJ	RS526	DJ	RS526	DJ	RS526
Phylum	13*	25	21	24	10	12
Class	55	141	46	74	35	50
Order	124	251	170	175	79	107
Family	273	485	330	366	190	245
Genus	565	864	643	731	424	494
Species	1154	1383	1302	1418	772	882

* The number of taxa that are present in Dongjin rhizosphere soil but not in RS526

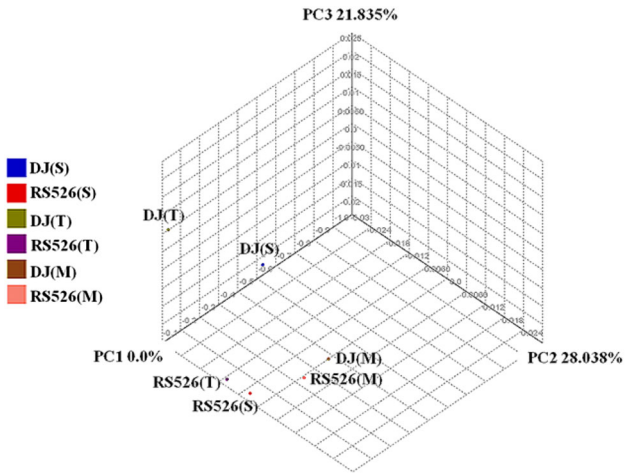

Fig. 2 PCoA analysis of 16S rRNA genes in the Dongjin (DJ) and RS526 soil based on OTUs sharing 97 % similarity (Bray-Curtis distance). *DJ*(*S*) rhizosphere soil of Dongjin collected at seedling stage, *RS526*(*S*) rhizosphere soil of RS526 collected at seedling stage, *DJ*(*T*) rhizosphere soil of Dongjin collected at tillering stage, *RS526*(*T*) rhizosphere soil of RS526 collected at tillering stage, *DJ*(*M*) rhizosphere soil of Dongjin collected at maturity stage, *RS526*(*M*) rhizosphere soil of RS526 collected at maturity stage

bacterial community change between GM and non-GM soils on the denaturing gel, while pyrosequencing is to analyze the difference between GM and non-GM soils through the 16S rDNA metagenomic sequencing analysis. Consequently, due to the analytical limit of DGGE analysis, we could not detect the difference between GM and non-GM soils.

Community-level physiological profiling (CLPP) analysis

To investigate the effect of RS526 cultivation on the soil microbial flora community, a CLPP analysis was carried out. Over the cultivation period on the Biolog Ecoplate, 31 types of substrate utilization were shown on Table 3 on a 5 day standard. For the June soil, it was found that

substrate utilization was generally high except for 6 substrates (D,L-α-glycerol phosphate, 2-hydroxy benzoic acid, γ-hydroxybutyric acid, α-ketobutyric acid, glycyl-L-glutamic acid, putrescine). For the tillering and maturity stage soils, the use of six substrates was shown to be high. Of the substrates, D-mannitol and L-asparagine were found to have high substrate utilization for all of seedling, tillering, and maturity stage soils.

The substrate utilization of RS526 and Dongjin rice soil microbial community for different time periods was comparatively analyzed by AWCD for each well (Fig. 3). The AWCD value for the seedling stage soil showed similar substrate utilization (based on final substrate utilization measure date on the 9th) between RS526 and Dongjin rice, the tillering and maturity stage soils also displaying similar results (Fig. 3A, B, C). These results show that the RS526 rice cultivation does not affect the substrates used by microorganisms. It has been reported that the bacteria present in rhizosphere soil of transgenic alfalfa with malate dehydrogenase transgene to show significantly more substrates utilization via AWCD, catabolic richness and evenness compared to non-GM alfalfa, showing a more

Table 3 Substrate utilization after 5 days in soil samples

Substrate	Jun		Aug		Oct	
	DJ	RS526	DJ	RS526	DJ	RS526
Pryuvic Acid Methyl Ester	5	5	4	4	4	4
Tween 40	5	6	5	5	6	6
Tween 80	5	5	5	5	5	5
α-Cyclodextrin	6	5	6	6	6	6
Glycogen	5	6	5	5	6	6
D-Cellobiose	5	6	6	6	5	6
α-D-Lactose	5	5	5	5	5	6
β-Methyl-Dglucoside	6	6	5	4	3	4
D-Xylose	7	5	6	6	4	5
i-Erythritol	5	5	3	3	3	4
D-Mannitol	6	6	6	6	6	5
N-Acetyl-D-Glucosamine	5	6	5	4	4	5
D-Glucoaminic Acid	5	5	4	5	5	4
Glucose-1-Phophate	5	4	4	3	2	3
D,L-α-Glycerol Phosphate	3	3	2	2	2	3
D-Galactonic Acid γ-Lactone	4	5	5	5	3	4
D-Galacturonic Acid	5	6	5	5	5	5
2-Hydroxy Benzonic Acid	3	1	4	2	2	1
4-Hydroxy Benzonic Acid	6	6	5	5	4	5
γ-Hydroxybutyric Acid	2	3	4	4	1	2
Itaconic Acid	5	6	5	6	4	5
α-Ketobutyric Acid	3	5	4	3	2	2
D-Malic Acid	4	5	4	4	4	4
L-Argenine	5	5	5	5	4	3
L-Asparagine	6	6	6	6	5	6
L-Phenylalanine	6	6	4	4	4	6
L-Serine	6	6	5	5	5	5
L-Threonine	6	6	4	3	2	2
Glycyl-L-Glutamic Acid	3	5	4	4	4	4
Phenlylethyl-amine	4	6	5	4	3	4
Putrescine	3	4	4	4	3	4

*OD<0.5 | 1 | 0.5<OD<1.0 | 2 | 0.5<OD<1.0 | 3 | 1.5<OD<2.0 | 4 | 2.0<OD<2.5 | 5 | 2.5<OD<3.0 | 6 | 3.0<OD | 7

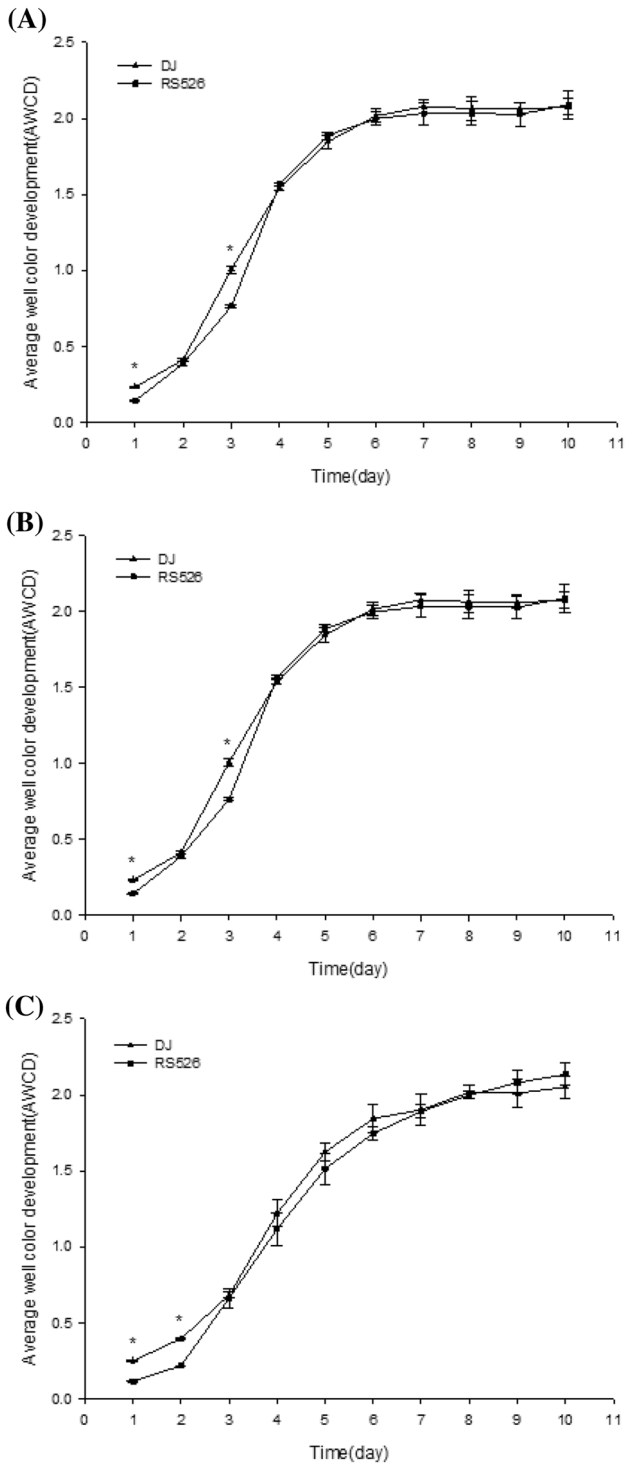

Fig. 3 Comparative analysis of average well color development (AWCD) between DJ and RS526 soil. (**A**) Seedling stage (**B**) tillering stage and (**C**) maturity stage soil. *Values* represent mean and standard deviation of the AWCD of three replicates. The *asterisk* denotes a significant difference (*t* test, $p < 0.05$)

diverse functionality (Tesfayeam et al. 2003). However, it has been shown to have no difference on bacterial population that can be grown on rhizosphere soil. In the case of *Bt* rice, it has been shown that there is no significant difference between the two soils (Mulder et al. 2006; Wei et al. 2012).

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