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ORIGINAL PAPER

Rhizoremediation of diesel-contaminated soil using the plant growth-promoting rhizobacterium *Gordonia* sp. S2RP-17

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Abstract A plant growth-promoting rhizobacterium (PGPR) was isolated and identified as *Gordonia* sp. S2RP-17, which showed ACC deaminase and siderophore synthesizing activities. Its maximum specific growth rate was 0.54 ± 0.12 d^{-1} at 5,000 mg L^{-1} of total petroleum hydrocarbon (TPH), and its maximum diesel degradation rate was 2,434.0 ± 124.4 mg L^{-1} d^{-1} at 20,000 mg L^{-1} of TPH. The growth of *Zea mays* was significantly promoted by the inoculation of *Gordonia* sp. S2RP-17 in the diesel-contaminated soil. Measured TPH removal efficiencies by various means were 13% by natural attenuation, 84.5% by planting *Zea mays*, and 95.8% by the combination of *Zea mays* and *Gordonia* sp. S2RP-17. The S2RP-17 cell counts were maintained at 1 × 10^6 CFU g-soil^{-1} during the remediation period, although they slightly decreased from their initial numbers (2.94 × 10^7 CFU g-soil^{-1}). These results indicate that rhizoremediation using both *Zea mays* and *Gordonia* sp. S2RP-17 is a promising strategy for enhancing remediation efficiency of diesel-contaminated soils.

Keywords Rhizoremediation · Plant growth-promoting rhizobacterium · Diesel-contaminated soil · *Gordonia* sp. · *Zea mays*

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Introduction

Total petroleum hydrocarbon (TPH) is a persistent environmental organic contaminant (Huang et al. 2005). Soil contamination with TPH is caused by the extraction, transportation, refining, and consumption of petroleum (Mcnicoll and Baweja 1995). Remediation of TPH-contaminated soil is generally a slow and expensive process (Huang et al. 2005; Macek et al. 2000). Rhizoremediation, employing the interaction of plant roots and rhizobacteria, has been investigated because it is an environmentally-friendly and cost-effective technology for the remediation of contaminated soils (Gerhardt et al. 2009). Rhizoremediation is especially suitable for soils contaminated with TPH in a low concentration range TPH (Zhuang et al. 2007). The key mechanism of

rhizoremediation is the rhizospheric effect enhancing biodegradation (Gerhardt et al. 2009; Newman and Reynolds 2004).

Rhizobacteria play important roles in rhizoremediation, and can be classified into deleterious rhizobacteria (DRB) and plant growth-promoting rhizobacteria (PGPR) (Nehl et al. 1996; Johnson et al. 2005). PGPR can colonize near growing roots, and grow using root exudates as carbon sources. Some rhizobacteria can protect plants from pathogenic bacteria by making antibiotics, provide nitrogen to plants by fixing N_2 in soil air, produce plant-growth-promoting hormones, and solubilize minerals such as phosphorus (Zhuang et al. 2007; Johnson et al. 2005; Koo and Cho 2009). In addition, rhizobacteria improve the tolerance of plants against contaminants such TPH, heavy metals, etc., and promote significantly the accumulation of contaminants into plants (Huang et al. 2005). A variety of plants have been reported as having been used for remediation of diesel-contaminated soils: Grass (*Lolium multiflorum lam*) (Palmroth et al. 2002; Liste and Felgentreu 2006), legume (*leguminosae*) (Palmroth et al. 2002; Johnson et al. 2004), poplar (Palmroth et al. 2002; Tesar et al. 2002), ryegrass (*Lolium multiflorum*) (Johnson et al. 2004; Etsuko et al. 2006), tall fescue (*Festuca arundinacea*) (Huang et al. 2005), alfalfa (*Medicargo sativa* L) (Kim et al. 2006), barnyard grass (*Echinochloa crusgalli*) (Kim et al. 2007) and maize (*Zea mays*) (Liste and Felgentreu 2006; Toshitomi and Shann 2001). In particular, maize has higher tolerance to diesel than ryegrass (Liste and Felgentreu 2006), and can produce excellent root exudation, one of the most important factors in successful rhizoremediation (Toshitomi and Shann 2001).

The growth of plants is inhibited in soils contaminated with high-strength diesel, which hinders the efficiency of remediation using plants alone. One of promising methods for enhancing plant growth in diesel-contaminated soils is the use of rhizobacteria having diesel-degrading ability as well as plant growth-promoting ability. Therefore, a new diesel-degrading PGPR, *Gordonia* sp. S2RP-17, was isolated and characterized in this study. In addition, the inoculation effects of *Gordonia* sp. S2RP-17 on the growth of plants (maize) and remediation efficiency were evaluated in contaminated soil with diesel (10,000 mg-TPH kg-soil^{-1}).

Materials and methods

Isolation, identification and characterization of a diesel-degrading rhizobacterium

Field horsetail (*Equisetum arvense* L.) grown in oil-contaminated soil at a petroleum refinery facility in South Korea was carefully sampled. The soil adhering loosely to the root was removed by shaking the plant, and the soil firmly adhering to the root was collected by brushing as a rhizosphere soil sample. After washing the root with distilled water (DW) several times, the washed root was dried at room temperature for 1 day, cut, and ground with a mortar to obtain a rhizoplane sample. One-g-fresh weight of the rhizosphere soil or ground rhizoplane sample was added into a 100 mL flask with 9 mL of sterilized DW, and then the flask was shaken at 250 rpm for 30 min. The resulting suspension solution was decimally diluted ($10^0 \sim 10^{-6}$) with sterilized DW. The dilutions (100 μL) were spread on the LB-agar medium (Difco), and then the plates were incubated at 30°C for 3 days. After cultivation, 28 colonies from the plates inoculated with the rhizosphere soil and 18 colonies from the rhizoplane were screened on the basis of their morphological properties. The plant growth-promoting activities (PGPA) of the screened colonies were investigated using a DF-salts minimal medium supplemented with 3 mM 1-aminocyclopropane-1-carboxylic acid (ACC) for an ACC deaminase activity test (Dell'Amico et al. 2005), and a chrome azurol S blue agar plate for siderophore(s) synthesis ability test (Schwyn and Neilands 1987). In order to evaluate the diesel-utilizing ability of the screened colonies, they were inoculated into 5 mL of BH medium supplemented with 10,000 mg L^{-1} of diesel as a sole carbon source, and then their growth was monitored after aerobically incubating for 1 week at 30°C, 180 rpm. The composition of the BH medium was 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.05 g $FeCl_3$, and 0.3 g Cholesterol per 1 L (pH 7.0–7.2). A bacterium having ACC deaminase activity and siderophore(s) synthesis ability as well as diesel-utilizing ability was selected, and named S2RP-17. For the identification of the isolate, its 16S rDNA was partially sequenced in the same manner as described previously (Koo and Cho 2009). All experiments were repeated at least twice and analyzed triplicate.

Diesel degradation by S2R17 was characterized by liquid shaking cultures, which used 5 mL of BH medium with 100 μL of S2R17 culture broth (final cell concentration: $OD_{600nm} = 0.2$) in a 18 mL-screw type test tube. The diesel concentrations were 5,000, 10,000, 20,000, 30,000 and 40,000 mg L^{-1}, and culture temperature was 30°C. Its diesel degrading abilities were also measured at the different pHs (5.0, 6.3, 7.1, and 7.9) and temperature (15, 25, 30, 37, and 42°C) (Hong et al. 2005). The remaining diesel concentrations were estimated by the amounts of total petroleum hydrocarbons (TPHs) extracted by hexane measured by gas chromatograph (5890 series II, Hewlett Packard, USA) (Pepper et al. 1995).

Effect of the isolate S2RP-17 on the growth of *Zea mays* and diesel removal

Soil was sampled in a forest area of Ewha Womans University, Seoul, South Korea. The soil was sieved using a 2-mm sieve, and mixed with sand in a 2:1 (w/w) ratio, and further with fertilizer in a 3:2 (w/w) ratio. The fertilizer was comprised of 25% pig excrement, 35% chicken excrement, 5% cattle excrement, and 25% sawdust, and the ratio of carbon to nitrogen was less than 50. Some of the soil mixed with fertilizer was also mixed with diesel (10,000 mg kg-$soil^{-1}$) and kept in a cool place for 4 days with thorough mixing once a day between the top and bottom.

After S2RP-17 was cultivated in 8 L of LB medium (Difco, USA) for 3 days at 30°C, the culture solution was centrifuged at 10,000 rpm for 10 min to harvest cells to be re-suspended with sterile water and centrifuged in the same way. After repeating this step two more times to the wash cells, the cells were suspended in 700 mL of sterile water. The strain suspension was injected into 70 kg of the diesel-contaminated soil and mixed thoroughly.

The effects of the inoculation of *Gordonia* sp. S2RP-17 on the growth of *Zea mays* and on diesel removal in diesel-contaminated soil were studied using mesocosm systems. Each mesocosm container was made of a PVC cylindrical column of 300 mm in diameter and 550 mm in height (Fig. 1). For irrigation, the three layers consisted of a sand layer (50 mm), gravel layer (50 mm), and empty space (50 mm) below them. About 14 kg of raw soil not amended with fertilizer, diesel, or S2RP-17 was added to the bottom level, and then 17.5 kg soil mixed with fertilizer was added to the middle level. About 17.5 kg of the diesel-contaminated soil inoculated with S2RP-17 prepared as mentioned above was added to the top level. A 2 week-old maize plant (*Zea mays*) with an average stem length of approx. 30 cm was planted. For the control test planted without S2RP-17, 17.5 kg of the diesel-contaminated soil without S2RP-17 was added to the top level, and then planting was done in the same manner as described above. In another control test without plants and S2RP-17, 17.5 kg of the diesel-contaminated soil was added to the top level. All tests were conducted in four replicates.

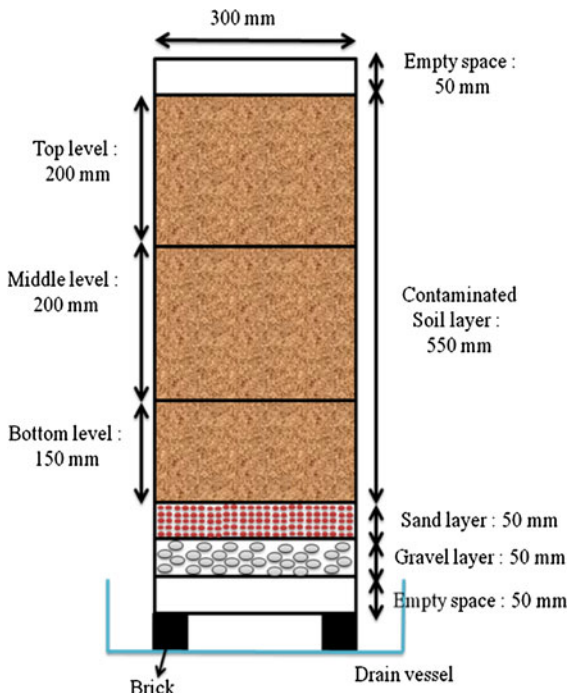

Fig. 1 Schematic diagram of a soil mesocosm

The period of the experiment was 46 days, and all mesocosms were placed in a greenhouse at temperatures ranging from 20 to 35°C, and were watered once a day for the first 20 days and once every 2 days for the remaining 25 days.

Analysis of plant growth, microbial activity and TPH concentration

While cultivating, plant growth was estimated by measuring the stem length, leaf length and leaf

numbers. On the 46th day, eight plants were carefully sampled from the mesocosms, and the plant samples were then oven-dried at 70°C for 3 days, and were divided into the shoots and roots whose biomasses would be measured. Soil samples from the top and middle levels of each mesocosm were collected and mixed well, and microbial activities were assayed using a dehydrogenase activity method (Pepper et al. 1995). To measure the TPH concentration, 5 g of the soil sample was mixed with hexane–acetone solution (1:1, v/v) in a test tube and shaken for 30 min at 30°C and 200 rpm in a shaker, and the test tubes were deposited for 30 min before analysis. TPH analysis was done using gas chromatography (5890 series II, Hewlett Packard, USA) (Ryu et al. 2006). Finally, measurement data were analyzed statistically with ANOVA and two sample *t*-tests using SPSS 14.0 (SPSS INC., Korea).

Quantification of strain S2RP-17 in soil

To design the TaqMan probe and primers, 16S rDNA of strain S2RP-17 was subjected to PCR using 27f (AGA GTT TGA TCM TGG CTC AG; M = C:A) and 1492r (TAC GGY TAC CTT GTT ACG ACTT; Y = C:T) primers (Martin-Laurent et al. 2001). PCR conditions and reagents used have been described previously (Ryu et al. 2006). The amplified 16S rDNA was purified using a PCR purification kit (QIAGEN GmbH, Hilden, Germany), and then sequenced (Ryu et al. 2006). Identified sequences were analyzed for similarities by using the Basic Local Alignment Search Tool (BLAST), a network service at Genbank (Hristova et al. 2001). Based on the National Center for Biotechnology and Information (NCBI) Blast search results, the most unique partial sequences of S2RP-17 were identified, and then the novel TaqMan probe and primers for S2RP-17 were designed. The TaqMan primers were 5′-CCATGCGAGTATCAGTCATA-3′ for the forward primer and 5′-CCTGGGAAACTGGGTCTAA TACC-3′ for the reverse primer. The probe sequence was 5′-TATGACTGATACTCGCATGG-3′ and was 5′ labeled with FAM (6-carboxyfluorescein).

The purified genomic DNA samples were obtained from the soil sampled from the top level using the Fast DNA spin kit (Q-BIOgene, USA), and then qRT-PCR was performed. qRT-PCR (Applied Biosystems 7300 real time PCR system, Applied Biosystems, USA) was performed using 25 μL samples in the MicroAmp Optical 96-well reaction plate and MicroAmp Optical Caps (Applied Biosystems, USA). PCR conditions and data analysis were conducted by the same methods described in the previous study (Lee et al. 2009). After qRT-PCR, the cycle threshold (C_t) values were converted into DNA concentration and S2RP-17 CFUs (colony forming unit) using a standard curve. All experiments were performed in triplicate.

Results and discussion

Isolation, identification and diesel degradation activity of a diesel-degrading rhizobacterium

The rhizobacterium S2RP-17 was isolated from the rhizoplane of *Equisetum arvense* (field horsetail) that had inhabited diesel-contaminated soil for a long period, and verified to have ACC deaminase activity and siderophore(s) synthesizing ability (data not shown). The ACC deaminase can degrade ACC, a precursor of ethylene produced by plant stress (Glick 2003), and the siderophore(s) captures irons present in soil (Koo and Cho 2009). Based on the 16S-rDNA partial sequences, S2RP-17 was identified as *Gordonia* sp. (accession number, GRP1815786).

The effects of diesel concentrations, pHs, and temperatures on diesel degradation rates by S2RP-17 are shown in Fig. 2. The diesel concentrations were equivalent to 5,000, 10,000, 20,000, 30,000 and 40,000 mg L^{-1} in TPH. Among them, the best growth occurred at 5,000 mg L^{-1} with 0.54 ± 0.12 d^{-1} (specific growth rate), but the maximum degradation rate was shown at 20,000 mg L^{-1} with 2434.0 ± 124.4 mg L^{-1} d^{-1} (TPH degradation rate; Fig. 2a). Even though TPH removal rate increased with increasing TPH concentration in the ranges of 5,000 to 20,000 mg L^{-1}, TPH removal rate as well as the specific growth rate of S2RP-17 decreased at high TPH concentration due to TPH toxicity.

To determine the influence of pH on the relative specific growth rates and degradation rates of S2RP-17, we measured them at the different pHs of 5.0, 6.3, 7.1, and 7.9 (Fig. 2b). Because the specific growth rate and the degradation rate of S2RP-17 were highest at pH 7.1, these two rates at this pH were defined as a

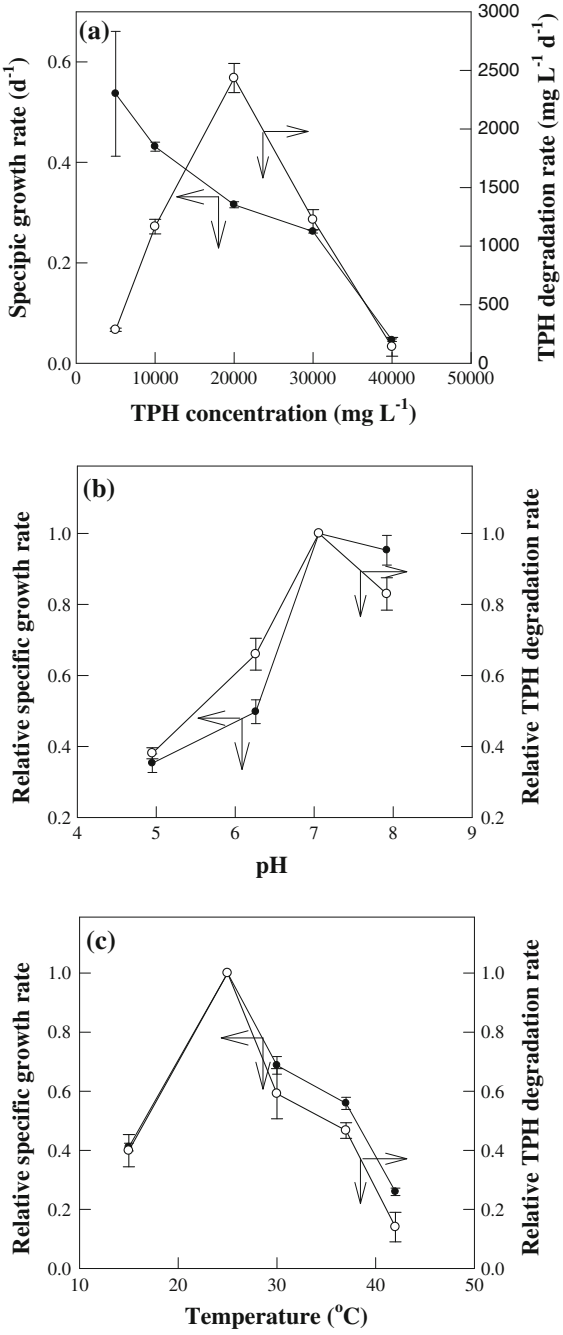

Fig. 2 Effects of diesel concentration (**a**), pH (**b**), and temperature (**c**) on the diesel degradation and specific growth rates of S2RP-17. *Open circle* diesel degradation rate, *filled circle* specific growth rate

default value (= 1), and the other rates were obtained by relative comparison to that value (Fig. 2). The relative specific growth rates and degradation rates of S2RP-17 were distributed in the following order; pH $5.0 < 6.3 < 7.9 < 7.1$.

The influence of temperature on the biodegradation of hydrocarbons was also determined by measuring the relative values of the specific growth rates and diesel degradation rates of the S2RP-17 strain at 15, 25, 30, 37, and 42°C (Fig. 2c). The specific growth rate as well as the diesel degradation rate of the S2RP-17 was the highest at 25°C .

Several species in the genus *Gordonia* have been known as petroleum-based hydrocarbon degraders, such as *Gordonia* sp. He4 isolated from phenanthrene-contaminated soil (Liu et al. 2007), and *Gordonia* spp. M22, BS25 and BS29 isolated from long-term diesel-contaminated soil (Franzetti et al. 2008). Joo et al. (2001) reported that the diesel degradation rate by *Pseudomonas stutzeri* Y2G1 in a liquid culture was 5,640 mg L^{-1} d^{-1}, 2.3 times that of S2RP-17 (2,434 mg L^{-1} d^{-1}). The diesel degradation rate (400 mg L^{-1} d^{-1}) of *Pseudomonas aeruginosa* IU 5 is lower than that of S2RP-17 (Hong et al. 2005).

The optimal values of pH and temperature for the diesel degradation rate as well as growth rate of S2RP-17 were 7.1 and 25°C, respectively. *Pseudomonas aeruginosa* WatG showed bacterial growth and optimal petroleum hydrocarbon removal at pH 6.5 (Wongsa et al. 2004), and *Pseudomonas aeruginosa* IU 5 showed maximum growth at pH 7.5 and 30°C (Hong et al. 2005). Rahman et al. (2002) demonstrated the optimal culture conditions for a crude-oil-degrading consortium to be pH 7.5 and 30°C. *Gordonia* sp. S2RP-17 displayed a maximum activity of growth and diesel degradation at a lower pH and temperature than these.

Inoculation effects of *Gordonia* sp. S2RP-17 on plant growth and TPH removal in diesel-contaminated soil

A study was performed to examine remediation efficiencies in soil contaminated with 10,000 mg kg-soil^{-1}, including the inoculation effect of S2RP-17 on the growth of *Zea mays*. Figure 3 shows that the stem of *Zea mays* grew much faster in soil with S2RP-17 than without it during the period of growth (46 days). They were 163.6 ± 2.1 cm with S2RP-17, and 139 ± 4.2 cm without S2RP-17, respectively, at 46 days (Fig. 3a). The effect of the inoculation of

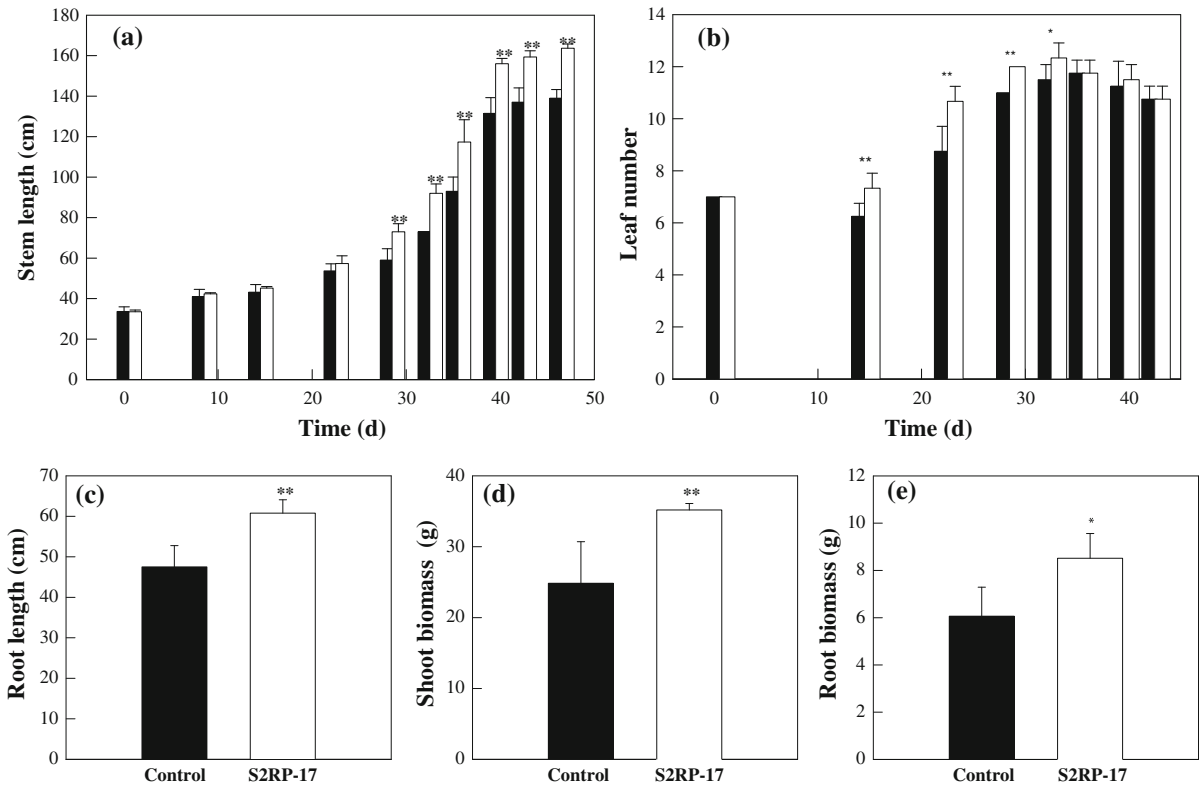

Fig. 3 Effects of the inoculation of *Gordonia* sp. S2RP-17 on the growth of *Zea mays* in soil mesocosms. **a** Stem length, **b** leaf numbers, **c** root length, **d** shoot biomass, and **e** root biomass. *Filled square* control (without S2RP-17), *open square* with S2RP-17; * signifies statistically significant differences from control (*P value < 0.1; **P value < 0.05)

S2RP-17 on the leaf length of *Zea mays* was also studied. The leaf length was slightly longer with S2RP-17 inoculation than without it, although not significantly different (data not shown). The leaf numbers were greater in the S2RP-17-inoculated soil after 14 days, and this trend continued until 35 days. From the 39th day, however, no further significant differences were observed between them (Fig. 3b). At harvesting time (46 days), the lengths and biomasses of roots and biomasses of shoots (Fig. 3c–e) were measured. The results showed that the inoculation of S2RP-17 promoted the growth of roots; the average main root length of *Zea may* was 47.5 ± 5.2 cm in the control system (without S2RP-17), and 60.8 ± 3.3 cm in the S2RP-17 inoculated system. The same result was indicated by shoot biomass measurements: 24.8 ± 5.9 g without S2RP-17 and 35.2 ± 0.9 g with S2RP-17, and also apparently by root biomass: 8.5 ± 1.1 g with S2RP-17 and 6.1 ± 1.2 g without S2RP-17. Therefore, the inoculation of S2RP-17 stimulated to increase both the shoot and root growth of *Zea mays*.

As one of its plant-growth-promoting abilities, the isolate S2RP-17 has an ACC deaminase activity that degrades ACC (1-aminocyclopropane-1-carboxylic acid), a precursor of ethylene that is a secondary metabolite caused by plant stress such as contaminants (Deikman 1997). It is also considered to prevent root growth, resulting in limiting phytoremediation effects (Arshad et al. 2007). S2RP-17 can also synthesize siderophore(s), which help to capture irons that are present in small amounts in soil but are indispensable to plant metabolism. We showed that S2RP-17 is a plant growth-promoting rhizobacterium, through a mesocosm experiment that resulted in S2RP-17's promotion of stem, leaf, and root growth (Fig. 3).

The effect of *Zea mays* and/or S2RP-17 on diesel removal is shown in Fig. 4. The residual TPH concentration was $9{,}807.3 \pm 2{,}768.1$ mg-TPH kg-soil^{-1} in

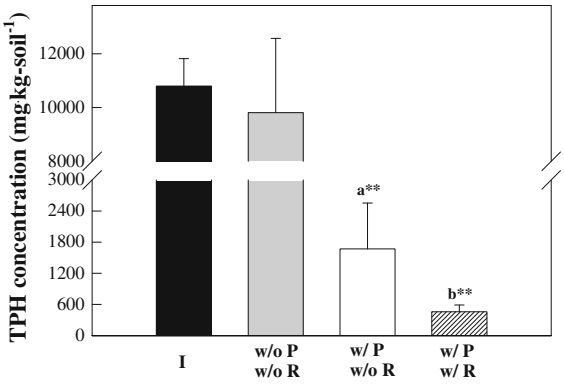

Fig. 4 Effects of *Zea mays* and *Gordonia* sp. S2RP-17 on diesel removal in soil mesocosms. *I* Initial, *w/o P* without plant, *w/o R* without S2RP-17, *w/P* with plant, *w/R* with S2RP-17; * means the statistically significant differences from control (**P value < 0.05)

control columns without *Zea mays* and S2RP-17, showing a natural attenuation of approximately 13%. However, the residual TPH concentration in the top level of planted and inoculated systems was 458.7 ± 131.2 mg-TPH kg-soil^{-1}, whereas that in the control system without S2RP-17 was 1,670.2 ± 884.0 mg-TPH kg-soil^{-1}. This result indicates that diesel removal can be enhanced by the inoculation of soil with S2RP-17.

Table 1 shows the dehydrogenase activity and cell numbers of S2RP-17 in diesel-contaminated soils of mesocosm systems. In the control system without *Zea mays* and S2RP-17, the dehydrogenase activity after 46 days was similar to that at the first day (125.4 ± 1.1 μg-TPF g^{-1} d^{-1}). However, the dehydrogenase activities increased to 397.7 ± 39.7 in soils amended with *Zea mays,* and 478.4 ± 84.0 μg-TPF g^{-1} d^{-1} in soils amended with *Zea mays* and S2RP-17 simultaneously. The cell numbers of S2RP-17 after 46 days could be maintained 1×10^6 CFU g-soil^{-1}, although they slightly decreased from their initial numbers (2.94×10^7 CFU g-soil^{-1}).

In addition, triphenylformazan (TPF) values (dehydrogenase activity) of S2RP-17's correlated exactly to cell numbers and diesel removal efficiencies, as shown in Table 1 and Fig. 4. Based on such reliable data, we may conclude that the isolate (S2RP-17), a plant growth-promoting bacterium, enhanced development of roots as well as other parts of plants, which in turn promoted more microbial activity. A similar research result presented that *R. leguminosarum* bv. *Trifolii* degrading PAH promoted the growth of clover (*Trifolium repens* L.), and italian ryegrass (*Lolium multiflorum* L.), and stimulated the growth and activity of other microorganisms around the rhizosphere as its roots developed (Johnson et al. 2004). Other researchers have also reported similar results (Gunther et al. 1996; Siciliano and Germida 1997).

According to this study, TPH removal efficiency, as well as plant growth, was improved by the presence of diesel-degrading rhizobacteria. There have been many research cases in diesel-contaminated soil in which removal efficiency increased due to inocula promoting plant growth. One example is *Enterobacter cloacae* UW4 and CAL2, which induced the growth of tall fescue (*Festuca arundinacea*), further enhancing TPH degradation (Huang et al. 2005). Also, a microbial consortium was shown to stimulate the growth of erect Milkvetch (*Astragalus adsurgens* Pall), and increase the removal of diesel fuel (Lin et al. 2008). Endophytic bacteria can also help plants to overcome contaminant-induced stress responses, leading plant growth and more efficient degradation (Weyens et al. 2009). Recently, studies by Gerhardt et al. (2009) and Golubev et al. (2009) have also supported this fact.

To overcome the disadvantage of phyoremediation in which the growth of plants is inhibited by contaminants, rhizoremediation employing plants and rhizobacteria simultaneously has been recently

Table 1 Comparison of dehydrogenase activities and cell numbers of *Gordonia* sp. S2RP-17 in soil mesocosms

Samples	Dehydrogenase activities (μg-TPF g^{-1} d^{-1})	Cell numbers of S2RP-17 (CFU g-soil^{-1})
Initial soil w/S2RP-17 (just before starting)	125.4 ± 1.1	2.94×10^7
Soils w/o *Zea mays*, w/o S2RP-17 after 46 days	131.6 ± 2.5	ND
Soils w/*Zea mays*, w/o S2RP-17 after 46 days	397.7 ± 39.7	ND
Soils w/*Zea mays*, w/S2RP-17 after 46 days	478.4 ± 84.0	1×10^6

TPF triphenylformazan, *w/o* without, *w/* with, *ND* not determined

suggested as a promising strategy. In this study, a new rhizobacteria displaying diesel-degrading and plant growth-promoting abilities was successfully isolated and identified as *Gordonia* sp. S2RP-17. Through soil mesocosm tests, it was shown that the inoculation of this isolate can enhance remediation efficiency as well as promote the growth of *Zea mays* in diesel-contaminated soil. The synergistic effects of *Gordonia* sp. S2RP17 and *Zea mays* should therefore lead to more successful remediation of diesel-contaminated soils in the field.

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