Spanish Journal of Agricultural Research
14(2), e0803, 13 pages (2016)
eISSN: 2171-9292
http://dx.doi.org/10.5424/sjar/2016142-6848
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Effects of rhizobacteria on the respiration and growth of *Cerasus sachalinensis* Kom. seedlings

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Abstract

In this study, we investigated the influence of rhizosphere microorganisms on seed germination and root metabolism in *Cerasus sachalinensis* Kom. We inoculated *C. sachalinensis* plants with suspensions of dominant bacterial strains isolated from their rhizosphere. Four bacterial strains each with significant growth-promoting or growth-inhibiting effects were screened from the efficient root-colonizing microorganisms. The number of actinomycetes increased and that of fungi decreased significantly in the seedling rhizospheres after rhizobacteria treatment. The growth-promoting bacteria slightly affected the respiration rates and respiratory pathway enzymes, but significantly improved root viability, root carbohydrate concentration and seedling growth. *Bacillus cereus*, *Staphylococcus* sp. and *Pseudomonas fluorescens* were identified as the growth-promoting rhizobacteria; one strain could not be identified. After inoculation with the growth-inhibiting bacteria, the number of fungal colonies in the seedling rhizospheres increased and root viability and respiration rate as well as starch and sucrose accumulation in the roots significantly decreased. The glycolysis, pentose phosphate and alternative oxidase pathways became the major pathways of respiratory metabolism after inoculation with the growth-inhibiting bacteria. The height, leaf number, growth and dry weight of the seedlings decreased significantly in plants inoculated with the growth-inhibiting bacteria. Inoculation of *C. sachalinensis* rhizosphere with growth-promoting and growth-inhibiting bacteria affected the soil environmental factors such as microbial group composition, nutrient concentration and seedling biomass.

Additional key words: rhizosphere microorganisms; seed germination; respiratory pathways; key respiratory enzymes; root.

Abbreviations used: AOX (alternative oxidase pathway); COX (cytochrome oxidase pathway); EMP (glycolysis); FW (fresh weight); G-6-PDH (glucose-6-phosphate dehydrogenase); 6-GPDH (6-phosphate glucose dehydrogenase); HK (hexokinase); IAA (indole-3-acetic acid); IDH (isocitrate dehydrogenase); MDH (malate dehydrogenase); PCR (polymerase chain reaction); PFK (phosphofructokinase); PGPR (plant growth-promoting rhizobacteria); PK (pyruvate kinase); PPP (pentose phosphate pathway); SDH (succinodehydrogenase); Str. (bacterial strains); TCA (tricarboxylic acid cycle); TTC (triphenyl tetrazolium chloride).

Authors' contributions: Conceived and designed the experiments: DGL, SJQ, WJZ and ZXL. Performed the experiments and analyzed the data: SJQ, WJZ and ZXL. Contributed reagents, materials and obtaining funding: DGL and SJQ. Wrote the paper: SJQ, WJZ and ZXL. Critical revision of the manuscript for important intellectual content: SJQ and WJZ.

Citation: Qin, S. J.; Zhou, W. J.; Li, Z. X.; Lyu, D. G. (2016). Effects of rhizobacteria on the respiration and growth of *Cerasus sachalinensis* Kom. seedlings. Spanish Journal of Agricultural Research, Volume 14, Issue 2, e0803. http://dx.doi.org/10.5424/sjar/2016142-6848.

Received: 18 Sept 2014. **Accepted**: 28 Mar 2016

Funding: The National Natural Science Foundation of China (30871688, 30900967, 31572077); Specialized Research Fund for the Doctoral Program of Higher Education (20122103110012); Program for Higher Educational Excellent Talents of Liaoning Province (LJQ2014070).

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Modification of the plant root system is considered as a means for crop improvement in environments with marginal soils, particularly, low-nutrient and drought-prone agricultural areas (MacMillan *et al.,* 2006). However, many studies investigating root development and physiology have not considered the effects of the rhizosphere (Bakker *et al.,* 2013; Philippot *et al.,* 2013). Rhizosphere microorganisms are an essential part of the soil-plant ecological system; their community structure and activity are affected by soil properties and plant physiological processes, such as root secretions and soil organic matter (Picard & Bosco, 2008; Bulgarelli *et al*., 2013). Feedback from the microorganisms in the rhizosphere helps in regulating root function (Wasaki *et al*., 2005; Allison *et al*., 2008), influences plant growth and development by producing indole-

3-acetic acid (IAA; Zak *et al*., 2003), alters the nutritional status of the rhizosphere (Carvalhais *et al*., 2013) and can induce disease resistance (Annapurna *et al*., 2013). Various microbial species are present in the soil, but only few of them have certain advantages and significantly impact on plant growth (Kamal *et al*., 2015). Studies on the biological control of deleterious rhizosphere microorganisms, identification of plant growth-promoting rhizobacteria (PGPR) and beneficial and harmful effects of microorganisms on plants have received considerable attention in recent years (Dutta *et al*., 2008; Zhou *et al*., 2015). Recent studies have mainly investigated the biological relationships between plants and bacteria in the root zone; these relationships can have a positive or negative influence on plant growth (Dary *et al*., 2010). Elucidating the mechanisms underlying the effects of microorganisms on soil ecology and microbial regulation of plant growth and development are necessary for maximizing soil biological potential and improving plant-production capacity.

Root respiration is the metabolic center of below-ground plant growth; it involves the production of both root-borne substances and energy. The status of root respiration is an important indicator of root function under different environmental stress conditions. For example, continuous drought and waterlogging decrease glycolysis (EMP) and the activity of the cytochrome oxidase pathway (COX), and simultaneously increase the activities of the pentose phosphate pathway (PPP) and alternative oxidase pathway (AOX) in *Cerasus sachalinensis* Kom. roots (Qin *et al*., 2011). The beneficial effects of microbe–plant interactions have been exploited in sweet cherry (*Prunus avium* L.) and apple (*Malus domestica* L.) to enhance root growth and nutrient uptake (Esitken *et al*., 2006; Karlidag *et al*., 2007). Root length of canola (*Brassica campestris*) was significantly increased when inoculated with endophytic bacterial strains selected from date palm (*Phoenix dactylifera* L.; Yaish *et al*., 2015); this confirmed the close relationship between root function and rhizosphere microbial community. Further, the stem wood production of Norway spruce (*Picea abies* L.) was correlated with soil microbial community composition that represents the mole percentage of fungi, bacteria and actinobacteria (Blaško *et al*., 2013). However, the direct influence of rhizosphere microorganisms on root respiratory metabolism has rarely been reported. *C. sachalinensis*, which is native to northeastern China and northern Korea, is widely used as a sweet cherry rootstock in cold regions (*e.g.*, Dalian and Qinhuangdao) because of its high cold tolerance. In 2011, the planting area of sweet cherry in China was approximately 134,000 ha; in one-fourth of this area, *C. sachalinensis* was used as a rootstock. Nevertheless, long-term production of sweet cherry has shown that the root function of sweet cherry plants grafted onto *C. sachalinensis* declines rapidly when the trees bear fruit, leading to the premature loss of tree vigor. Lü *et al*. (2008) reported that the bacterial populations in the *C. sachalinensis* rhizosphere were significantly different from those of Mahaleb (*Cerasus mahaleb* L.), Colt (*Cerasus avium* L. × *Cerasus pseudocerasus* Kom.), and *C. pseudocerasus*; *Pseudomonas*, *Flavobacterium* and *Bacillus* were found to be the major bacterial groups in the *C. sachalinensis* rhizosphere (Lü *et al*., 2008). A previous study showed that bacterial diversity and richness indices in the rhizosphere of cultivated cherry were lower than those in the rhizosphere of wild cherry, and the rhizobacteria caused crown gall disease in cultivated plants (Lü *et al*., 2011).

This study aimed to identify the physiological mechanism of how *C. sachalinensis* roots respond to potentially growth-promoting rhizobacteria. Growth-promoting and growth-inhibiting bacterial strains were isolated, purified and identified. The effects of feedbacks on the soil environment and root function were used to elucidate the relationships between microbiological properties and root functions in the *C. sachalinensis* rhizosphere. The biological mechanism of functional decline of cherry root under culture conditions was determined by analyzing root respiration and responses of the soil biological environment by using a seed germination and inoculation test. Further, a theoretical basis was developed for formulating scientific soil management practices and improving the rhizosphere environment and root function.

Material and methods

Isolation of rhizospheric bacteria and seed germination experiment

Rhizospheric bacteria were isolated and purified from the rhizosphere soil of wild *C. sachalinensis*; 37 predominant strains were screened and cryopreserved. The sampling site was a mountainous area in Lianshanguan town, Benxi City, Liaoning Province, China (41°24’ N, 124°17’ E), located at an altitude of 512-546 m asl, with a land slope of 20° to 45°. The climate in this area is classified as humid in the northern temperate zone. Annual precipitation is approximately 800-900 mm and average annual temperature is 6.1°C to 7.8°C. The length of the frost-free season is approximately 156–172 d. The area has predominantly Brunisolic soil.

Seed germination experiments were conducted at a constant temperature of 20°C in an incubator (LHS-

150HC-I; Shanghaiyiheng, China). These experiments were used for the pre-screening of growth-promoting and growth-inhibiting rhizospheric bacteria. For the preliminary screening of bacteria, seeds were treated with the suspension of the 37 bacterial strains previously isolated from the *C. sachalinensis* rhizosphere soil. Uniform-sized plump, stratified and fresh seeds of *C. sachalinensis* were used for the preliminary selection of efficient root-colonizing bacterial strains. Shelled *C. sachalinensis* seeds were disinfected with 0.5% potassium permanganate solution and placed into sterilized Petri dishes containing a filter paper. Twenty *C. sachalinensis* seeds and 6 mL bacterial suspension (prepared as described in below) that was diluted five times were placed into each Petri dish and cultivated at a constant temperature (20°C). This experiment was replicated three times. Next, 1 mL sterile water was added to each Petri dish, including the control treatment (which did not include the bacterial suspension), every alternate day to ensure seed germination. Germination percentage and seedling growth were determined after 7 d. Strains with evident germination promoting or inhibiting activities were selected.

Physiological effects of bacterial inoculations on *C. sachalinensis* seedlings

The *C. sachalinensis* seeds were sown on April 25 in a shelter house with an open roof at the Shenyang Agricultural University (41°83' N, 123°56' E). The elevation, averages of accumulated temperature, annual sunshine hours, annual frost-free period and mean annual precipitation were 76.2 m, 3281°C, 2372 h, 146–163 d and 721 mm, respectively. The growth conditions were not controlled. Seedlings were planted in 12 cm × 13 cm feeding blocks and grown for 3 months in the culture substrate consisting of garden soil with 20% turfy soil. The basic physical and chemical properties of the substrate were as follows: pH, 7.18; soil organic matter, 76.61 g/kg; alkaline-hydrolyzable nitrogen, 296.3 mg/kg; available phosphorus, 35.51 mg/kg; and potassium, 32.17 mg/kg. Uniform-sized seedlings were selected for treatment with bacterial suspension (50 mL/pot) and 10 replicate seedlings were used for each treatment. The mean plant height was 24.5 ± 2.8 cm before treatment. Seedlings treated with an equal amount of sterile water were used as the control. Inoculations were performed every 3 d for 30 d. Morphological changes in seedling growth and development and related indices of respiratory metabolism and rhizosphere environment were determined at the end of the 30-d treatment.

Microbial procedures

Microbial counts. A dilution plate count method was used for estimating the number of microorganisms (Johnson & Curl, 1972). Bacteria, fungi and actinomycetes were counted on peptone beef, Martin Substratum and Gause 1 cultural media, respectively. All materials for the cultural medium were purchased from Sigma Chemical Company (St Louis, MO, USA). The numbers of colonies per plate were determined by diluting the samples in deionized water to 10^{-4}, 10^{-5} and 10^{-6} g/mL, and 0.1 mL soil solution was added to each plate. The plates were incubated at 28°C for 1 d for bacteria, 3 d for fungi and 7 d for actinomycetes, and then the developing colonies were counted. Three plates were used for each treatment.

Acquisition of efficient root-colonizing bacteria. Efficient colonizers are defined as those strains that yield >5 colonies at the highest dilutions. In this experiment, soil was diluted to 1:1,000,000 with three replicates. Bacterial strains were cultured in peptone beef medium for 2–3 d, and a single developing colony was purified by culturing in peptone beef for 2–3 times. The purified bacteria were stored at 4°C.

Preparation of bacterial suspension. The obtained bacteria were cultured in peptone beef medium at 28°C for 24 h and then inoculated in liquid medium (peptone beef medium without agar) with shaking. The supernatant was collected after centrifugation and colorimetric analysis was performed at 600 nm by using a spectrophotometer (Agilent HP 8453; Agilent Technologies, Waldbronn, Germany). The OD_{600} was adjusted to 0.4 with sterilized water and this bacterial suspension contained approximately 10^8 CFU/mL (Peng *et al*., 2007).

Identification of bacterial strains by using 16S rDNA gene sequence analysis. Total DNA was extracted as previously described (Laguerre *et al*., 1992). Universal primers were used for polymerase chain reaction (PCR) amplification (forward primer, 8-27F: 5'-AGAGTTTGATCMTGGCTCAG-3'; reverse primer, 1378-1401R: 5'-CGGTGTGTACAAGGCCCGGGAACG-3'). PCR amplification was performed in a 50 µL reaction volume: 5 µL Taq DNA polymerase, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM each of forward and reverse primers, 40 ng template DNA, 5 U of Taq DNA polymerase and 32 µL ddH_2O. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (1 min at 95°C), annealing (30 s at 60°C) and extension (1.5 min at 72°C) and a final extension at 72°C for 10 min. Amplified DNA was examined by gel electrophoresis on a 1.0% agarose gel. PCR products were purified using the PCR extraction kit (DNA Fragment Purification Kit vers. 2.0; Takara) and sent to Takara Biotechnology Co. Ltd., Dalian, for sequencing. The sequenc-

ing results were blasted to GenBank (http://www.ncbi.nlm.nih.Gov) for sequence alignment in order to obtain the closest sequence homology. Criterion for identification at the genus level was defined as a 16S rDNA sequence similarity of ≥97% with that of the prototype strain sequence in GenBank.

Measurement of root parameters

Root viability. The triphenyl tetrazolium chloride (TTC) method (Lindström & Nyström, 1987) was used to determine root viability. TTC is reduced mainly by dehydrogenases, which are associated with mitochondrial function in plants (Bagniewska-Zadworna, 2008). Higher root viabilities suggest higher physiological activities of dehydrogenases in the roots. Briefly, 500 mg (diameter <2 mm) of fresh fine roots were placed into beakers containing 5 mL 0.4% TTC solution and 5 mL Na_2HPO_4-KH_2PO_4 (pH 7.0) and incubated for 2 h at 37°C in the dark. Subsequently, triphenylformazan was extracted with ethyl acetate and the absorbance was measured at 485 nm. Root viability was calculated as $C/(1{,}000 \cdot W \cdot h)$ [mg TTF/(g·h)], where C represents reduction of tetrazolium, W represents root weight and h is time (hours).

Total root respiration rates. Root respiration rate was measured as oxygen consumption by using an Oxytherm oxygen electrode (Hansatech, England) according to a modified method by Bouma *et al*. (2001). The total respiration rate was determined by slicing new root tissue samples (1.5 mm wide, 2–3-cm long, 50 mg) into 2-mm pieces and setting aside for 15 min to eliminate the influence of wound respiration. Next, 0.05 g of each sample was weighed for measurement and six replicates were incubated at 25°C ± 1°C. Total respiration rates were determined as root O_2 uptake per unit of fresh weight (μmol O_2/min/g FW) in the assay buffer without inhibitors.

Root respiratory pathways. Root respiratory pathways were measured according to Yu & Pan (1996). The capacities of EMP, tricarboxylic acid cycle (TCA) and PPP pathways were determined using 0.5 M NaF, malonic acid and Na_3PO_4 as inhibitors, respectively. The COX and AOX pathways were inhibited using 0.1 M NaCN and salicylhydroxamic acid, respectively. Phosphate buffer (0.2 M, pH 6.8) was used as the reaction medium. Each respiratory pathway capacity was determined three times. The percentage of each respiratory pathway was calculated as [(Total respiration rate–Residual respiration rate)/Total respiration rate]. Residual respiration rates represented respiration rates after the addition of the corresponding inhibitor.

Key root respiratory enzymes. Fresh root tissues (0.5 g) were pulverized in 3 mL extraction buffer (100 mM Tris-HC1, pH 7.5) by using a mortar and pestle chilled in ice. The mixture was centrifuged for 30 min at 10,000 *g* at 4°C and the supernatant was assayed immediately for 3 min in 3.0 mL reaction mixtures. The activities of hexokinase (HK), pyruvate kinase (PK) and phosphofructokinase (PFK) were measured using the methods of Samuelov *et al*. (1991), Sridhar *et al*. (2000) and Ling *et al*. (1966), respectively. The malate dehydrogenase (MDH) and succinodehydrogenase (SDH) activities were measured according to Ouyang (1985). The isocitrate dehydrogenase (IDH) activity was measured according to Plaut (1969). The glucose-6-phosphate dehydrogenase (G-6-PDH) and 6-phosphate glucose dehydrogenase (6-GPDH) activities were measured as described by Lamed & Zeikus (1980).

Root respiratory substrates and intermediate products. Concentrations of sucrose and starch, pyruvic acid and citric acid were determined using anthrone colorimetry (Hodge, 1962), 2,4-dinitrophenylhydrazine (Katsuki *et al*., 1961) and enzymatic reaction colorimetry, respectively.

Biomass and soil properties. Seed germination and seedling growth were determined using a ruler and balance. The concentration of soil organic matter, alkali-hydrolyzable N, available P and available K were measured according to Shi *et al*. (1996).

Statistical analyses

Figures were drawn in Microsoft Excel 2003. Statistical analyses were performed using Statgraphics (STN). The response variables considered were: seed germination percentage; seedling height; fresh weight of seeds; root viability; root respiratory rates; percentage of each root biochemical respiratory pathway; key enzyme activities of EMP pathway, TCA pathway and PPP pathway; percentage of each root electron transport respiratory pathway; concentration of sucrose and starch; concentration of pyruvate and citrate of *C. sachalinensis* seedlings; increases of seedling height and seedling leaves; fresh weights of shoots and roots; dry matter weights of shoots and roots; number of bacteria, fungi and actinomycetes; percentage of treated bacteria in the total bacteria; alkali-hydrolyzable N concentration; and available P and K and organic matter concentrations. As explanatory variable for each analysis of variance (ANOVA) we used the bacterial strains. According to normality, the data fulfilled the presumptions of ANOVA. Differences between means were considered significant

when the p value of the ANOVA F test was <0.05. All p values obtained from multiple comparisons were corrected using Tukey's highly significant difference (HSD) method. p values of the one-way ANOVAs of strains (B) are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Results

Selection and species identification of efficient root-colonizing bacteria

Of the 37 bacterial strains, 31 were found to influence seed germination, seedling height and fresh weight either positively or negatively compared to the control treatment (Fig. 1). Seed germination rate, seedling height and fresh weight were significantly different between the control and cultures treated with Str. 6, 9, 12, 14, 30, 34, 35 and 37. Seed germination rate and seedling height and weight were significantly higher in plants cultured with Str. 12, 34, 35 and 37 than those of the control, indicating that these strains facilitated plant growth. In contrast, these indices were significantly lower in plants cultured with strains 6, 9, 14 and 30 than those of the control, indicating that these strains inhibited plant growth.

The taxonomic status of the efficient root-colonizing bacteria was identified and further experiments were performed to determine their effects on *C. sachalinen-*

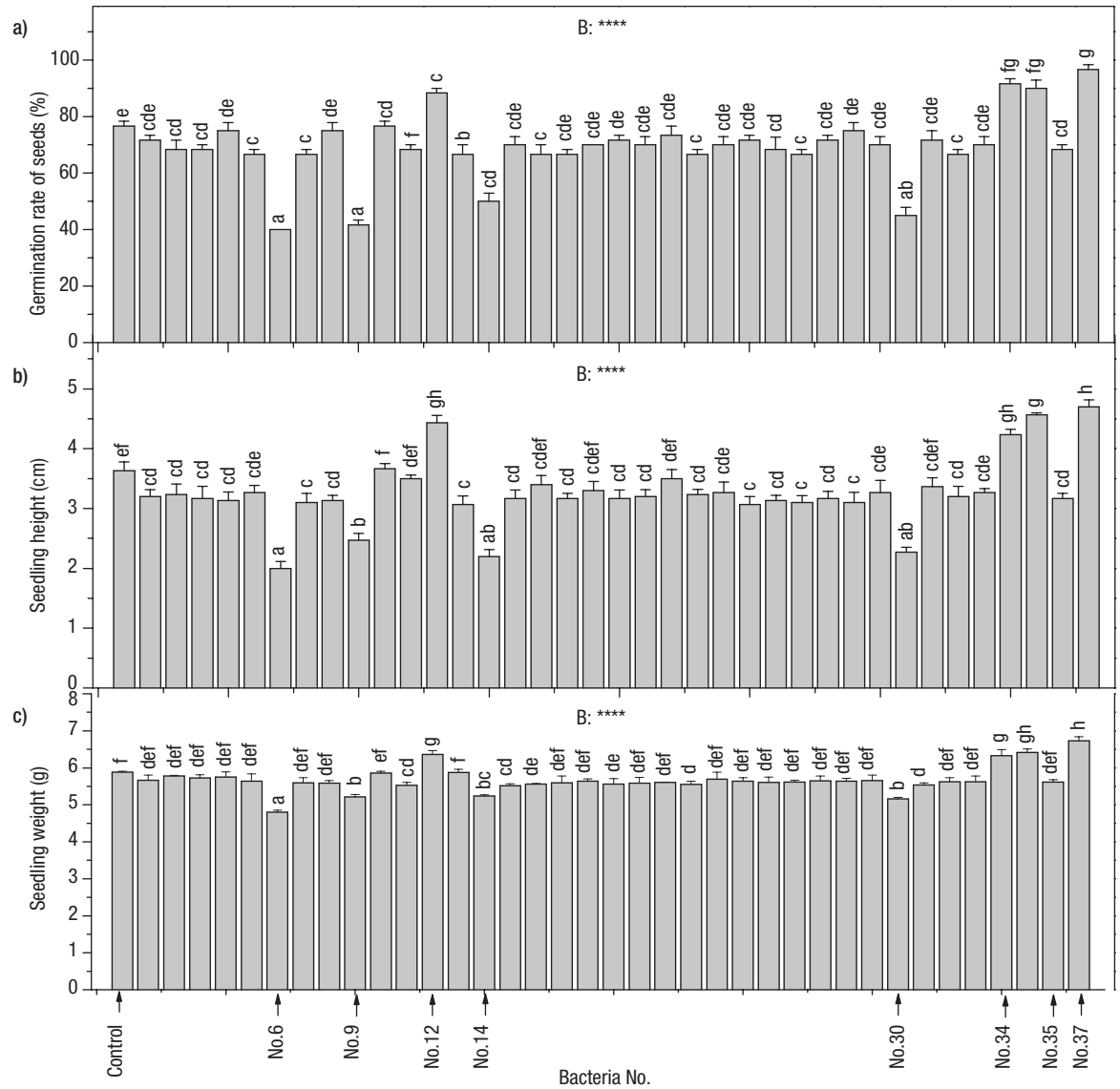

Figure 1. Germination percentage and seedling height and weight of *Cerasus sachalinensis* seeds at 7 d after inoculation with bacterial strains. Seeds in each culture plate were treated as one replicate. Data indicate means ± standard error (n = 3). Different letters on the bars indicate significant difference between the treatments. ****, p<0.0001 (one-way analysis of variances of bacterial strains, B).

sis seedlings. The 16S rDNA gene sequence analysis revealed that strains 34, 35 and 37 were *Bacillus cereus* (GenBank Acc. No. HM245778.1), *Staphylococcus* sp. (AY940424.1) and *Pseudomonas fluorescens* (HM008949.1), respectively. Strain 12 was an unknown bacterium. Growth-inhibiting Str. 6, 9, 14 and 30 were identified as *Stenotrophomonas maltophilia* strain *YLZZ-2* (EU022689.1), *B. cereus* (HM003208.1), *B. cereus* (HM055982) and *P. fluorescen*s (DQ095904.1), respectively.

Effects of inoculation with rhizobacteria on root viability and respiration rate

Root viability and respiration rate were significantly different among the eight dominant bacterial treatments and the control treatment (Fig. 2a). The viability of roots inoculated with growth-promoting Str. 12, 34, 35 and 37 was significantly higher than that of the control, with plants treated with growth-promoting Str. 37 showing the highest viability (42% higher than that of the control). The respiration rate of the control roots and those inoculated with growth-promoting Str. 12, 34, 35 and 37 were not significantly different (Fig. 2b). In contrast, the viability and respiration rates of roots inoculated with growth-inhibiting Str. 6, 9, 14 and 30 were significantly lower than those of the control, with plants treated with strain 6 showing the lowest viability (57.8% lower than that of the control) and respiration rate (59.5% lower than that of the control; Fig. 2b).

Effects of inoculation with rhizobacteria on root respiratory pathways, enzymes and electron transport

EMP and TCA were the dominant respiratory pathways in the roots of *C. sachalinensis* seedlings grown under normal conditions. TCA accounted for approximately 60% of the total respiratory rate, whereas PPP accounted for 24% (Fig. 3a). TCA, the major respiratory pathway in the control treatment, was dominant in the roots inoculated with the growth-promoting Str. 12 and 35. EMP was enhanced and TCA was reduced by the growth-promoting Str. 34. Roots inoculated with growth-promoting Str. 37 showed an increase in the proportion of TCA and reduction in PPP compared to those of the control. The activity of the TCA pathway was remarkably reduced in the roots inoculated with growth-inhibiting Str. 6, 9, 14 and 30. The proportion of EMP increased, but that of TCA declined significantly in the roots inoculated with growth-inhibiting Str. 9 and 14 compared to those of the control, indicating that these two bacterial strains inhibited normal root respiration in *C. sachalinensis*. In addition, the proportion of EMP remained unchanged in plants inoculated with Str. 6 and 30 compared to that in the control. Further, the EMP and PPP pathways were the dominant pathways that were affected by bacterial inoculations.

The proportion and direction of the basic biochemical pathways are regulated by various enzymatic activities. HK, PFK and PK are the key enzymes that

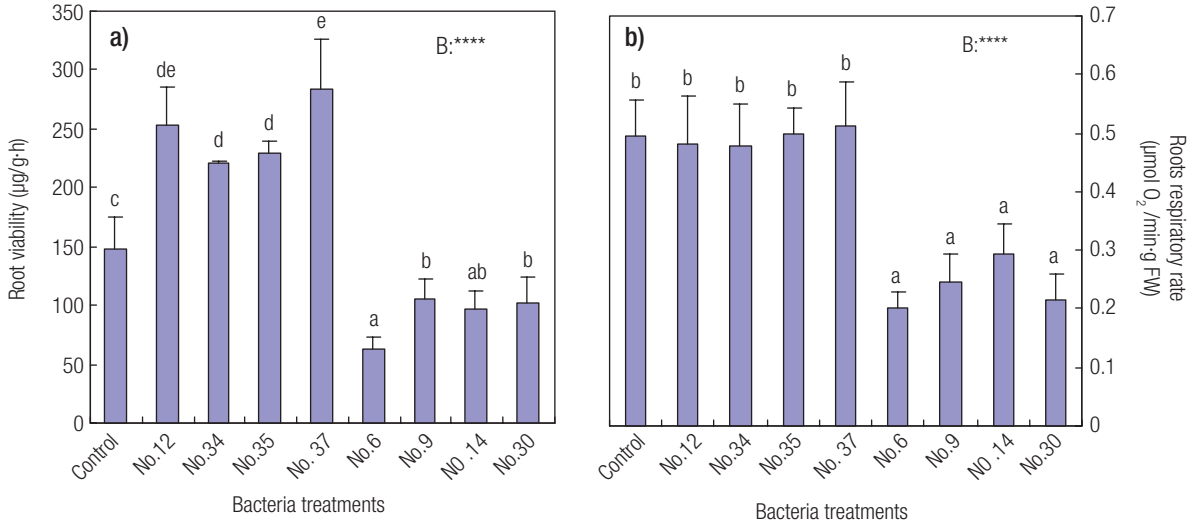

Figure 2. Root viability (a) and root respiratory rates (b) of *Cerasus sachalinensis* seedlings at 30 d after inoculation with growth-promoting and growth-inhibiting bacterial strains. *C. sachalinensis* seedlings were 4 months old at sampling. Strain nos. 12, 34, 35 and 37 are the growth-promoting bacterial strains and nos. 6, 9, 14 and 30 are the growth-inhibiting bacterial strains. Data indicate means ± standard error (n = 3). Different letters on the bars indicate significant difference between the treatments. ****, $p < 0.0001$ (one-way analysis of variances of bacterial strains, B).

regulate the EMP pathway. The key enzymatic activities in EMP were unchanged by the growth-promoting Str. 12, 34, 35 and 37 (Fig. 3b). In contrast, the activity of the key EMP enzymes changed significantly in the roots inoculated with the growth-inhibiting Str. 6, 9, 14 and 30. The activity of HK changed most significantly after inoculation with the growth-inhibiting bacterial strain 14, increasing by 4.4 times relative to that of the control. The activities of PFK and PK decreased significantly in the roots inoculated with Str. 6 and 30, but their activities were increased significantly by Str. 9 and 14.

TCA is mainly regulated by the activities of SDH, MDH and IDH. IDH activity was increased significantly by growth-promoting Str. 12, 34 and 35 and was decreased significantly by growth-inhibiting Str. 6, 9, 14 and 30. MDH activity was unchanged with the exception of a significant decrease in the presence of Str. 9 and a significant increase in the presence of Str. 34. SDH activity increased significantly in the presence of Str. 12, 35 and 37 and Str. 9 and 30 and was 3.9 times higher in the roots inoculated with Str. 37 than that in the control (Fig. 3c).

G-6-PDH and 6-GPDH are the key enzymes in the PPP pathway and G-6-PDH is the dominant enzyme. The activities of these enzymes decreased in the roots inoculated with the Str. 12, 34, 35 and 37, especially their activities decreased by 40.9% and 55.2%, respectively, in the presence of Str. 37 (Fig. 3d). The activity of G-6-PDH was increased by Str. 6, 9, 14 and 30 and especially by Str. 6 (82% increase). The activity of 6-GPDH was increased by Str. 6 and 9.

Under normal growth conditions, COX is the primary pathway of the respiratory electron transport chain (control; Fig. 3e). The proportion of COX was increased by Str. 34 and 37 and that of AOX was increased by Str. 35. The proportion of COX decreased significantly and that of AOX increased significantly in the roots inoculated with Str. 6, 9, 14 and 30.

Effects of inoculation with rhizobacteria on root respiratory substrates and intermediate products

Sucrose and starch are the primary substrates for respiratory metabolism under normal conditions. Sucrose concentration increased in the roots inoculated with Str. 35 and 37, but decreased in the roots treated with Str. 6, 9, 14 and 30 (Fig. 3f). Starch concentrations were significantly higher in the roots exposed to Str. 12 and 35 than those in the control, and they were significantly lower in the roots exposed to Str. 6, 14 and 30.

Pyruvic acid and citric acid are the key products of EMP and TCA. The pyruvic acid concentration decreased significantly in all the treatments, except for those treated with Str. 9 and 14. Citric acid concentration increased significantly in the roots exposed to the growth-promoting bacterial strain 37 and decreased significantly in the roots exposed to Str. 6, 9, 14 and 30. Other treatments did not affect citric acid concentration (Fig. 3g).

Effects of inoculation with rhizobacteria on seedling biomass

Compared with those of the control, the height, leaf number and shoot and root dry weights changed significantly in the seedlings inoculated with the eight strains of rhizobacteria (Table 1). All these variables increased in the seedlings whose roots were inoculated with Str. 12, 34, 35 and 37; the height and leaf number increased by 77.7% and 86.6%, respectively, in the *C. sachalinensis* seedlings treated with Str. 37. These variables decreased in the seedlings treated with Str. 6, 9, 14 and 30. The height and leaf number decreased by 58.7% and 63.6%, respectively, in the *C. sachalinensis* seedlings treated with Str. 6.

Effects of inoculation with rhizobacteria on soil environment

In the present study, the number of fungi significantly decreased, whereas the content of alkali-hydrolyzable N and available P increased in the rhizosphere soil after treatment with Str. 12, 34, 35 and 37. In contrast, after treatment with Str. 6, 9, 14 and 30, the fungal populations significantly increased, whereas the content of alkali-hydrolyzable N and available P varied slightly (Tables 2 and 3).

Discussion

Lü *et al*. (2011) suggested that a close relationship exists between the changes in root function and evolution of microbial community structure in the *C. sachalinensis* rhizosphere. Plant growth is also affected by the soil microenvironment, which is altered by microbial metabolic processes and organic matter decomposition (Zak *et al*., 2003). The bacteria significantly affect the plants when they are sufficient in number. Similarly to biological agents, bacterial inoculations need to reach an optimal amount to show an obvious effect (Yu *et al*., 2012; Qin *et al*., 2014; Kamal *et al*.,

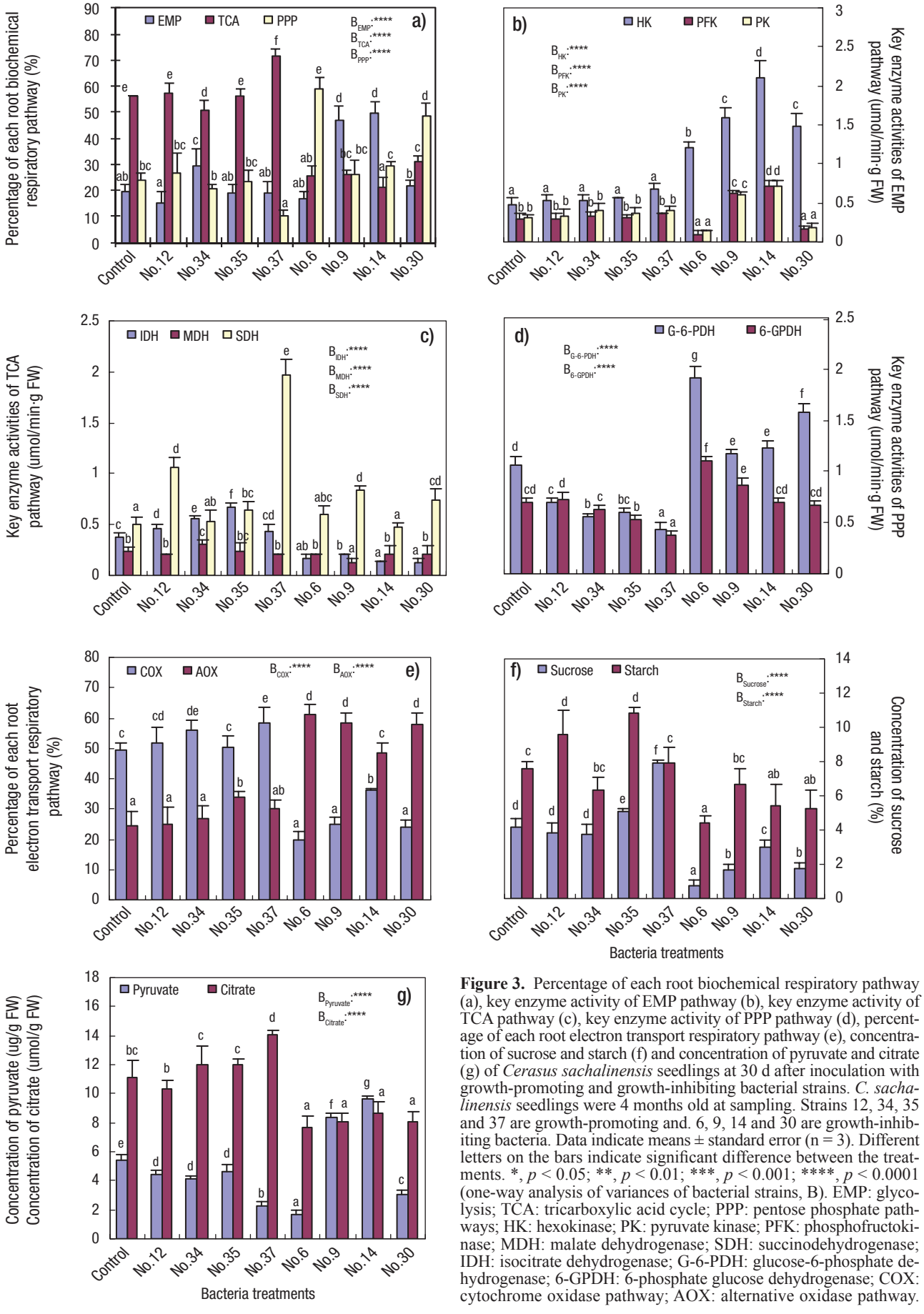

Figure 3. Percentage of each root biochemical respiratory pathway (a), key enzyme activity of EMP pathway (b), key enzyme activity of TCA pathway (c), key enzyme activity of PPP pathway (d), percentage of each root electron transport respiratory pathway (e), concentration of sucrose and starch (f) and concentration of pyruvate and citrate (g) of *Cerasus sachalinensis* seedlings at 30 d after inoculation with growth-promoting and growth-inhibiting bacterial strains. *C. sachalinensis* seedlings were 4 months old at sampling. Strains 12, 34, 35 and 37 are growth-promoting and. 6, 9, 14 and 30 are growth-inhibiting bacteria. Data indicate means ± standard error (n = 3). Different letters on the bars indicate significant difference between the treatments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (one-way analysis of variances of bacterial strains, B). EMP: glycolysis; TCA: tricarboxylic acid cycle; PPP: pentose phosphate pathways; HK: hexokinase; PK: pyruvate kinase; PFK: phosphofructokinase; MDH: malate dehydrogenase; SDH: succinodehydrogenase; IDH: isocitrate dehydrogenase; G-6-PDH: glucose-6-phosphate dehydrogenase; 6-GPDH: 6-phosphate glucose dehydrogenase; COX: cytochrome oxidase pathway; AOX: alternative oxidase pathway.

Table 1. Morphological changes in *Cerasus sachalinensis* seedlings treated with growth-promoting or growth-inhibiting rhizobacteria

Bacterial strain No.	Increases of:		Fresh weight (g) of:		Dry matter weight (g) of:	
	Seedling heights (cm)	No. of seedling leaves	Shoots	Roots	Shoots	Roots
Control	1.21 ± 0.10 c	2.71 ± 0.01c	10.86 ± 0.31 c	9.06 ± 0.61 c	3.64 ± 0.27 b	2.27 ± 0.20 c
6	0.40 ± 0.09 a	0.73 ± 0.04 a	7.35 ± 0.17 a	5.55 ± 0.36 a	2.56 ± 0.25 a	1.26 ± 0.06 a
9	0.68 ± 0.07 ab	1.69 ± 0.01 b	9.01 ± 0.39 b	7.24 ± 0.53 b	2.77 ± 0.18 a	1.71 ± 0.15 b
12	1.83 ± 0.09 d	3.72 ± 0.00 d	12.78 ± 0.45 d	10.98 ± 0.40 de	4.63 ± 0.16 c	2.78 ± 0.07 d
14	0.73 ± 0.10 b	1.58 ± 0.01 b	8.77 ± 0.56 b	6.97 ± 0.56 b	2.86 ± 0.37 a	1.74 ± 0.15 b
30	0.69 ± 0.08 b	1.58 ± 0.01 b	9.04 ± 0.45 b	7.37 ± 0.42 b	2.68 ± 0.30 a	1.64 ± 0.22 ab
34	1.89 ± 0.09 de	4.35 ± 0.00 de	13.00 ± 0.23 de	11.20 ± 0.35 de	4.47 ± 0.06 c	2.82 ± 0.10 d
35	1.93 ± 0.13 de	4.86 ± 0.00 e	12.74 ± 0.20 d	10.94 ± 0.20 d	4.56 ± 0.09 c	2.74 ± 0.11 d
37	2.15 ± 0.14 e	5.11 ± 0.00 e	14.03 ± 0.33 e	12.23 ± 0.24 e	4.73 ± 0.34 c	3.02 ± 0.07 d
p-value	****	****	****	****	****	****

Note: *C. sachalinensis* seedlings were 4 months old at sampling. Data are determined at 30 d after treatment. Strain nos. 12, 34, 35 and 37 are the growth-promoting bacterial strains and nos. 6, 9, 14 and 30 are the growth-inhibiting bacterial strains. Data indicate means ± standard error (n = 10). Different letters in the same column indicate significant difference between the treatments. ****, $p < 0.0001$ (one-way analysis of variances of bacterial strains).

Table 2. Microbial group changes in the rhizosphere of *Cerasus sachalinensis* seedlings treated with growth-promoting and growth-inhibiting rhizobacteria

Bacterial strain no.	No. of bacteria (10^6 CFU/g dry soil)	No. of fungi (10^3 CFU/g dry soil)	No. of Actinomycetes (10^4 CFU/g dry soil)	% of the treated bacteria in the total number of bacteria
Control	0.63 ± 0.02 a	3.93 ± 0.29 b	4.01 ± 0.28 ab	------
6	1.17 ± 0.05 b	4.95 ± 0.48 c	3.94 ± 0.48 ab	34.91 ±1.18 e
9	1.78 ± 0.13 cd	5.12 ± 0.16 cd	4.61 ± 0.30 bc	13.71 ± 0.54 ab
12	1.92 ± 0.18 d	2.75 ± 0.15 a	6.67 ± 0.60 d	18.68 ± 0.27 c
14	1.59 ± 0.14 cd	4.76 ± 0.15 c	3.09 ± 0.31 a	14.72 ± 0.34 b
30	1.48 ± 0.02 bc	5.89 ± 0.31 d	3.62 ± 0.55 ab	31.82 ±1.50 d
34	1.99 ± 0.21 de	2.44 ± 0.30 a	8.31 ± 0.62 e	11.84 ± 0.26 a
35	1.96 ± 0.20 d	2.87 ± 0.31 a	6.57 ± 0.38 d	32.13 ±1.00 d
37	2.38 ± 0.11 e	2.42 ± 0.17 a	5.92 ± 0.59 cd	38.98 ± 0.80 f
p-value	****	****	****	****

Data are determined at 30 d after treatment and indicate means ± standard error (n = 3). Strains 12, 34, 35 and 37 are growth-promoting and 6, 9, 14 and 30 are growth-inhibiting bacteria. Different letters in the same column indicate significant difference between the treatments. ****, $p < 0.0001$ (one-way analysis of variances of bacterial strains)

Table 3. Soil nutrient concentration of *Cerasus sachalinensis* seedling rhizosphere treated with growth-promoting and growth-inhibiting rhizobacteria

Bacterial strain no.	Alkali-hydrolyzable N concentration (mg/kg)	Available P concentration (mg/kg)	Available K concentration (mg/kg)	Organic matter concentration (g/kg)
Control	296.33 ± 5.09 ab	35.51 ± 0.36 a	32.17 ± 0.21 bc	76.61 ± 0.19 a
6	301.00 ±5.35 b	35.81 ± 0.47 a	29.69 ± 0.35 a	76.80 ± 0.57 a
9	291.67 ±2.33 ab	36.64 ± 0.58 ab	30.39 ±1.10 ab	77.18 ± 0.19 a
12	345.33 ± 2.33 c	38.71 ± 0.68 cd	34.09 ± 0.11 c	77.18 ± 0.50 a
14	299.83 ±1.17 b	36.94 ± 0.34 ab	31.62 ± 0.04 ab	77.37 ± 0.00 a
30	283.50 ±8.81 a	35.66 ± 0.34 a	32.31 ±1.30 bc	77.75 ± 0.83 a
34	344.17 ± 3.09 c	37.63 ± 0.00 bc	32.08 ± 0.63 bc	76.42 ± 0.83 a
35	336.00 ±3.50 c	38.86 ± 0.60 cd	31.84 ±1.20 ab	76.61 ± 0.38 a
37	360.50 ±3.50 d	39.99 ± 0.68 d	31.66 ± 0.40 ab	77.37 ± 0.00 a
p-value	****	****	**	ns

Note: Data are determined at 30 d after treatment. Strain nos. 12, 34, 35 and 37 are the growth-promoting bacterial strains and nos. 6, 9, 14 and 30 are the growth-inhibiting bacterial strains. Data indicate means ± standard error (n = 3). Different letters in the same column indicate significant difference between the treatments. *p* values of the one-way analysis of variances of bacterial strains (B) are indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (one-way analysis of variances of bacterial strains).

2015). Previously, we showed that soil sterilization, biological agents and PGPR inoculation could promote the growth of *C. sachalinensis* seedlings by altering the root respiration metabolism (Qin *et al*., 2014; Zhou *et al*., 2015). In this study, in order to simulate a field trial and to allow the growth of spontaneous microorganisms, we used a non-sterilized substrate. We conducted this study by referring to Beuchat *et al*. (1998), who used the same amount of sterilized water as that in controls to treat apples, tomatoes and lettuce leaves. Guentzel *et al*. (2008) and Lim *et al*. (2011) also used the same treatment method. Since no sterilized controls exist, the results need to be interpreted carefully in order to efficiently distinguish direct bacterial effects from the possible interaction effects caused by complex microbial interactions in the non-sterilized soil.

In the present study, eight of the 37 efficient bacterial colonizers in the *C. sachalinensis* rhizosphere were screened. These strains showed obvious promoting or inhibiting effects on seed germination and plant growth and development, suggesting high complexity of the rhizobacterial community. *Bacillus cereus*, *Staphylococcus* sp., *Pseudomonas fluorescens* and an unknown bacterium were the four PGPR in this study. *Staphylococcus* sp. could solubilize phosphate and produce IAA with antagonistic activity against pathogenic microorganisms (Kumar *et al*., 2011). *Bacillus* and *Pseudomonas* were the main PGPR used in agricultural practice (Pandey *et al*., 2005; Niu *et al*., 2011). *Pseudomonas* could promote plant growth by facilitating phosphate solubilization or antagonistic activity or by producing phytohormones, siderophores and hydrogen cyanide (Kumar *et al*., 2012). *Pseudomonas* also could increase biomass (Gholami *et al*., 2009), enhance yield (Rosas *et al*., 2009) and relieve the damage of water stress (Sandhya *et al*., 2010) and drought stress (Kohler *et al*., 2009) to the plants. For various PGPR, including *Azospirillum*, enhanced root proliferation has been shown to be related to bacterial IAA biosynthesis (Perrig *et al*., 2007; Naz *et al*., 2009), improved nitrogen fixation (Pedraza, 2008), enhanced mineral availability and altered environmental stress susceptibility (Vacheron *et al*., 2013). PGPR have also been found to control or minimize phytopathogenic effects through niche competition, antibiosis and systemic resistance induction (Sturz & Christie, 2003; Zhou *et al*., 2015). Inoculation with *Azospirillum brasilense* FT 326 was shown to increase the tomato shoot and root fresh weight, main root-hair length and root surface area, thereby resulting in improved plant growth (Ribaudo *et al*., 2006). In contrast, pathogenic microorganisms could inhibit root growth by damaging beneficial microbes, secreting toxins and infecting the root system. As a bacterial pathogen, *Pseudomonas* could infect the root of *Arabidopsis* (Bais *et al*., 2004). In this study, one *Pseudomonas* strain was found as a growth-inhibiting bacterial strain. This proved that bacterial strains of the same genus might have opposite effects.

Respiratory metabolism is the core of belowground growth and root development and provides raw materials and energy for plant physiological activity, which is sensitive to alterations in the rhizosphere environment (Bryla *et al*., 2001). In vitro tests and cellular analysis of root tips revealed inhibition of primary root growth caused by synergistic effects on bacterial consortia (Felici *et al*., 2008). Moreover, co-inoculation with mycorrhizae and rhizobia from different bean genotypes (three wild genotypes *Phaseolus filiformis*, *P. acutifolius* and *P. vulgaris* and two commercial genotypes *P. vulgaris* 'Pinto Villa' and *P. vulgaris* 'Flor de Mayo') reduced trehalose content, suggesting that rhizobial or mycorrhizal inoculation should be performed separately (Ballesteros *et al*., 2010). The mechanisms underlying the beneficial effects of microorganisms to improve plant rooting are only partially understood and discriminating the direct effects on specific or total activities and indirect effects due to the enhanced availability of nutrients and growth regulators is difficult. In previous studies, PGPR were found to improve soil environment, stimulate growth and increase the yields of mulberry (*Morus alba*), sweet cherry (*Prunus avium* L.), raspberry (*Rubus idaeus* Heritage) and apple (*Malus domestica* L.) fruits (Sudhakar *et al*., 2000; Esitken *et al*., 2006; Orhan *et al*., 2006; Huseyin *et al*., 2007). However, the positive effects of inoculation with efficient microorganisms on physiological functions (*e.g.*, respiratory metabolism) of roots are not sufficiently understood in fruit trees. In this study, although significant differences were not noted in seedling root respiration rate between growth-promoting rhizobacterial strains and the control, the bioavailability of nutrients in the rhizosphere, soil biological characteristics and root absorption capacity were enhanced and *C. sachalinensis* seedling biomass was increased after inoculation with the growth-promoting rhizobacterial strains.

Because of their physiological adaptability and metabolic versatility, bacteria in the plant root zones play important roles in changing soil agroecosystems (Sturz & Christie, 2003). An increase in fungi and decrease of bacteria in the soil were associated with a decline in soil quality (Ruan *et al*., 2003). In this study, we could not confirm whether the changes in number of fungal taxa and nutrient content were caused by the inoculation of growth-promoting and growth-inhibiting bacterial strains or interactions between exogenous bacterial strains and indigenous microorganisms be-

cause the soils were not sterilized. The complexity of plant–soil–microbial interactions is extensive and elucidating all the relationships involved seems highly unlikely (Sturz & Christie, 2003). The composition and size of microbial communities in the rhizosphere are regulated by root metabolic activity and environmental factors such as temperature and moisture. The increase in the number of bacteria might be related to the bacterial suspension used. Therefore, one of the possible mechanisms by which efficient root-colonizing microorganisms enhanced or inhibited seed germination and root metabolism might have been by affecting the available nutrients. Similar results have been reported by Watanabe *et al*. (2004) for apple trees (*M. domestica* Borkh.).

Microorganisms of agricultural importance represent an alternative and ecological strategy for disease management in agricultural crops. Although the mechanism of interaction between rhizosphere microorganisms and their effects on plant growth are not yet completely understood, microorganisms with obvious growth-promoting and growth-inhibiting functions might exist among the rhizosphere. Therefore, the orchard management strategy of screening beneficial bacterial from the plant rhizosphere and then using them as biological agents to regulate rhizosphere microenvironment and improve root function and plant growth and development is feasible approach.

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