The Journal of Microbiology (2012) Vol. 50, No. 1, pp. 45–49
DOI 10.1007/s12275-012-1415-z

Growth Promotion of *Xanthium italicum* by Application of Rhizobacterial Isolates of *Bacillus aryabhattai* in Microcosm Soil

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(Received August 22, 2011 / Accepted October 25, 2011)

This study was conducted using rhizobacteria, which are able to exert beneficial effects upon plant growth in the infertile soil collected from barren lakeside areas. Four strains of plant growth promoting bacteria were isolated from the rhizosphere of a common wild plant, *Erigeron canadensis*. Isolated strains LS9, LS11, LS12, and LS15 were identified as *Bacillus aryabhattai* by 16S rDNA sequence analysis. *B. aryabhattai* LS9, LS11, LS12, and LS15 could solubilize 577.9, 676.8, 623.6, and 581.3 mg/L of 0.5% insoluble calcium phosphate within 2 days of incubation. Production of indole acetic acid, a typical growth promoting phytohormone auxin, by strain LS15 was 471.3 mg/L in 2 days with the addition of auxin precursor L-tryptophan. All the strains also produced other phytohormones such as indole butyric acid, gibberellins, and abscisic acid, and strain LS15 showed the highest production rate of gibberellin (GA_3), 119.0 μg/mg protein. Isolated bacteria were used in a microcosm test for growth of wild plant *Xanthium italicum*, which can be utilized as a pioneer plant in barren lands. Seed germination was facilitated, and the lengths of roots, and shoots and the dry weights of germinated seedlings after 16 days were higher than those of the uninoculated control plants. Root lengths of seedlings of *X. italicum* increased by 121.1% in LS11-treated samples after 16 days. This plant growth-promoting capability of *B. aryabhattai* strains may be utilized as an environmentally friendly means of revegetating barren lands, especially sensitive areas such as lakeside lands.

Keywords: plant growth promotion, rhizobacteria, *Xanthium italicum*, *Bacillus aryabhattai*

Introduction

The beneficial effects of some rhizobacteria on plant growth are well known, and so-called plant growth promoting rhizobacteria (PGPR) have been utilized for several decades, although their mechanisms of plant growth promotion have not been completely elucidated (Babalola, 2010). Some of the important mechanisms include direct phytohormonal action, plant disease suppression, increase of plant nutrient availability, and the enhancement of other plant-beneficial microorganisms (Lucy *et al.*, 2004; Ryu *et al.*, 2005; Dodd et *al.*, 2010). To date, many studies on the introduction of PGPR have focused on some economically important agricultural crops (Bertland *et al.*, 2001; Kokalis-Burelle *et al.*, 2006) and trees (Chanway, 1997; Lucas García, 2004), but wild flora have not been considered as an important research target. Recently, PGPR application to 3 grass species was reported for phytoremediation of creosote contaminated soil (Huang *et al.*, 2004). The growth enhancement of wild flora may be an alternative tool for the revegetation of non-vegetated barren areas. Throughout the world, many barren land areas have recently appeared due to various reasons, such as deforestation and construction projects. These barren areas spoil the beauty of the landscape; moreover, they are prone to erosion and even collapse by heavy rainfall and wind. Revegetation of such areas by either natural or artificial means is expensive, and sometimes may be difficult for geological, topographical, or biological reasons. Barren lakeside land is one such area due to fluctuations of temperature, and low water and clay mineral content due to loss of clay minerals by lake water leaching. Revegetation of such wild land may be initiated with some pioneer wild plants that can tolerate arid and infertile conditions. PGPR are expected to help the establishment of such pioneer plants. In this study, the enhancement of the growth of *Xanthium italicum*, a common wild plant in barren lakeside soil in Korea, was examined after application of some isolated rhizobacterial strains that have several plant growth-promoting mechanisms.

Materials and Methods

Isolation and identification of rhizobacterial isolates

Soil samples containing rhizospheres of a common wild plant, horseweed (*Erigeron canadensis*), from a barren lakeside area at Lake Paro, Korea, were collected, diluted in sterile distilled water, and spread on a minimal medium (KH_2PO_4 2.99 g, Na_2HPO_4 5.96 g, NH_4Cl 1.02 g, NaCl 0.53 g, $MgSO_4 \cdot 7H_2O$ 0.15 g, $CaCl_2 \cdot 2H_2O$ 0.013 g, and glucose 0.99 g per 1 L DW). Among many isolated bacterial strains that showed high growth rates, 4 strains were selected based on the production rate of the phytohormone auxin and solubilization rate of insoluble phosphate. Their 16S rDNA sequences were analyzed (Macrogen Inc., Korea) and the identification of each strain was carried out using BLAST analysis (Basic

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Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic trees of isolates were constructed by the neighbor-joining method based on the Jukes-Cantor model (Jukes and Cantor, 1969) using their 16S rDNA sequences.

Plant growth promoting capability of rhizobacterial isolates

Among many mechanisms of plant growth promotion exhibited by bacteria, solubilization of insoluble phosphate by isolated bacteria was tested after incubation in Nutrient Broth medium (Difco Lab., USA) containing 0.5% tricalcium phosphate. Soluble phosphate in the culture supernatant was analyzed by using the vanadomolybdophosphoric acid colorimetric method (Clesceri *et al.*, 1995). Bacterial production of several phytohormones such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), gibberellins (gibberellic acid; GA_3), and abscisic acid (ABA) were determined by high performance liquid chromatography (HPLC). After incubation of PGPR in 100 ml brain heart broth medium (3 mM L-tryptophan was added as a precursor of IAA and IBA) for 2 days, bacterial cells were removed from the culture medium by centrifugation (3,000×g, 30 min). The pH of the culture supernatant was adjusted to 2.5, and 15 ml of ethyl acetate was added. Extraction of phytohormones from the mixture using an extraction shaker (Recipro shaker RS-1, Jeio Tech, Korea) at 300 stroke/min for 10 min was repeated 3 times, and a total of 45 ml of ethyl acetate was recovered. Ethyl acetate was dried in a rotary vacuum evaporator, dissolved in 5 ml of methanol and filtered through a 0.45 μm pore membrane filter. Extracted phytohormones were analyzed by HPLC (Model Breeze, Waters, USA). Analytical conditions were as follows; Luna 5μ C18 column (250×4.6 mm), flow rate of 1 ml/min of 35% methanol (in 1% acetic acid) for IAA and IBA, 30% methanol (0.1 M H_3PO_4) for GA_3, and 55% methanol (0.1 M acetic acid) for ABA. The wavelengths of the UV detector were 280, 208, 254, and 265 nm for IAA, IBA, GA_3, and ABA, respectively.

Microcosm test for growth promotion of *X. italicum*

Promotion of plant growth by isolated bacteria was examined in the microcosm containing soils collected from a bare lakeside area of Lake Paro. Surface soils (~15 cm depth) were collected, and sieved through a 2 mm diameter mesh to remove large debris. The soil texture was sandy with a low organic content (1.2%) measured by the loss-on-ignition method (Ball, 1964). Sieved soils (200 g) were poured into 5 small plastic cups [7 (top diameter)×5 (bottom diameter)×10 (H) cm]. Seeds of the wild plant, clotbur (*Xanthium italicum*) which had been collected from nearby areas during the previous autumn, were disinfected with ethanol for 1 min, followed by 1% sodium hypochlorite for 10 min, and washed twice with sterile distilled water. Four seeds were evenly distributed on the soil surface and covered with a 1–2 cm layer of the same soil. The cultured bacterial cells were harvested by centrifugation (3,000×g, 30 min), washed with sterile distilled water, and cells suspended in distilled water were sprayed on the surface soil at 10^7 cells/g soil. The inoculated and uninoculated control microcosms were maintained in a plant growth chamber with an air temperature of 28°C for 12 h during day conditions (3,000 lux), and at 23°C for 12 h during night conditions; distilled water (25 ml) was sprayed every day. After 16 days of growth, all the plants grown in the pots were cautiously harvested without root loss, and washed with distilled water to remove the remaining soil particles. Plants were dried in an oven (80°C) for 24 h, and the root and shoot length of each plant and the dry weights of whole plants were measured.

Analysis of variance (ANOVA) was performed on all experimental data, and means were compared using the SYSTAT software. The level for statistical significance was set at $p<0.05$.

Results and Discussion

Isolation of rhizobacterial isolates

Among 48 isolated rhizobacteria, 4 strains that showed a high production rate of either the phytohormone auxin or a high solubilization rate of insoluble phosphate were selected for this study. All the isolates (LS9, LS11, LS12, and LS15) were aerobic Gram-positive, rod-shaped, bacteria that showed similarities of 97.7, 99.6, 99.3, and 99.0%, respectively with *Bacillus aryabhattai* based on the sequences of 16S rDNA in the GenBank database; the bacteria were named *B. aryabhattai* LS9, LS11, LS12, and LS15. The phylogenetic tree of these strains constructed by using their

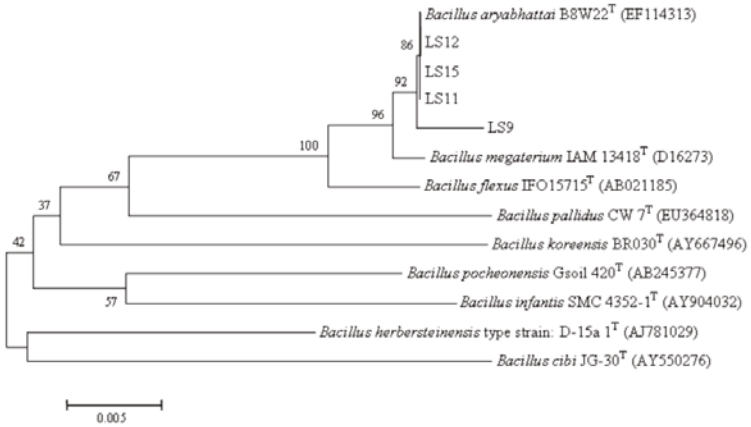

Fig. 1. Phylogenetic tree constructed by NCBI blast program using approximately 1,400 bp of 16S rDNA sequences of rhizobacterial isolates. The scale bar represents the expected number of substitutions.

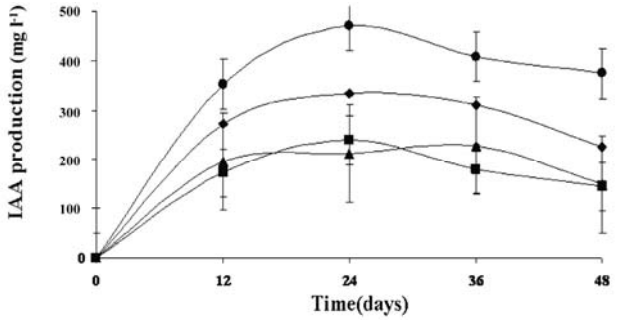

Fig. 2. **IAA production by isolates *B. aryabhattai* LS9 (◆), LS11 (■), LS12 (▲), and LS15 (●) in brain heart broth medium in which 3 mM L-tryptophan was added as IAA precursor.**

16S rDNA sequences (Fig. 1) showed that they were quite similar to the common soil bacterium, *B. megaterium*.

Production of plant growth promoting substances by rhizobacterial isolates

Phosphate is one of the most important plant growth limiting nutrients. Although phosphates are abundant in many environments, most of them are insoluble forms that cannot be easily utilized by plants; therefore, the addition of bioavailable phosphate may have a significant effect on plant growth, especially in infertile land. Many bacteria can solubilize the insoluble phosphate by production of either acidic metabolites or phosphatases (Hilda and Reynaldo, 1999). When the solubilization of insoluble tricalcium phosphate by rhizobacteria was measured, all the strains of *B. aryabhattai* showed high and similar levels of soluble phosphates (577.9, 676.8, 623.6, and 581.3 mg/L for strains LS9, LS11, LS12, and LS15, respectively) at 12 h incubation. For most strains, soluble phosphates showed the highest concentrations at 12 h of incubation and then maintained similar levels until 48 h. The highest phosphate solubilization of 676.8 mg/L by strain LS12 was much higher than those shown by many soil bacteria (11–395 mg/L), which were reported in several similar studies (Rodríguez and Fraga, 1999). Therefore, these *B. aryabhattai* strains may stimulate plant growth through providing phosphate in a bioavailable form.

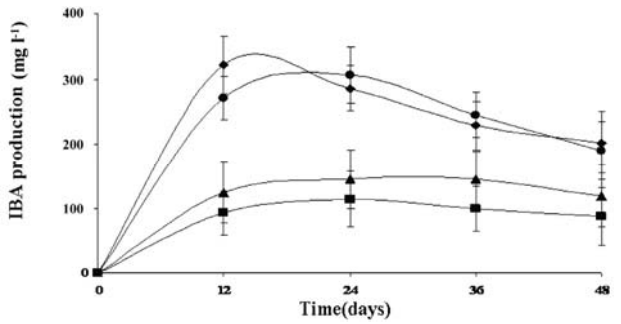

Fig. 3. **IBA production by isolates *B. aryabhattai* LS9 (◆), LS11 (■), LS12 (▲), and LS15 (●) in brain heart broth medium in which 3 mM L-tryptophan was added as IBA precursor.**

Table 1. **Production of phytohormones by isolated strains of *B. aryabhattai* after 2 days of incubation in brain heart broth medium**

Strain	Production of phytohormone (µg/mg protein)	
	Gibberellin	ABA
LS9	88.5	1.77
LS11	53.3	1.39
LS12	107.1	1.78
LS15	119.0	1.63

Besides solubilization of insoluble phosphates, production of some growth stimulating phytohormones is another important growth enhancing mechanism of PGPR (Dodd *et al.*, 2010). The production rates of IAA and IBA, which are typical groups of auxins that stimulate the growth of plant roots and shoots, are shown in Figs. 2 and 3, respectively. All the strains were able to produce auxin and maintained this capacity for 48 h. The LS15 strain demonstrated the highest IAA production of 471.3 mg/L at 24 h incubation, which was much higher than the 61 mg/L production by *Rhodobacter sphaeroides* (Watanabe *et al.*, 2000), and the 57–288 mg/L production demonstrated by *Bacillus thuringiensis* and *Pseudomonas aeruginosa* (Shahab *et al.*, 2009). A large amount of IBA, another auxin compound showing an effect similar to IAA, was also produced by all strains of *B. aryabhattai*. Strains LS9 and LS15 showed similar high production of IBA, and the highest production was 321.3 mg/L by strain LS9 at 12 h incubation (Fig. 3). IBA production by these strains was much higher than the 22–34 mg/L shown by *B. thuringiensis* and *P. aeruginosa* (Shahab *et al.*, 2009). Other phytohormones, gibberellins, and ABA were also produced by *B. aryabhattai* strains (Table 1). Production of gibberellin (GA_3), which stimulates plant elongation, was higher in the cultures of strains LS12 and LS15, and their GA_3 production was also higher than those (1.2–3.0 µg/mg protein) demonstrated under the same conditions by *Bacillus* sp., which shows high growth promotion of tomato plants (Lee and Song, 2007). Production of ABA, which controls water stress and mediates root elongation (Dodd *et al.*, 2010) was quite similar when compared to production of other phytohormones among *B. aryabhattai* strains. Although ABA is one of the important phytohormones, there are not many reports on bacterial ABA production (Perrig *et al.*, 2007; Dodd *et al.*, 2010). The concentration range of ABA in the culture media of *B. aryabhattai* strains, (~500–600 ng/ml) was higher than the values of 73 ng/ml in culture medium and 235 ng/ml in 100 mM NaCl-added culture medium produced by *Azospirillum brasilense* (Cohen *et al.*, 2008). Although the culture conditions and analytical methods were different from each other, the production capacities for several phytohormones by *B. aryabhattai* strains in this study seemed to be higher as compared to other reported PGPR. These production capabilities of soluble phosphate and some phytohormones by *B. aryabhattai* strains may be useful for the enhancement of plant growth.

Microcosm test for growth promotion of *X. italicum*

Plant growth promotion by isolated *B. aryabhattai* strains was tested in a soil microcosm with *X. italicum*, a common

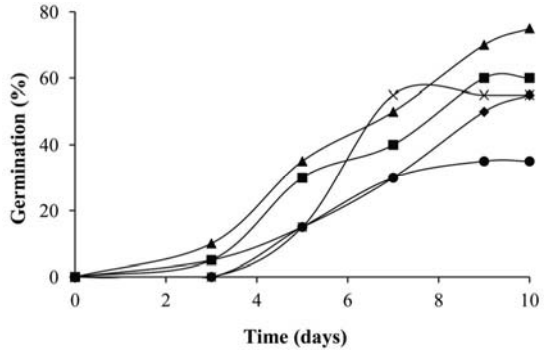

Fig. 4. Germination of seeds (n=20) of *X. italicum* treated with *B. aryabhattai* in a microcosm soil (◆, LS9; ■, LS11; ▲, LS12; ×, LS15; ●, control).

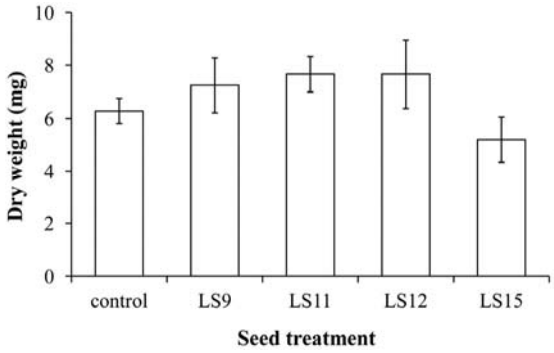

Fig. 6. Dry weight of *X. italicum* seedlings treated with *B. aryabhattai* LS9, LS11, LS12, and LS15 after 16 days of growth in microcosm soil.

wild plant found in many barren lakeside areas in Korea. This plant was chosen because of tolerance to water stress and high temperature. *X. italicum* can grow on hot sunny lakeside soil during summer; furthermore, their seeds can germinate after several months of submergence of lakeside areas (Cheon *et al.*, 2010). Because water levels of most lakes in Korea show a significant fluctuation, the germination of submerged seeds is an important characteristic for the selection of plants to revegetate a naked lakeside area. When *B. aryabhattai* strains were applied to the microcosm soils, they enhanced the germination rates of seeds of *X. italicum* (Fig. 4). Strain LS12 increased the seed germination by 2-fold compared to the uninoculated control at 10 days. Although the effects of application of *B. aryabhattai* on the shoots of germinated seedlings grown for 16 days did not show statistical significance, strains LS11 and LS12 increased the shoot lengths by 29.8 and 26.1%, respectively, when compared to the control (Fig. 5). Moreover, all strains of *B. aryabhattai* significantly increased the root lengths of the germinated seedlings, and strains LS11 and LS12 showed 121.1% and 117.4% root elongation, respectively, compared to the control. The growth stimulation of roots of *X. italicum* by *B. aryabhattai* was significantly higher than that of the shoots, and this might be due to water deficiency and high temperature of surface soil in lakeside areas. Root development may be a good mechanism for the tolerance of these harsh conditions. The dry weights of total biomass of *X. italicum* harvested after 16 days were also increased by strains LS9, LS11, and LS12, but not by LS15, and the highest enhancing effect (31.8%) was exhibited by strain LS12 (Fig. 6). Plants themselves can synthesize phytohormones, but they can also utilize exogenous sources such as microbially produced phytohormones (Vessey, 2003), and it may be one of the important mechanisms for plant growth promotion by microorganisms, especially when plants are in poor growth conditions. Although strain LS15 showed the highest production rates of some phytohormones, strains LS11 and LS12 were better for the growth promotion of *X. italicum*. Strains LS11 and 12 may have other direct or indirect growth stimulating mechanisms than those examined in this study. Siddikee *et al.* (2010) reported a >40% increase in root elongation and dry weight of salt-stressed canola seedlings by *B. aryabhattai* RS341, compared to an uninoculated control. Several species of *Bacillus* have been reported to inhibit plant pathogens and stimulate plant growth, and have great potential for use in agriculture (Pérez-García *et al.*, 2011).

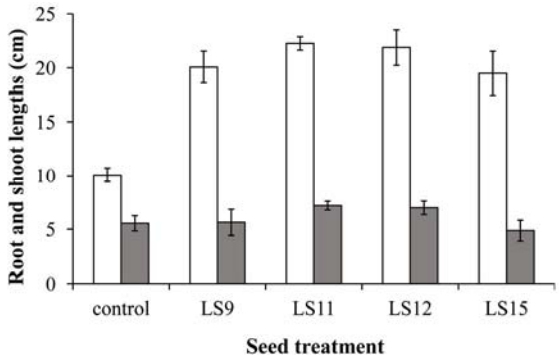

Fig. 5. Comparison of shoot and root length of *X. italicum* seedlings treated with *B. aryabhattai* LS9, LS11, LS12, and LS15 (open bar, root; closed bar, shoot) after 16 days of growth in microcosm soil. The data were subjected to one-way ANOVA and the values with different letters are significantly different ($P \leqq 0.05$), according to Fisher's LSD test. n=4

A growth promoting effect on the wild plant *X. italicum* was demonstrated by *B. aryabhattai* strains in a microcosm, and it might be caused mainly by production of soluble phosphate and certain phytohormones, both of which are mechanisms for promoting plant growth. *B. aryabhattai* strains can be utilized as an efficient and environmentally friendly means for revegetation of barren lands through growth promotion of wild plants that may act as pioneer plants. There may be other growth stimulating mechanisms related to PGPR, such as production of phytohormone cytokinins, iron supply by siderophores, N_2 fixation, and antifungal effects (Vessey, 2003), and they should be investigated in a further study. The contents of phytohormones in the grown plants should be determined to confirm the effects of bacterial inoculation. Molecular biological techniques, such as denaturing gradient gel electrophoresis or real time PCR are necessary to investigate the survival and fate of inoculated PGPR and their influence on the indigenous bacterial community for their application in the field.

Acknowledgements

This study was supported by the CAER (Center for Aquatic Ecosystem Restoration) of Eco-STAR project from MOE (Ministry of Environment, Republic of Korea).

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