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Plant Growth Promotion in Soil by Some Inoculated Microorganisms

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The inoculation of some microorganisms into a microcosm containing soil from a barren lakeside area at Lake Paro in Kangwon-do enhanced plant growth significantly. The direct and viable counts of soil bacteria and soil microbial activities measured by electron transport system assay and fluorescein diacetate hydrolysis assay were higher in inoculated soil. The plant growth promoting effect of this inoculation may be caused by phytohormone production and the solubilization of insoluble phosphates by the inoculated bacteria. Three inoculated strains of *Pseudomonas fluorescens* produced several plant growth promoting phytohormones, including indole-3-acetic acid (auxin), which was confirmed by thin layer chromatography and GC/MS. *P. fluorescens* strain B16 and M45 produced 502.4 and 206.1 mg/l of soluble phosphate from $Ca_3(PO_4)_2$ and hydroxyapatite, respectively. *Bacillus megaterium* showed similar solubilization rates of insoluble phosphates to those of *Pseudomonas* spp. We believe that this plant growth promoting capability may be used for the rapid revegetation of barren or disturbed land.

***Key words*:** plant growth promoting rhizobacteria, phytohormones, phosphate solubilization, revegetation

Because of the enormous numbers of microbial populations and species in the soil, especially in the rhizosphere, intensive and extensive interactions have been established between soil microorganisms and various other soil organisms, including plant roots, and plant growth promotion by rhizosphere microorganisms is well established (Bashan, 1998). In spite of the deleterious effects of some microorganisms on plants, the beneficial effects are usually greater, and the overall results are usually demonstrated by growth promotion and faster germination (Atlas and Bartha, 1998). To utilize the positive effects of microorganisms, some useful microorganisms have been isolated from soil, cultured and inoculated into rhizosphere soil (Glick, 1995). Although there may be some artificial and trivial effects on plant growth promotion induced by the inoculation of some soil bacteria, overall evidence showing significant plant growth effects induced by rhizosphere microorganisms is overwhelming (Gerhardson and Wright, 2002). The mechanisms of plant growth promotion by non-pathogenic, plant-associated bacteria have not been completely elucidated, but the important mechanisms include direct phytohormonal action, plant disease suppression, enhancement of plant nutrient availability, and the enhancement of other plant beneficial microorganisms (Gerhardson and Wright, 2002).

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To date many studies on the inoculation of plant growth promoting rhizobacteria (PGPR) have focused on some economically important agricultural crops, and wild flora has not been considered as a research target (Glick, 1995; Bashan, 1998). Although much wild land in Korea has been covered with plants as a result of the afforestation policy by government since the 1960s, many large barren land areas have been produced due to many different reasons for example, water in artificial reservoirs may be drained, and large submerged areas exposed. These depleted areas spoil the beauty of the lake landscape. Moreover, bare lakeside lands are prone to erosion and collapse. Natural or artificial revegetation of such lakeside lands is difficult for geological, topographical and biological reasons. In this study, the enhancement of the plant growth and revegetation in lakeside soil by the inoculation of soil microorganisms, previously shown to promote plant growth in fire-simulated soil microcosms (Ahn *et al.*, 2002) was examined, and the mechanisms of plant growth promotion, such as phytohormone production and the solubilization of insoluble phosphates were investigated.

Materials and Methods

Microorganisms

Pseudomonas fluorescens strains MC07, B16 and M45, *Bacillus megaterium* and *Azotobacter vinelandii* were iso-

lated from various soils in Kangwon-do area, and identified in the Korean Collection of Type Culture (Ahn *et al*., 2002). White rot fungus, *Irpex lacteus* isolated in Kangwon-do was obtained from the Mycology Laboratory, Kangnung National University. All bacteria and fungi were cultivated in Nutrient Broth medium (Difco Lab., USA) or YMG broth medium (yeast extract 10 g, malt extract 4 g, glucose 10 g, distilled water 1 liter) on a rotary shaker (160 rpm, 30°C), harvested and washed with sterile distilled water, and utilized as an inoculum.

Microcosm study

Plant growth promotion was investigated in the microcosm containing the surface soil from a bare lakeside area of Lake Paro in Kangwon-do. Soil was collected, and sieved through a mesh with 4 mm diameter. Sixty kg of sieved soils was poured into six plastic containers [1.2 (L)×0.6 (W)×0.5 (H) m]. Six kinds of wild plant seeds were also collected from the same area, and evenly distributed on the surface soil and covered with the same soil to a depth of 1~2 cm. The names of three identified plants were *Torilis japonica*, *Rorippa islandica*, *Calystegia sepium* var. *americana*. Three unidentified plants were assigned to the families *Gramineae* and *Cyperaceae*. The washed microbial biomass (10^6 cells/g soil for each strain) was inoculated into the surface soil of the three microcosms. The three inoculated and the three uninoculated control microcosms were maintained in a greenhouse in which water was sprayed regularly. Soil microorganisms and plant growth were monitored for 70 days. Every 10~15 days about 50 g of composite soil samples were collected from each microcosm and the followings were determined; acridine orange direct count (AODC), viable *Pseudomonas* sp. count, total microbial activity as determined by electron transport system (ETS) assay (dehydrogenase activity) and fluorescein diacetate (FDA) hydrolysis assay. The experimental methods have been previously described in detail (Ahn *et al*., 2002). After 70 days of incubation, all the plants in the microcosms were pulled out without root loss and washed with distilled water to remove soil. Remaining water was removed by placing the plants in a drying oven (80°C) for 24 h, and whole plant weights were measured.

Analysis of plant growth promoting phytohormone production

Production of plant growth promoting phytohormones was examined in the cultures of 3 strains of *Pseudomonas fluorescens*. Bacteria were grown at 30°C in 10 ml of 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, $CaCl_2$ 0.013 g, glucose 0.99 g in 1 l distilled water) supplemented with 0, 50, 100, 200 and 500 mg/l of L-tryptophan, a precursor of indole acetic acid (IAA). IAA production was examined and quantified periodically in replicate cultures using the methods described by Patter and Glick (2002). The effect of glucose concentration on the amount of IAA produced was investigated using 500 mg/l of L-tryptophan and 0.5, 1.0, 2.5 and 10 g/l of glucose. In addition to IAA, the productions of other plant growth promoting phytohormones were examined in the culture supernatant of *P. fluorescens* MC07 by thin layer chromatography (TLC) and gas chromatography (GC) (Mordukhova *et al*., 2000). Each phytohormone was analyzed by GC/MS.

Solubilization of insoluble phosphates

The solubilization of insoluble phosphate was examined in cultures of *Pseudomonas fluorescens* strains (MC07, B16, M45) and *Bacillus megaterium* grown in the Pikovskaya (PVK) medium (glucose 10 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.2 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $MnSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.5 g, yeast extract 0.5 g in 1 l distilled water) supplemented with insoluble phosphate, $Ca_3(PO_4)_2$ or hydroxy- apatite (final concentration, 0.5%). After incubation at 30°C for 5 days on a rotary shaker (200 rpm) the pH of the cultures was measured, and the cultures were centrifuged (17,400×g, 20 min). The concentration of soluble phosphate in the supernatant was measured using a vanado-molybdophosphoric acid colorimetric method (Clesceri *et al*., 1998).

Results and Discussion

Plant growth promotion and microbial populations in the microcosm

Plant growth began 10 days after establishing the microcosms, continued for 40 days, and then stopped due to a decrease of temperature in late autumn. During this study, plant growth in the inoculated microcosm appeared to be more favorable than that in the uninoculated microcosms. After a 70 days study period, most plants died, and the total plant biomass in the microcosm was measured. The dry weight of the plant biomass in the inoculated microcosms (1.73±0.17 g) was almost 3 times higher than that

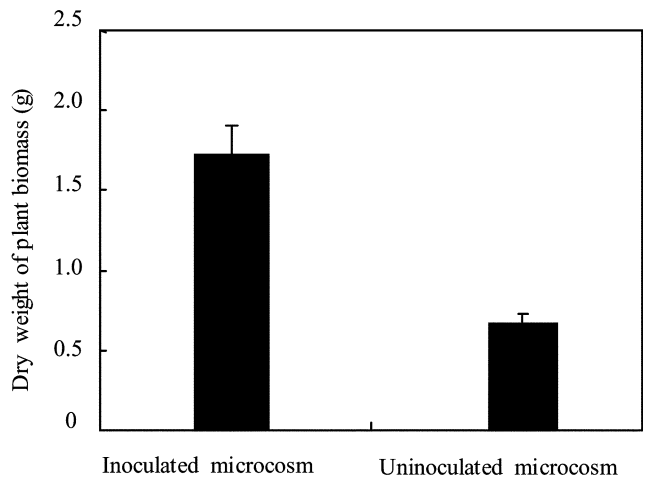

Fig. 1. Comparison of dry plant biomass weights in the inoculated and uninoculated microcosms.

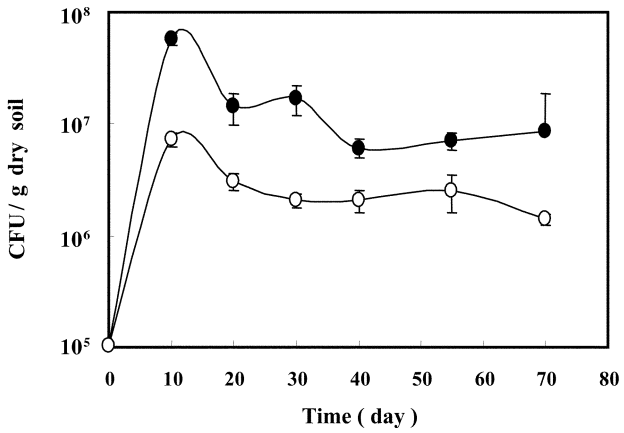

Fig. 2. Changes of populations of *Pseudomonas* sp. in uninoculated (○) and inoculated (●) microcosm soils.

in the uninoculated microcosms (Fig. 1). Plants grown in the microcosms were mainly sown species such as *Torilis japonica*, *Rorippa islandica*, *Calystegia sepium* var. *americana*, and three unidentified plants belonging to the families *Gramineae* and *Cyperaceae.*

Since plant growth promotion seems to be affected at least partially by the direct and indirect stimulation of the inoculated microorganisms, the microbial community was analyzed by plate counting and by microbial activity measurements. Total bacterial population measured by AODC ranged 5.2×10^7~10^8 cells/g soil in the uninoculated soils and $2\sim5\times10^7$ cells/g soil in the inoculated soils. This result suggests that the inoculated microorganisms maintain higher levels in the soil micrococosms over 2 months. Of the inoculated microorganisms, *Pseudomonas fluorescens* is known as a typical PGPR and changes in the *Pseudomonas* sp. population were monitored. After inoculation *Pseudomonas* counts greatly increased from 10^5 CFU/g soil to 5.9×10^7 CFU/g soil, and slightly decreased after 20 days to maintain 10^7 CFU/g soil (Fig. 2). The pattern of population change of *Pseudomonas* sp. detected by viable counting in this study was similar to that of *P. fluorescens* strains inoculated in different soils, which were monitored by fluorescent *in situ* hybridization (Ahn *et al*., 2002). The number of *Pseudomonas* sp. in the uninoculated soil also increased markedly because of increased levels of indigenous *Pseudomonas* sp. caused by the soil preparation procedures such as sieving and rewetting (Lund and Goksøyr, 1980). The same phenomenon was observed in the AODC results (data not shown). However, the populations of *Pseudomonas* sp. in the inoculated microcosms were always higher than those in the uninoculated microcosms.

Microbial activities measured by ETS assay (dehydrogenase activity) and FDA hydrolysis assay, which have been used to detect total microbial activity in soil (Prosser, 1997) were elevated for 70 days in both microcosms (Fig. 3), which might also have been due to the soil preparation

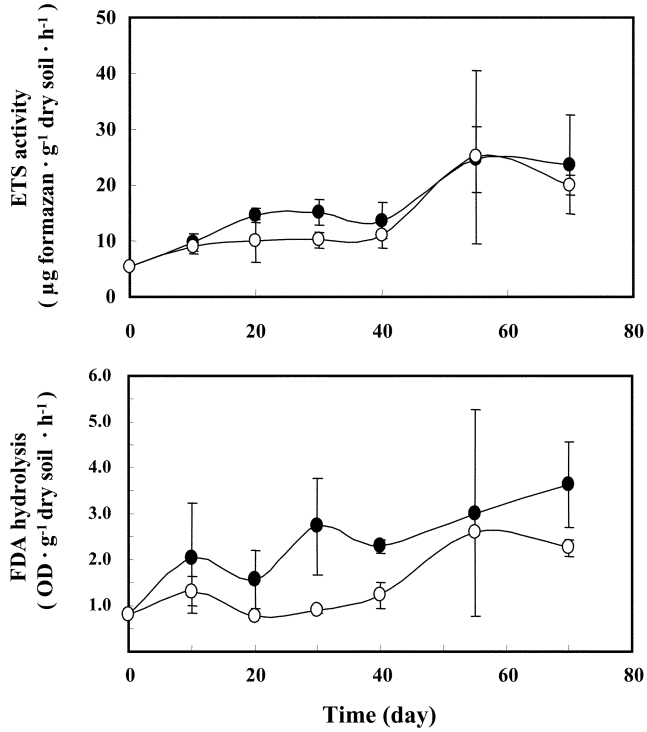

Fig. 3. Changes of total microbial activities measured by ETS (A) and FDA hydrolysis (B) assays. Symbols are as described in Fig. 2.

procedures (Lund and Goksøyr, 1980). The total microbial activities in the inoculated microcosms were higher than those in the uninoculated microcosms, which might promote directly and indirectly plant growth together and account for the increased numbers of total bacteria and *Pseudomonas* sp.

Bacterial production of plant growth promoting phytohormone

Since plant growth was enhanced in the inoculated microcosms, plant growth promoting phytohormones were analyzed in cultures of *Pseudomonas fluorescens* strains, which are known to produce phytohormones as the dominant PGPR (Misko and Germida, 2002; Gamalero *et al*., 2003). When the culture extract of *P. fluorescens* strain MC07 was analyzed by TLC, several spots appeared on the TLC plate. These spots were identified as indole, indole-3-acetic acid (auxin) and indole-3-acetamide by GC/MS (Fig. 4). Besides these auxin compounds some other compounds presumed to be phytohormones were also produced, but their structures were not identified completely (Table 1). Koga (1995) also reported that several plant growth-promoting hormones, in addition to IAA, are produced during IAA biosynthesis. And it has been reported that the ability of plants to produce self-growth regulating substances may be impeded, especially under less than ideal environmental conditions, and that under such conditions plants are more dependent on exogenous

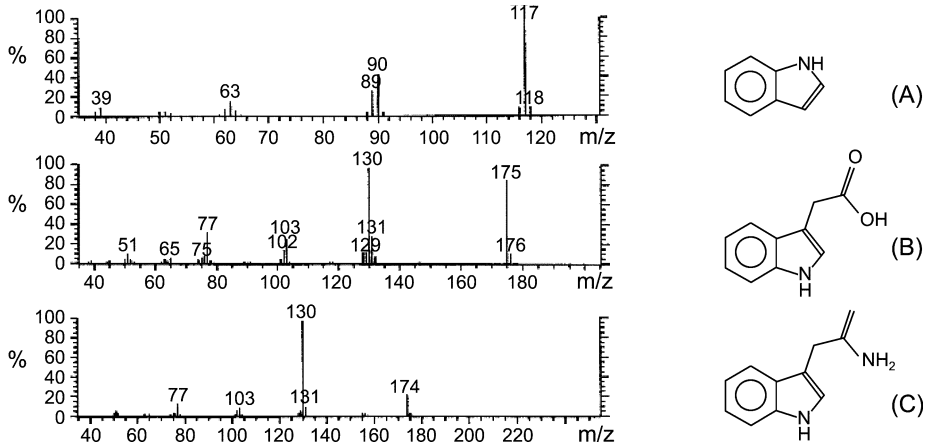

Fig. 4. Identification of indole (A), indole-3-acetic acid (B) and indole-3-acetamide (C) by GC/MS.

Table 1. Compounds produced by *Pseudomonas fluorescens* strain MC07, which may be involved in plant growth promotion*

Compound	Name	Chemical composition	Molecular mass (Da)	GC retention time (min)
1	Indole-3-acetic acid	$C_{10}H_9NO_2$	175	16.04
2	Indole-3-acetamide	$C_{10}H_{10}N_2O$	174	9.48
3	Indole	C_8H_7N	117	8.50
4	Acymidol (presumed)	$C_{15}H_{16}N_2O_2$	256	16.20
5	Kinetin (presumed)	$C_{10}H_9N_5O$	214	11.19
6	6-Benzylaminopurine (presumed)	$C_{12}H_{11}N_5$	226	9.50
7	Phloroglucinol (presumed)	$C_6H_8O_3$	128	4.15
8	Maleic acid hydrazide (presumed)	$C_4H_4N_2O_2$	110	4.50

*These compounds were identified by GC/MS analysis.

sources such as microbially produced phytohormones (Gerhardson and Wright, 2002). In spite of the plant growth-promoting ability, the survival and maintenance of activity may limit the usefulness of inoculated microorganisms. However, most inoculated *Pseudomonas* sp. were found to be as culturable subpopulation and 'viable but nonculturable' subpopulation in the rhizosphere. Moreover, the growth and metabolic activity of *Pseudomonas* inoculants were higher and were maintained for a longer time in the rhizosphere than in the bulk soil (Normander *et al*., 1999, Sørensen *et al*., 2001). Therefore, the various phytohormones produced appear to have been involved in plant growth promotion in the present study. Among the three strains of *P. fluorescens* tested, the productions of IAA in strains M45 and MC07 were increased according to the increase of the concentration of IAA precursor, L-tryptophan, but strain B16 did not produce IAA (Table 2). IAA production rates of *P. fluorescens* strains M45 and MC07 were somewhat lower than those of *P. putida* GR12-2 (Patten and Glick, 2002). However, primary root growth was stimulated by applying relatively low levels of IAA, typically between 10^{-9} and 10^{-12} M, and inhibited at higher IAA concentrations (Pilet and Saugy, 1987). Therefore, IAA production rates in this study may have been sufficient to stimulate plant growth. Since it has been

Table 2. Production of indole-3-acetic acid (IAA) by three *Pseudomonas fluorescens* strains in the presence of various concentration of L-tryptophan

Strain	Tryptophan concentration (μg/ml)	IAA production (μg/ml/OD_{600})[a]
P. fluorescens B16	0	0.4±0.1
	50	0.3±0.1
	100	0.5±0.0
	200	0.7±0.1
	500	0.7±0.0
P. fluorescens M45	0	1.0±0.1
	50	7.6±0.0
	100	8.6±0.2
	200	14.9±0.5
	500	17.7±0.8
P. fluorescens MC07	0	4.5±0.8
	50	10.0±3.8
	100	18.4±0.5
	200	19.5±0.2
	500	22.7±0.3

[a]Average ± standard error from triplicate samples

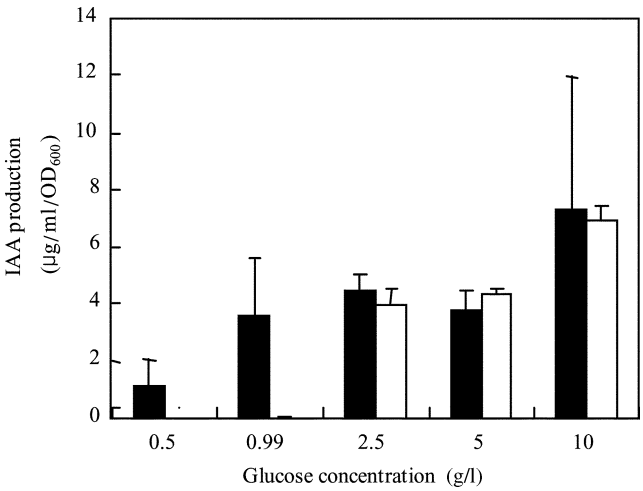

Fig. 5. Effect of glucose concentration on the production of indole-3-acetic acid (IAA) by *Pseudomonas fluorescens* strain MC07. Closed bar, 48 h incubation; open bar, 72 h incubation.

reported that glucose is the optimum carbon source for IAA production (Mordukhova *et al.*, 2000), the effect of glucose concentration on IAA production by *P. fluorescens* strain MC07 was examined. IAA production was found to be almost proportional to the glucose concentration up to 10 g/l and was peaked after 48 hours of incubation (Fig. 5).

Solubilization of insoluble phosphates

Plant growth is frequently limited by an insufficiency of phosphates, which are considered one of the most important growth-limiting environmental factors. The low solubilities of common phosphates, such as $Ca_3(PO_4)_2$ hydroxyapatite and aluminum phosphate cause low phosphate availability. However, because some bacteria can solubilize insoluble phosphates, they may promote plant growth (Rodríguez and Fraga, 1999). We studied the solubilization of $Ca_3(PO_4)_2$ by 3 strains of *P. fluorescens* and *Bacillus megaterium* at initial pHs of 5 and 7. After 5 days of incubation, over 400 mg/l of soluble phosphate was produced from $Ca_3(PO_4)_2$ by *P. fluorescens* strains and *B. megaterium*, and the pH values of the cultures were reduced from 7 to 4~4.4 (Table 3). As acidic initial condition (pH 5) did not significantly increase phosphate solubilization, the final pH values were similar in both cultures starting from pH 7. Son *et al.* (2003) reported that 698 mg/l of soluble phosphate was produced from $CaHPO_4$ by *Pantoea agglomerans* R-38, and that the pH decreased from an initial 7.5 to a final 2.6. In their study, phosphate solubilization was mainly due to the acidification of the culture by bacterium, however, this high level of phosphate solubilization may not be achievable in soil because most soils have a great pH buffering capability, and such low pHs are not found frequently in mineral soils (Tate, 2000). Although the phosphate solubilization rates by *Pseudomonas fluorescens* and *Bacillus megaterium* were somewhat lower in this study than those of $CaHPO_4$ by *P. agglomerans* R-38, they were higher than the 360 mg/l of soluble phosphate production from $Ca_3(PO_4)_2$ by *Penicillium radicum*, and hydroxyapatite solubilization was similar to the 230 mg/l achieved by *Rahnella aquatilis* (Kim *et al.*, 1997). Moreover, the final pH did not reduce to the strongly acidic levels. It is known that the production of organic acids by soil microorganisms and commensurate pH decrease is the major mechanism of inorganic phosphate solubilization (Whitelaw *et al.*, 1999). In addition to organic acids, inorganic acids and chelating agents can increase phosphate solubilization. Chelation of Al^{+3} by gluconic acid may also be involved in the solubilization of colloidal aluminum phosphate (Whitelaw *et al.*, 1999). Mineralization of organic phosphorus is another mechanism of phosphate solubilization, and several phosphatases (also called phosphohydrolases) are involved in the mineralization of various organic phosphorus compounds (Rodríguez and Fraga, 1999). Since the final pH values in the present study were not as low as those in the study of Son *et al*. (2003), some mechanism other than those described above may be responsible for the phosphate solubilization. Further study is necessary to determine the precise solubilization mechanism.

Other plant growth promoting effects

In addition to phytohormone production and phosphate solubilization some other plant growth-promoting effects may be involved. The term 'mycorrhiza helper bacteria' refers to certain soil bacteria, mainly *Pseudomonas* spp., which have the ability to enhance mycorrhizal formations significantly in plants (Garbaye and Bowen, 1989). Since most flowering plants form mycorrhizal relationships with fungi, which contribute to plant nutrition and produce

Table 3. Solubilization of insoluble phosphates by *Pseudomonas fluorescens* and *Bacillus megaterium*

bacteria	$Ca_3(PO_4)_2$ (initial pH 7.0)		$Ca_3(PO_4)_2$ (initial pH 5.0)		Hydroxyapatite (initial pH 7.0)	
	soluble phosphate (mg/l)	final pH	soluble phosphate (mg/l)	final pH	soluble phosphate (mg/l)	final pH
P. fluorescens MC07	458.3	4.2	442.7	4.6	149.0	4.3
P. fluorescens M45	447.6	4.0	499.6	3.9	206.1	4.0
P. fluorescens B16	427.7	4.4	502.4	4.4	199.7	4.2
B. megaterium	489.4	4.4	437.4	4.0	205.1	4.2

other useful effects (Atlas and Bartha, 1998), the inoculated *Pseudomonas* strains could also benefit plant growth indirectly. Although the nitrogen fixation rate by *Azotobacter vinelandii* was not determined and the roles of white rot fungus *Irpex lacteus* were not investigated, they may promote plant growth. White rot fungus seemed to affect plant growth indirectly via the degradation of recalcitrant humic compounds and toxic aromatics and the formation of extracellular polymers contributing to the formation of soil aggregates, which are very important for soil productivity and stability (Tate, 2000). Besides the effects of inoculated microorganisms described above, synergistic interactions that may benefit plant growth may occur between the soil microbial community and plant roots (Bashan, 1998). Even though the supplementation of extra nutrients in the inoculum may affect plant growth, the overall evidence that significant plant growth promotion effects are induced by inoculated PGPR is overwhelming (Gerhardson and Wright, 2002). To apply plant growth promotion by microbial inoculants to barren ground areas the roles of microorganisms and the method of formulating the microbial inoculant should be further investigated. Field-testing is also warranted.

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