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Isolation and Characterization of a Plant Growth-Promoting Rhizobacterium, *Serratia* sp. SY5

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The role of plant growth-promoting rhizobacteria (PGPR) in the phytoremediation of heavy-metal-contaminated soils is important in overcoming its limitations for field application. A plant growth-promoting rhizobacterium, *Serratia* sp. SY5, was isolated from the rhizoplane of barnyard grass (*Echinochloa crus-galli*) grown in petroleum and heavy-metal-contaminated soil. This isolate has shown capacities for indole acetic acid production and siderophores synthesis. Compared with a non-inoculated control, the radicular root growth of *Zea mays* seedlings inoculated with SY5 can be increased by 27- or 15.4-fold in the presence of 15 mg-Cd/l or 15 mg-Cu/l, respectively. The results from hydroponic cultures showed that inoculation of *Serratia* sp. SY5 had a favorable influence on the initial shoot growth and biomass of *Zea mays* under non-contaminated conditions. However, under Cd-contaminated conditions, the inoculation of SY5 significantly increased the root biomass of *Zea mays*. These results indicate that *Serratia* sp. SY5 can serve as a promising microbial inoculant for increased plant growth in heavy-metal-contaminated soils to improve the phytoremediation efficiency.

Keywords: Plant growth-promoting rhizobacteria (PGPR), phytoremediation, rhizoremediation, *Serratia* sp., heavy metal

With increasing industrialization, ecosystems are exposed to a variety of pollutants, with the risk of environmental pollution and human health issues [20, 35]. The contamination of soils with heavy metals has been accelerated by the deposition of atmospheric pollutants, landfill drainage, the use of pesticides and fertilizer, and residues from metalliferous mines and smelting industries, *etc*, with the problem of inducing secondary pollution, such as groundwater contamination [31]. Heavy metals are easily adsorbed by soil particles, and remain within ecosystems for a long period [38]. Heavy metals in soils are transferred to humans, and can then cause DNA damage and carcinogenic effects.

The remediation of soils contaminated with heavy metals can be performed using chemical, physical, and biological techniques. Chemical and physical methods have the advantage of a short remediation time, but are expensive and cause secondary pollutants [30]. Phytoremediation, the process of utilizing plants to absorb, accumulate, and detoxify heavy metals in soil, is considered an alternative strategy for the remediation of soils contaminated with heavy metals [20, 32]. This method is ecologically sound, safe, and cost effective, but its remediation efficiency is mostly affected by limiting factors, such as meteorological factors and the toxicity of pollutants [20, 30].

To promote the uptake efficiency of heavy metals by plants, many investigations have focused on the close relationship between plants and plant growth-promoting rhizobacteria (PGPR). Some rhizobacteria can reduce the toxicity of heavy metals, resulting in the stimulation of plant growth [8, 11, 15]. Some PGPR have a capacity to enhance the growth of the host plant by nitrogen fixation, mineral solubilization, as well as the transformation of nutrients, production of phytohormones and siderophores, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase [22]. Furthermore, some rhizobacteria can excrete organic acids to enhance the bioavailability of heavy metals [2]. Several established studies indicated that PGPR can promote the growth of plants under the toxicity of Ni, Pb, or Zn [10, 11, 23]. In addition, a variety of bacteria (mainly PGPR) have been reported as phytoextraction assistants; *Pseudomonas* spp. [9, 18, 33, 41], *Bacillus* spp. [33, 40], *Mesorhizobium* sp. [25], *Microbacterium* spp. [1, 41], *Rhizobium* spp. [1, 37], *Variovorax* sp. [5], *Rhodococcus* sp. [5], *Psychrobacter* spp. [31, 33], *Flabobacterium* sp. [5], *Sinorhizobium* sp. [16], and *Achromobacter* sp. [32].

Recently, microbe-assisted phytoremediation has appeared as a more successful approach for the remediation of soils

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contaminated with heavy metals. Therefore, the exploration of new microbial resources, including PGPR, is still necessary for the development of *in situ* remediation strategies under multifarious conditions. Moreover, a better understanding of the interaction between PGPR and their host plants is important for enhancing the efficiency of microbe-assisted phytoremediation. Therefore, in this study, a PGPR, *Serratia* sp., was isolated from heavy-metal-contaminated soils and then characterized. The plant growth-promoting abilities of the isolate were evaluated, and the effects of its inoculation on the growth of *Zea mays* determined under both heavy metal-contamination and noncontamination conditions.

Materials and Methods

Isolation and Identification of a PGPR, Strain SY5

Echinochloa crus-galli, grown in petroleum and heavy-metal-contaminated soil at a petroleum refinery facility in South Korea, was carefully sampled, and the soil adhering loosely to the root removed by shaking the plant. The soil firmly adhering to the root was collected by brushing (rhizosphere soil sample). After washing with distilled water (DW) several times, the root was also collected by cutting, and ground using a mortar (rhizoplane sample). One g fresh weight of rhizosphere soil, or the ground rhizoplane, was added to a 100-ml flask with 9 ml of sterilized DW, and the flask was then shaken at 250 rpm for 30 min. The resulting suspension was decimally diluted ($100\sim10^{-6}$) with sterilized DW. The diluted media were spread on LB-agar medium (Difco), with the plates incubated at 30°C for 3 days. After cultivation, 47 colonies from the plates inoculated with the rhizosphere soil and 24 colonies from the rhizoplane were screened for their morphological properties. The plant growth-promoting activities (PGPA) of the screened colonies were investigated, as follows:

To test the productivity of indol acetic acid (IAA) by the isolates, each isolate was inoculated in 5 ml of modified DF medium, supplemented with 0.5 mg/ml of L-tryptophane [17], and incubated on a rotary shaker (180 rpm) at 30°C for 5 days. The composition of the modified DF medium was as follows: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 4 g; $Na_2HPO_4{\cdot}12H_2O$, 15 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $FeSO_4{\cdot}7H_2O$, 1.0 mg; B (as H_3BO_3), 10 μg; Mn (as $MnSO_4{\cdot}H_2O$), 11 μg; Zn (as $ZnSO_4{\cdot}7H_2O$), 125 μg; Cu (as $CuSO_4{\cdot}5H_2O$), 78 μg; Mo (as $Na_2MoO_4{\cdot}2H_2O$), 17 μg; distilled water, 1 l. The resulting culture broth was mixed with Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled water, 7.5 ml of 0.5 M $FeCl_3{\cdot}6H_2O$) with a 1:2 (v/v) ratio, and allowed to stand at room temperature for 20 min. The pink color that developed, indicating IAA production, was measured at 530 nm with a spectrophotometer (8453 UV-Visible Spectrophotometer, Agilent Technologies, U.S.A.). The absorbance was converted into the concentration of IAA using a standard curve prepared with 3-indole acetic acid (C_8H_6N-CH_2COOH; Showa Chemical Co., Japan). Each test was carried out in triplicate.

The activity of the siderophore produced by the isolates was determined using blue agar plates, containing chrome azurol S (CAS) [39]. An orange halo around the colony on the CAS agar was indicative of siderophore excretion. Each colony was inoculated on the center of a CAS agar plate, with the increasing surface area of the orange halo around the colony periodically measured during incubation at 30°C

To compare the activities of the IAA and siderophore produced by the isolates, the bacterium showing the highest activities was named as SY5. Strain SY5 was identified by a 16S rDNA analysis. The colony of SY5 on the LB-agar plate was suspended with 30 μl of 0.05 M NaOH and then treated at 95°C for 30 min for extraction of its genomic DNA. The genomic DNA sample was used as the template for a polymerase chain reaction (PCR). PCR and sequencing were performed as described in a previous study [27], using 27f (5'-AGA GTT TGA TCM TGG CTC AC-3') and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT-3') primers. The 16S rDNA sequences of SY5 were compared with those present in the GenBank database using a BLAST (basic local alignment search tool) search, and then deposited (Accession No. AY125647).

Root Elongation Assay

The plant root elongation promoting activity of SY5 under heavy-metal-contaminated conditions (15 mg/l of Cd or Cu) was determined using *Zea mays*. The experiment was divided into three sets of treatment conditions: treatment 1, neither the addition of tryptophane nor SY5 inoculation; treatment 2, tryptophane addition; treatment 3, the addition of 30 mg/l of Cd or Cu solution, prepared with $CdSO_4$ or $CuSO_4$, and 1 g/l of tryptophane solution, filtrated using a sterilized filter (0.2 μm pore size). The SY5 colonies grown on the LB-agar plates were suspended in sterilized water to a final optical density of 0.8 at 600 nm. For treatment 1, 3 ml each of the metal solution and sterilized water were mixed. For treatment 2, 3 ml each of the metal solution and tryptophane solution were mixed. For treatment 3, 3 ml each of the metal solution and SY5 cell suspension were mixed. The 6 ml of solution prepared for each treatment was added to a sterilized glass Petri dish employing 2 layers of filter papers. The seeds of *Zea mays* were surface-sterilized with a mixture of ethanol and 30% H_2O_2 (1:1) for 20 min, washed with sterile water, and placed on the wetted filter paper, with 10 seeds per Petri dish. The primary and radicular root lengths of the seedlings were measured after incubation of the closed Petri dishes for 8 days at room temperature in the dark. The assay was duplicated for each treatment. A two-sample *t*-test was carried out using SPSS (version 12.0) ($p<0.05$).

Hydroponic Culture Test

The effect of SY5 inoculation on the growth of *Zea mays* was studied under Cd-contaminated and noncontaminated conditions using a hydroponic culture system. Eight liters of dechlorinated tap water, either with or without 5 mg/l of Cd, was added to a 10-l bottle, with an air pump employed for aeration. SY5 culture broth (100 ml), precultured in LB medium for 2 days at 30°C, was centrifuged at 7,000 ×*g* for 10 min, with the harvested cells washed twice with sterilized water. The washed cells were resuspended in 10 ml of sterilized water (OD=17 at 600 nm). Eight ml of the cell suspension was added to the bottle. The seeds of *Zea mays*, which had been surface-sterilized by the same method described above, were placed on a filter paper, wetted with sterilized water, in the glass Petri dish, and then cultured for 7 days at room temperature in the dark. The resulting seedlings were transferred to the bottle (10 seedlings per bottle), and cultured for 10 days at room temperature under natural lighting. During the culture periods, the hydroponic solution was continuously aerated, with the water lost because of

evaporation compensated for by supplying with sterilized water. The shoot length was measured every 2 days during the hydroponics. After 10 days, the seedlings were harvested, dried at 60°C, and cut into two parts (shoots and roots), and the dry biomass of the shoots and roots was then measured.

RESULTS

Isolation and Identification of a PGPR, Strain SY5

SY5, isolated from the rhizoplane of the *Echinochloa crus-galli* in petroleum and heavy-metal-contaminated soil, was identified by comparison of the 16S rDNA gene sequences in the NCBI database using a BLAST analysis. The closest relatives of strain SY5 was found to be *Serratia plymuthica* (AJ233433), with 99% similarity (1135/1139). SY5 was assigned an accession number of EF364430 in GenBank.

SY5 possessed the ability for both IAA production and siderophores synthesis (Fig. 1). The IAA concentration in the culture broth increased according to the growth of SY5, with 38 mg/l of IAA accumulated during 5 days (Fig. 1A). The area of the orange halo around the SY5 colony in the CAS-agar plate increased linearly during the incubation, indicating that SY5 could synthesize siderophores (Fig. 1B).

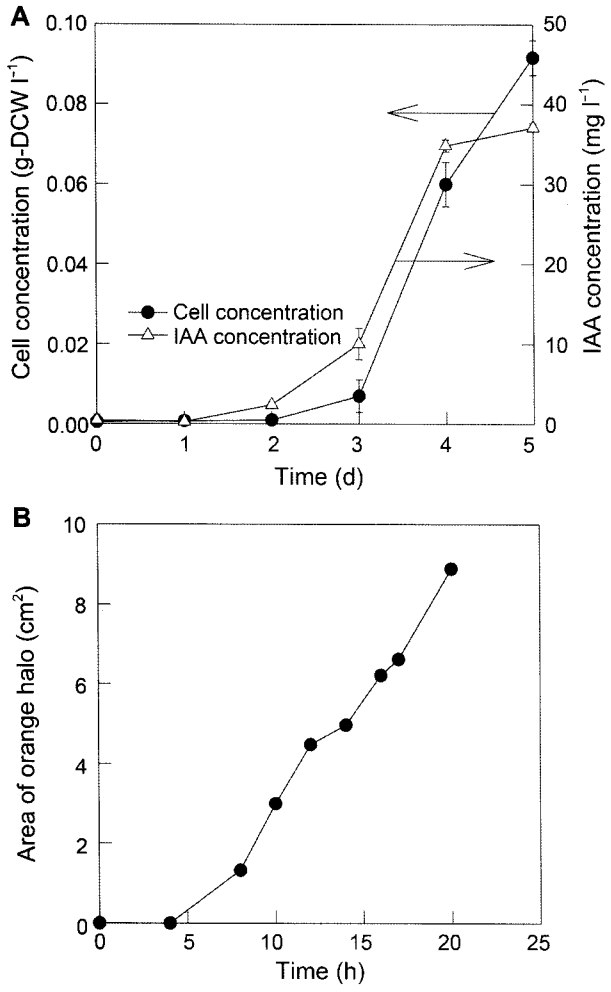

Fig. 1. Time course of the IAA concentration in the modified DF medium containing L-tryptophane (**A**) and orange halo area in the CAS-agar plate (**B**).

Root Growth-Promoting Ability of SY5

The effect of the inoculation of *Serratia* sp. SY5 on the root growth of *Zea mays* was determined under either Cd or Cu contamination conditions (Fig. 2 and 3). The effect of the addition of tryptophane was simultaneously tested as a positive control. Under contamination conditions (15 mg/l of Cd or Cu), the development of radicular roots of *Zea mays* was remarkably inhibited (Fig. 2, 3, and 4). The growth of primary root, on inoculation of SY5 or addition

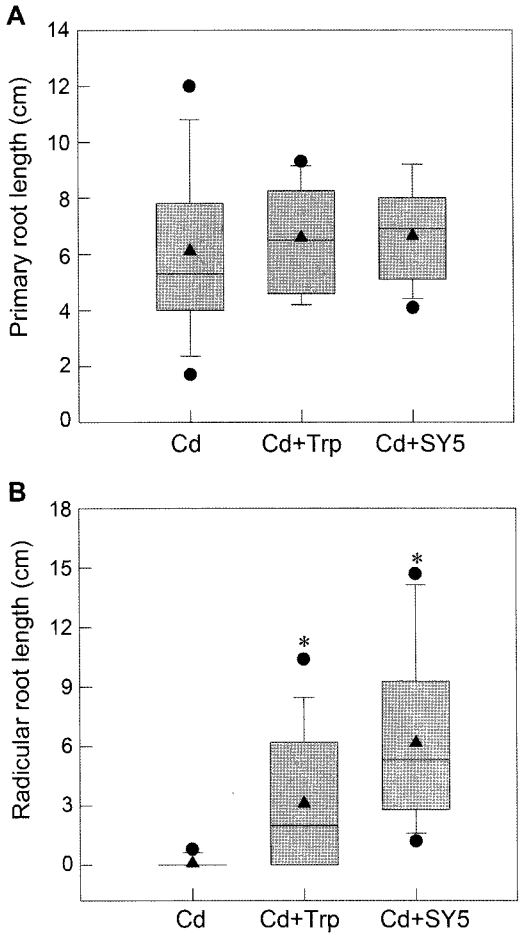

Fig. 2. Effect of the inoculation of SY5 on the root growth of corn seedlings in a 15 mg/l Cd solution.

The seedlings were cultivated for 8 days. **A**. Primary root length. **B**. Radicular root length. Cd control, no addition of tryptophane and no inoculation of SY5; Cd+Trp, addition of tryptophane; Cd+SY5, inoculation of SY5. Plots of the median, 10th, 25th, 75th, and 90th percentiles as vertical boxes, with error bars. Circles indicate out-of-range values; triangles indicated mean values. An asterisk (*) means significantly different to the control ($p<0.05$).

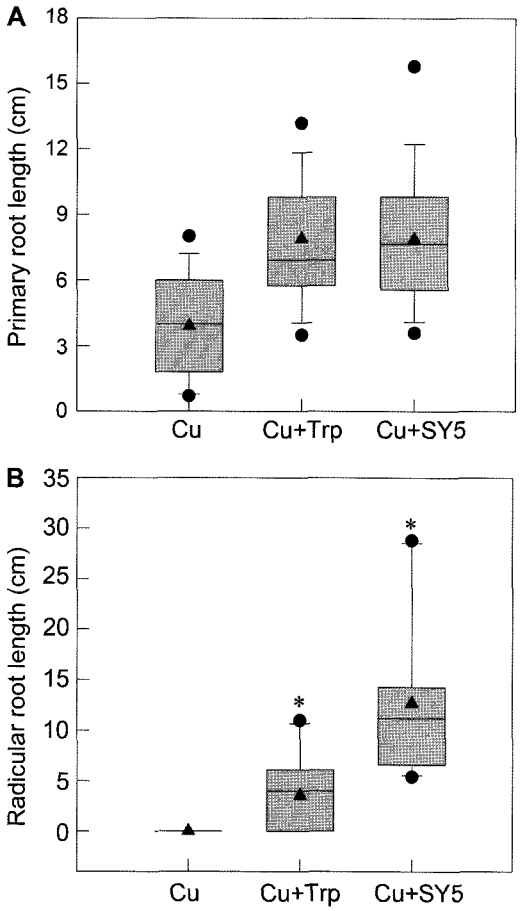

Fig. 3. Effect of SY5 inoculation on the root growth of corn seedlings in a 15 mg/l Cu solution.
The seedlings were cultivated for 8 days. **A.** Primary root length. **B.** Radicular root length. Cu control, no addition of tryptophane and no inoculation of SY5) Cu+Trp, addition of tryptophane; Cu+SY5, inoculation of SY5. Plots of the median, 10th, 25th, 75th, and 90th percentiles as vertical boxes, with error bars. Circles indicate out-of-range values; triangles indicated mean values. An asterisk (*) means significantly different to the control ($p<0.05$).

of tryptophane treatment under heavy-metal toxicity was not significantly different from that with nontreatment ($p>0.05$). However, both treatments significantly promoted the growth of radicular roots. On treatment by SY5 inoculation, the radicular root lengths with 15 mg-Cd/l and 15 mg-Cu/l were promoted 27.0- and 15.4-fold that of the control, whereas the radicular root lengths with tryptophane were increased 16.8- and 10.2-fold, respectively. The effects on primary root growth of the two treatments were similar, whereas the radicular root-promoting effect of SY5 inoculation was 2.5-fold better than that of tryptophane treatment. Fig. 4 shows that the inoculation of SY5 or addition of tryptophane had a beneficial effect on the radicular root length of *Zea mays*, and the effect of SY5 was better than that of tryptophane. With 30 mg/l Cd or Cu treatment, the root growth was similar to that with 15 mg/l treatment (data not shown).

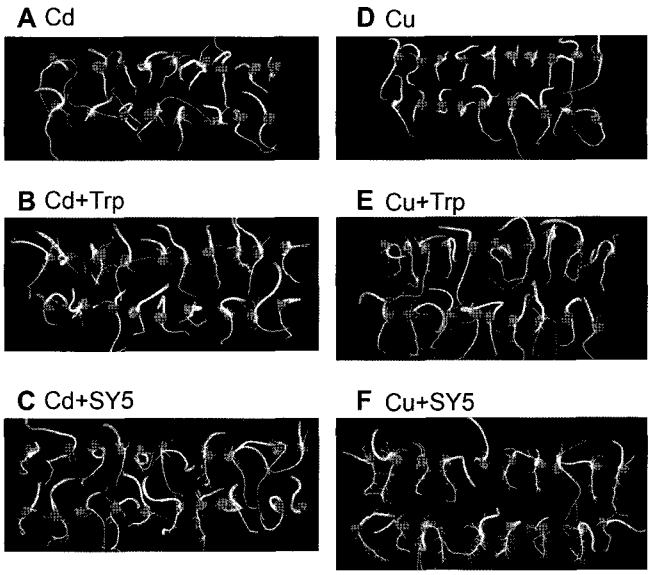

Fig. 4. Seedlings of *Zea mays* cultivated in the presence of 15 mg/l Cd or Cu solution for 8 days.
A. Cd control, with 15 mg/l Cd solution. **B.** Cd+Trp, the addition of tryptophane with 15 mg/l Cd solution. **C.** Cd+SY5, the inoculation of SY5 with 15 mg/l Cd solution. **D.** Cu control, with 15 mg/l Cu solution. **E.** Cu+Trp, addition of tryptophane with 15 mg/l Cu solution. **F.** Cu+SY5, inoculation of SY5 with 15 mg/l Cu solution.

Plant Growth-Promoting Ability of SY5 in Hydroponic Culture

The plant growth-promoting ability of SY5 was determined under Cd-contaminated and noncontaminated conditions in hydroponic cultures. Under noncontaminated conditions, the inoculation of SY5 significantly increased the shoot growth during the early growth period compared with the non-inoculated control ($p<0.05$); whereas, no significant difference in shoot lengths was observed during the later growth period between the control and inoculated conditions (Fig. 5A). Comparison of the shoot biomasses between the two sets of conditions also showed that the shoot biomass increased with SY5 inoculation ($p<0.05$, Fig. 6A). The root biomass under SY5 inoculated conditions was greater than that of the control, but without significant difference.

Under 5 mg/l Cd contaminated conditions, the early shoot growth was also promoted and the shoot biomass increased by SY5 inoculation, but these improvements were not statistically significant ($p>0.05$, Fig. 5B and 6B). However, the root biomass was considerately increased by SY5 inoculation ($p<0.05$, Fig. 6B).

Discussion

Plant growth-promoting rhizobacteria (PGPR) colonizing the surface or inner part of roots play an important positive role that directly or indirectly influences plant growth and development [20, 22]. In this study, a new PGPR, *Serratia*

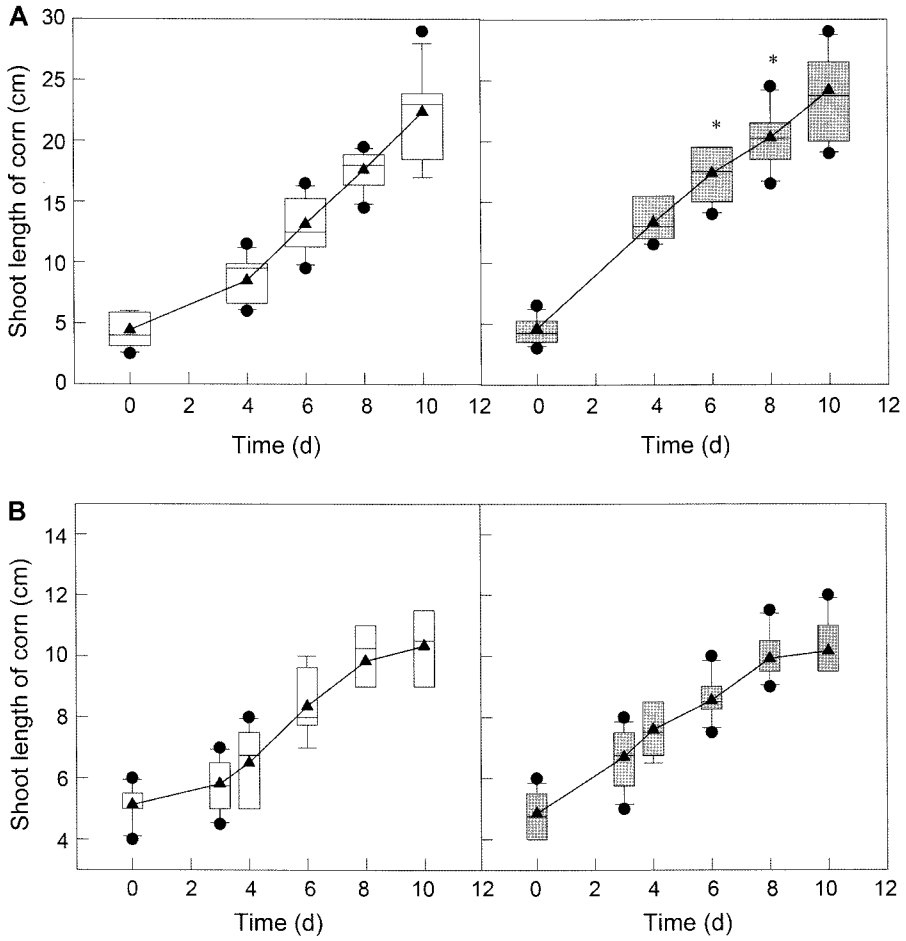

Fig. 5. Effect of the inoculation of SY5 on the growth of corn shoots.
A. Noncontaminated conditions. **B**. Five mg/l Cd-contaminated conditions. White box, without inoculation of SY5; Gray box, with inoculation of SY5. Plots of the median, 10th, 25th, 75th, and 90th percentiles as vertical boxes, with error bars. Circles indicate out-of-range values; triangles indicate mean values. An asterisk (*) means significantly different to the control ($p<0.05$).

sp. SY5, was isolated from the rhizoplane of *Echinochloa crus-galli*; this bacterium also showed both the ability for IAA productivity (Fig. 1A) and siderophores synthesis (Fig. 1B). *Serratia plymuthica*, the closet relatives of SY5, is a Gram-negative bacterium frequently associated with plants [6, 7, 12, 13]. *S. plymuthica* has been isolated from the rhizosphere of wheat, oat, cucumber, maize, oil-seed rape, and potato [4, 24, 28]. *S. plymuthica* has the ability of siderophores synthesis and antibiosis, and also possesses fungal cell-wall degrading enzymes, such as chitinases and β-1,3-glucanases [7, 13, 28]. The endophytic bacterium, *S. plymuthica,* could impart beneficial effects for the protection of cucumber plants against the fungal plant pathogen, *Pythium* [6, 34]. Therefore, this antifungal bacterium has been used as a biological control agent against fungal plant pathogens in agriculture to help prevent disease in plants [6, 29, 34, 42].

IAA is a common metabolite of tryptophane by several microorganisms, including PGPR [19], and is absorbed to the roots as a plant growth regulator. Therefore, close symbiotic relationships are developed between plants and IAA-producing rhizobacteria. In the study of Poonguzhali *et al.* [36], the PGPR from the rhizosphere of *Brassica campestris* ssp *pekinensis* was shown to produce 6.02–29.75 μg/ml of IAA. The heavy-metal-resistant isolates from the rhizosphere of *Brassica juncea* L. Czern. have shown IAA productivities of 0.4–43 μg/ml [5], and Ni mobilizing plant growth-promoting bacteria have IAA productivities of 14–111 μg ml^{-1} [33]. Therefore, the *Serratia* sp. SY5 exhibited relatively higher IAA productivity than those isolated in previous studies.

Owing to the physiochemical properties of iron, which exists in the trivalent state as the oxyhydroxide under aerobic and biological pH, it is virtually insoluble [39]. Heavy-metal contamination of soil causes iron deficiency in plants [9]. Iron deficiency inhibits the synthesis of chloroplasts and chlorophyll, and induces the production of ethylene in plant tissue, which acts as stress matter to

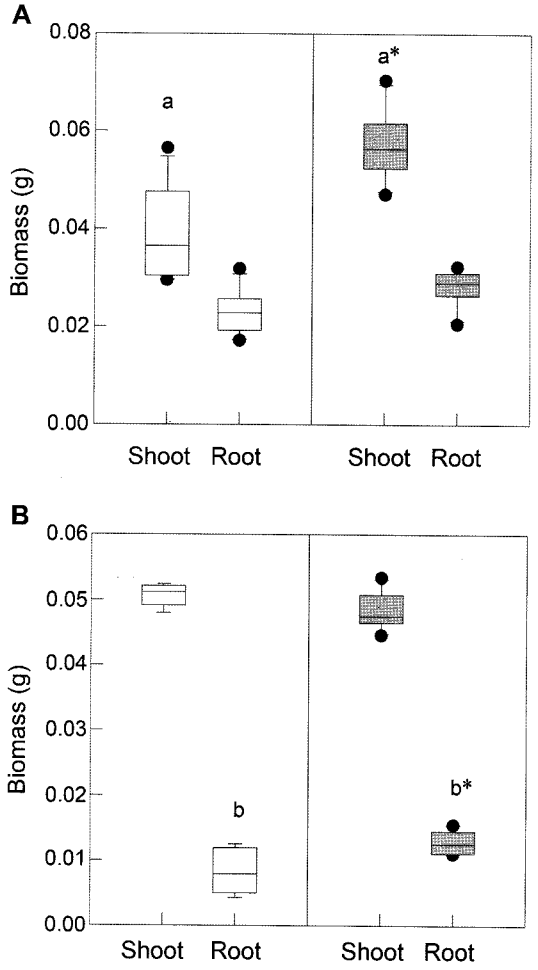

Fig. 6. Effect of the inoculation of SY5 on the corn biomass after 10 days of cultivation.
A. Noncontaminated conditions. **B.** 5 mg/l Cd-contaminated conditions. White box, without SY5 inoculation; gray box, with SY5 inoculation. Plots of the median, 10th, 25th, 75th, and 90th percentiles as vertical boxes, with error bars. Circles indicate out-of-range values; triangles indicate mean values. An asterisk (*) means significantly different to the control ($p<0.05$).

plants, eventually leading to decreased remediation efficiency [21, 26]. Many soil microbes use a ferric-specific ligand, termed "siderophores," with a high affinity and low molecular-weight, for the take up of the essential element iron [21, 26]. These microbial iron–siderophores complexes, which bind iron, can be taken up by plants as an iron source [43]. Therefore, siderophores synthesis by *Serratia* sp. SY5 may cause growth promotion of corn.

The growth of plant roots is generally inhibited owing to heavy-metal toxicity. The addition of SY5 or tryptophane could promote the growth of roots, especially radicular roots ($p<0.05$), and the effect of SY5 was relatively higher than that of tryptophane (Fig. 2 and 3). The production of IAA was found to be tryptophane concentration dependant, and the production of IAA increased with increasing tryptophane concentration [3]. The effects of IAA on root growth differed with concentration: Primary root growth was promoted at low IAA concentrations, whereas a high level of IAA stimulated radicular root growth [14].

Based on the hydroponic culture results (Fig. 5 and 6), the inoculation of *Serratia* sp. SY5 exhibited a favorable influence on the initial growth of shoots as well as shoot biomass under noncontaminated conditions. Interestingly, the beneficial effect of inoculating *Serratia* sp. SY5 was markedly displayed by the increased root biomass under Cd-contaminated conditions. The enhancement of root growth on SY5 inoculation may have been caused by the protection of the roots against the toxic effect of heavy metals; however, further study is required to understand why these effects on corn growth are different under temperate (non-contaminated) and stress (contaminated) conditions.

Our study has demonstrated a PGPR, *Serratia* sp. SY5, which could be applied as a promising microbial inoculant for the direct stimulation of plant biomass production, and to indirectly enhance heavy-metal uptake by plants in the phytoremediation of heavy-metal-contaminated soils.

Acknowledgments

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