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Effects of Application of *Rhodopseudomonas* sp. on Seed Germination and Growth of Tomato Under Axenic Conditions

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Abstract Purple nonsulfur bacteria were isolated from river sediments and their growth promoting capabilities on tomato were examined. Isolated strains KL9 and BL6 were identified as *Rhodopseudomonas* spp. by 16S rDNA sequence analysis. *Rhodopseudomonas* strain KL9 maximally produced 5.56 mM/min/mg protein and 67.2 μM/min/mg protein of indole-3-acetic acid (IAA) and 5-aminolevulinic acid (ALA), respectively, which may be one of the mechanisms of plant growth enhancement. The germination percentage of tomato seed, total length, and dry mass of germinated tomato seedling increased by 30.2%, 71.1%, and 270.8%, respectively, compared with those of the uninoculated control 7 days after inoculation of strain KL9. The lengths of the root and shoot of germinated seedling treated with 3 mM tryptophan, a precursor of IAA, increased by 104.4% and 156.5%, respectively, 7 days after inoculation of strain KL9. *Rhodopseudomonas* KL9 increased 123.5% and 54% of the root and shoot lengths of germinated seedling, respectively, treated with 15 mM glycine and succinate, precursors of ALA. This plant growth promoting capability of purple nonsulfur bacteria may be a candidate for a biofertilizer in agriculture.

Keywords: Purple nonsulfur bacteria, tomato, plant growth promotion, seed germination, phytohormone

Chemical nitrogen and phosphate fertilizers are used worldwide to increase the yield of agricultural crop plants. Chemical fertilizers have been successful to improve crop production, but these compounds have induced some environmental problems, such as eutrophication of receiving waters, and contributed to a number of human and animal health problems. The negative effects of the enormous use of chemical fertilizers have been challenged to be solved by many scientists and are being substituted by biofertilizers without negative effects. There are many reports on plant growth promotion and yield enhancement by plant growth promoting bacteria (PGPB), as well as growth promoting mechanisms such as solubilization of insoluble phosphates [24], nitrogen fixation [15], production of phytohormones [6], and lowering of ethylene concentration by 1-aminocyclopropane-carboxylate deaminase [9]. However, to date, most studies have been focused on some plant growth promoting rhizobacteria (PGPR) [20, 22, 25, 26]. Recently, purple nonsulfur bacteria (PNSB) are being used in agriculture, although the precise mechanisms of growth promotion have not been elucidated [7].

Among plant growth promoting phytohormones, 5-aminolevulinic acid (ALA) has been well known as a precursor of tetrapyrroles such as vitamin B_{12}, heme, and bacteriochlorophyll. Recently, ALA has received great attention as a biodegradable herbicide [3]. In addition, ALA has been reported to promote the growth and yield of agricultural crops at low concentration and to improve the salt tolerance of cotton seedlings by the enhancement of plant photosynthesis and chlorophyll content [12, 28]. Indole-3-acetic acid (IAA) is a typical phytohormone, and influences various cellular functions and regulation of plant growth and development. IAA is involved in the stimulation of cell division and induction and elongation of root and shoot growth [14]. PNSB can also synthesize IAA, like various plant-associated bacteria [23]; however, the effect of IAA produced by PNSB on plant growth promotion has not been investigated.

In the present study, many strains of PNSB were isolated from the river sediment, and then, some mechanisms for plant growth promotion including the production of ALA and IAA were examined. Their efficacy of seed germination and growth enhancement was tested on tomato under axenic conditions.

Materials and Methods

Enrichment and Isolation of Purple Nonsulfur Bacteria

Sediment samples were collected from Gongji-cheon, Korea. For enrichment culture, 1 g of river sediment was inoculated into 15 ml of modified Biebl and Pfennig's medium [1] [composition in l^{-1}: 0.5 g KH_2PO_4, 0.2 g

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$MgSO_4 \cdot 7H_2O$, 0.4 g NaCl, 1.2 g NH_4Cl, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.3 g yeast extract, 3 g organic acids (1.0 g each of malate, succinate, and acetate), 5 ml ferric citrate (0.1% w/v), 1 ml SL7 {(composition in mg/l): 25% (v/v) HCl 1 ml, $ZnCl_2$ 70, $MnCl_2 \cdot 4H_2O$ 100, H_3BO_3 60, $CoCl_2 \cdot 6H_2O$ 200, $CuCl_2 \cdot H_2O$ 20, $NiCl_2 \cdot 6H_2O$ 20, $NaMoO_4 \cdot 2H_2O$ 40}, pH 6.8–7.0 before autoclaving] in a 15-ml screw cap tube, and solid medium was prepared with addition of 2% agar. The cultures were incubated under anaerobic conditions in the presence of light (light intensity of 3,000 lux from fluorescent lamps) at 30°C for 10 days. When the culture showed redish purple color, a 5 ml inoculum was transferred into a fresh medium and incubated again under the same conditions. The enrichment culture was then spread onto Biebl and Pfennig's agar plates and overlaid with paraffin wax [1]. The plates were incubated in an anaerobic chamber (Model 1025 Anaerobic System, Forma Scientific, U.S.A.) under lights at 30°C for 10 days, and 64 colonies were transferred to separate fresh liquid medium and incubated as described above. Among 64 isolated strains, 35 strains that showed a different colony morphology and higher growth rate were selected for the following experiments. The isolates were purified by the repeated paraffin wax-overlay method.

Identification of Purple Nonsulfur Bacteria

For the sequence analysis, bacterial genomic DNA was extracted and purified using a G-spin genomic DNA extraction kit (iNtRON, Korea). Universal primers 518F (5'-CCAGCAGCCGCGGTAAT-3') and 800R (5'-TACCAGGGTATCTAATCC-3') were used for 16S rDNA amplification of the strains KL9 and BL6, which showed the highest growth rate among 35 isolated strains. PCR products were purified with a PCR purification kit (PCR quick-spin, iNtRON, Korea) and directly sequenced in an automated sequencing process (Macrogen, Korea). The sequences were compared with BLAST analysis (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/).

Determination of Indole-3-Acetic Acid and 5-Aminolevulinic Acid

Indole-3-acetic acid (IAA) concentrations in the culture supernatants grown anaerobically were determined with the Salkowski's reagent [10]. After incubation for 42 h in Biebl and Pfennig's medium containing 3 mM L-tryptophan, bacterial cells were removed from the culture medium by centrifugation (10,000 ×g, 15 min). A 1-ml aliquot of the supernatant was mixed with 4 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$) and incubated at room temperature for 20 min. The red color resulting from the chemical reaction was quantified at 535 nm with a Shimadzu UV-1700 spectrophotometer (Shimadzu Co., Japan). A standard curve was prepared from serial dilution of a 100 mg/l IAA (Sigma Chemical Co., U.S.A.) stock solution in ethanol.

Production of 5-aminolevulinic acid (ALA) by PNSB was quantified by the method of Mauzerall and Granick [19]. To 0.5 ml of culture supernatant, 0.5 ml of 1 M sodium acetate buffer (pH 4.7) and 50 µl of acetylacetone were added, and then tubes were boiled in a water bath for 15 min. After cooling, 3.5 ml of modified Ehrlich's reagent [1 g *p*-dimethylaminobenzaldehyde, 42 ml of glacial acetic acid, 8 ml of 70% (v/v) perchloric acid] were added. The absorbance of the mixture was measured at 556 nm after 20 min at room temperature. The concentration of ALA in the culture supernatant was determined by comparison with a standard curve.

The solubilization of insoluble phosphate by PNSB was tested after 7 days of incubation in the $Ca_3(PO_4)_2$ (200–800 mg/l) containing Biebl and Pfennig's medium at 30°C, 150 rpm. Soluble phosphate in the culture supernatant was analyzed by using the vanadomolybdophosphoric acid colorimetric method [4].

Tomato Seed Germination and Growth Promotion Assay

The germination and root elongation assay was performed with tomato (*Lycopersicon esculentum* Mill. cv. Poongyoung) seed to estimate effects of seed treatment with purple nonsulfur bacteria. Seeds of tomato (Zeus) were purchased from Dongbu HiTek Co., and disinfected by agitation for 1 min in 70% ethanol and 10 min in 1% sodium hypochlorite solution. Bacterial cells were washed twice with sterile distilled water, and the inoculation density of the bacterial suspension was adjusted to 1×10^7 cells/ml. Five ml of the suspension of live bacteria, autoclaved bacteria (killed control) or sterile water (uninoculated control) was added to filter paper in a glass Petri dish on which 10 disinfected tomato seeds were placed. Germination of tomato seeds was examined 3 days after inoculation, and the root and shoot lengths of the germinated tomato seedlings were measured after 7 days of incubation at 25°C beginning with a cycle of 10 h dark followed by 14 h of light with 280–325 µE m^{-2} s^{-1} of photosynthetically active radiation. The assay was repeated three times with 10 tomato seeds per Petri dish for each treatment.

The effect of KL9 inoculation on the growth of tomato seed was also investigated in the germination test with the supplement of 3 mM tryptophan, a precursor of IAA, or 15 mM succinate and glycine, precursors of ALA. Incubation and measurement of root and shoot lengths of tomato seedlings grown were the same as the above experiment.

Measurement of Length and Dry Mass of Germinated Tomato Seedling

The lengths of the shoot (leaves+stem) and root of germinated tomato seedling were measured separately. The dry mass

of whole tomato seedling was determined after drying at 80°C for 7 days.

All experiments were carried out in triplicate, and the data were analyzed by Student's *t*-test.

RESULTS AND DISCUSSION

Isolation and Identification of Purple Nonsulfur Bacteria

Thirty-five photosynthetic purple nonsulfur bacterial strains were isolated from river sediments, and then, the fastest growing strains, KL9 and BL6, were selected and identified. Both strains, Gram-negative rod-shaped bacteria, formed purple-colored colonies on a paraffin wax-overlayed PNSB agar plate and were a purple color in PNSB liquid medium. Analysis of the 16S rDNA sequence was performed for the identification. In the case of matching in the NCBI nucleotide Blast for sequenced 16S rDNA, KL9 (GenBank Accession No. EF204997) and BL6 (GenBank Accession No. EF221638) were matched between type strains and phylogenetically identified by the neighbor-joining method based on the Jukes-Cantor model [13] as *Rhodopseudomonas faecalis* gc^T (GenBank Accession No. AF123085), with the similarity of 98.69% and 99.27%, respectively. Various morphological and biochemical tests are necessary for the precise identification to the species level.

Production of IAA and ALA

There are many mechanisms for plant growth promotion by PGPB, including production of phytohormones. Among several phytohormones, IAA and ALA production rates by the isolated strains of purple nonsulfur bacteria were measured. The highest concentration of IAA in the culture of *Rhodopseudomonas* KL9 strain reached to 51.8 mg/l after 36 h of incubation; however, the production rate based on bacterial protein increased until 48 h of incubation and showed 5.56 mM/min/mg protein. IAA production by *Rhodopseudomonas* BL6 strain was much lower and slower than that of KL9 (Fig. 1A), and showed the maximal production rate, 0.44 mM/min/mg protein, after 72 h of incubation. The IAA production rate of *Rhodopseudomonas* KL9 strain was much higher than that (14.5–32.7 μg/ml) of *Pseudomonas putida* GR12-2, which enhanced 35% to 50% root elongation in canola seeds [21], but slightly lower than 61 mg/l by another purple nonsulfur bacterium, *Rhodobacter sphaeroides*, under the same experimental conditions [28]. ALA production in the KL9 culture began quickly and maintained a high level during several days (Fig. 1B). The highest concentration of 8.57 mg/l in the culture was achieved after 48 h of incubation, and the production rate based on bacterial protein reached 67.2 μM/min/mg protein. In contrast, ALA production was very low in the BL6 culture. There is a report on plant growth promoting properties of ALA at low concentrations (0.01–10 mg/l)

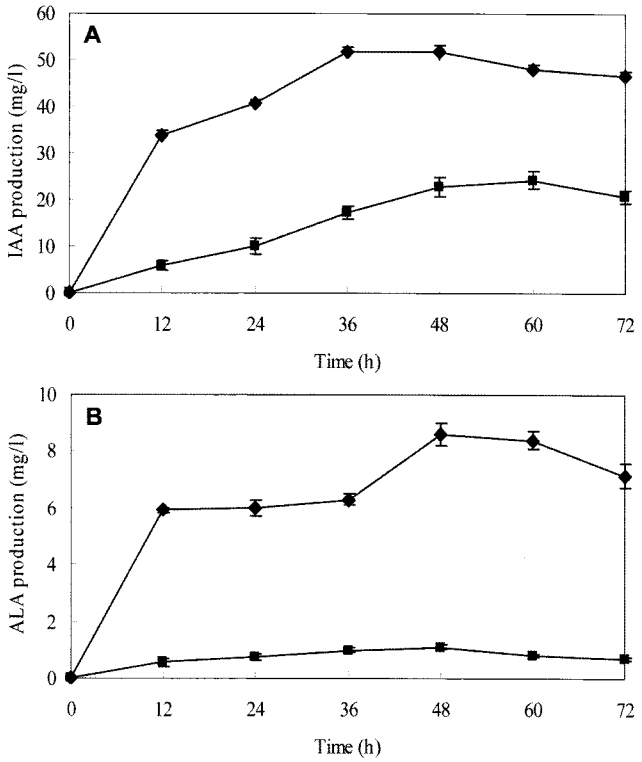

Fig. 1. Production of IAA (**A**) and ALA (**B**) by *Rhodopseudomonas* KL9 (◆) and BL6 (■).
Tryptophan (3 mM) was added for IAA production, and 15 mM glycine and succinate were supplemented for ALA production.

[12]. However, it can suppress plant growth at higher concentrations (>2 mM), and recently, it has received attention as a biopesticide [3]. The production range of ALA by *Rhodopseudomonas* KL9 strain may be in the appropriate range for plant growth promotion.

Plants themselves can synthesize phytohormones, but they can also utilize exogenous sources such as microbially produced phytohormones [9], and it may be one of the mechanisms of plant growth promotion by microorganisms. There have been many reports on the microbial production of phytohormones. However, most have focused on the rhizobacteria, and there are a few reports on the IAA production by purple bacteria [23]. ALA production by purple bacteria has not been studied at all. Although the production of IAA and ALA by *Rhodopseudomonas* strains was analyzed under anaerobic culture condition, whereas the growth promotion assay was carried out under aerobic condition in this study, those *Rhodopseudomonas* strains could grow well even under aerobic conditions and produce IAA and ALA at levels of 60–70% compared with those under anaerobic condition (data not shown). This study shows that, besides rhizobacteria, purple nonsulfur bacteria can also produce phytohormones that may be utilized for plant growth promotion.

Other mechanisms for plant growth promotion of *Rhodopseudomonas* KL9 and BL6 strains were also

investigated. KL9 and BL6 strains produced 90.0–95.0 mg/l and 63.6–70.0 mg/l of soluble phosphate, respectively, from 200–800 mg/l of insoluble $Ca_3(PO_4)_2$. The initial pH of the medium was 7.0 and the final pH of the medium was 6.5–6.7. These solubilization rates of insoluble phosphate by PNSB were not higher than those of other reports [22, 29], because the PNSB medium pH could not be lowered by KL9 and BL6 during the incubation periods. Decrease of pH down to around 4 has been reported as a major solubilizing mechanism of inorganic phosphate by PGPB [29]; however it may not be probable in natural environments. Nitrogen fixation has been well known as a major mechanism for plant growth promotion by some N-fixing bacteria [15, 18]. Since most purple nonsulfur bacteria can fix dinitrogen, nitrogen fixation by KL9 and BL6 was measured by the micro-Kjeldahl method [4]. However, N-fixation by KL9 and BL6 was not detected, and it may be due to a low amount of nitrogen fixed by these bacteria, which could not be detected by the micro-Kjeldahl method. A more sensitive method, such as acetylene reduction assay, is necessary for the quantification of nitrogen fixed by KL9 and BL6. Production of other phytohormones, such as gibberellins and cytokinin, by purple nonsulfur bacteria should be investigated. Common metabolites such as various amino acids and organic acid, and some other growth factors produced by bacteria, could be a nutrient supplement for plants and they can also stimulate some beneficial indigenous bacteria, which ultimately results in plant growth promotion.

Effects on Tomato Germination and Growth by PNSB Under Axenic Conditions

The effects of each PNSB strain on germination of tomato seeds under axenic conditions are shown in Table 1. The germination percentage of the tomato seeds treated with *Rhodopseudomonas* KL9 strain (96.8%) was higher compared with the uninoculated control (73.4%) and killed KL9 control (76.2%); however, the inoculation of *Rhodopseudomonas* BL6 did not show a high increase of germination. Inoculation of strains KL9 and BL6 increased the germination percentage of uninoculated control by 31.8% and 7.6%, respectively. The increase of seedling emergence of tomato by *Rhodopseudomonas* KL9 was higher than the 26% by *Azotobacter chroococcum*, *Azospirillum* sp., and *Pseudomonas fluorescens* [11], and much higher than the germination rate of canola inoculated with *Methylobacterium fujisawaense* [17]. This result also indicates that seed germination increased through bacterial metabolism, since the germination rates by killed controls were similar to that of the uninoculated control. It seems that purple nonsulfur bacteria can increase the germination rate of tomato seeds better than typical plant growth promoting rhizobacteria.

Table 1. Effects of inoculation of *Rhodopseudomonas* strains KL9 and BL6 on germination and growth of tomato under axenic conditions at 25°C.

Inoculation	Germination (%)[b]	Plant length (mm)[c]	Dry mass (mg)[d]
CTL[a]	73.4±1.3	28.8±0.1	24.6±1.8
Killed BL6	71.0±0.7	27.7±0.1	48.8±4.2
BL6	79.0±0.7	39.3±0.2	67.5±2.4
Killed KL9	76.2±0.8	31.2±0.1	36.7±3.0
KL9	96.8±1.0	49.2±0.3	89.5±2.5

Data were analyzed by Student's *t*-test (n=3).
[a]CTL: uninoculated control.
[b]Germination percentage was determined 3 days after inoculation of bacteria.
[c,d]Length and dry mass of germinated tomato seedling were measured 7 days after inoculation of bacteria.

Germinated tomato seedlings, which were treated with either *Rhodopseudomonas* strains KL9 and BL6, showed significantly longer lengths of seedlings than those of killed controls and uninoculated control (Table 1). KL9 strain increased the whole length of tomato seedling by 70.8% and 57.7% compared with the uninoculated control and autoclaved control, respectively, and strain BL6 increased these by 36.5% and 41.9%, respectively. Elongation effects of germinated tomato by *Rhodopseudomonas* KL9 and BL6 were higher than that (6–8%) by 2 strains of *Pseudomonas fluorescens*, although they were inoculated to tomato seedling in a greenhouse [27]. The elongation effect on tomato seedling by KL9 was also higher than the root elongation of canola (64.8% and 62.2%) by *Pseudomonas putida* [16].

In addition, the dry mass of the germinated tomato seedlings increased by 174.4% and 263.8% with treatment of *Rhodopseudomonas* BL6 and KL9, respectively (Table 1). The inoculation of tomato seeds by *Azotobacter chroococcum*, *Azospirillum* sp., and *Pseudomonas fluorescens* increased the total plant weight from 40% to 60% as compared with the control after 7 days [11]. *Azospirillum* and *Pseudomonas fluorescens* 313 could increase fresh weights of germinated tomato root by 16%–21% [2]. In a 60-day greenhouse study, only a 4%–5% increase of plant fresh weight of tomato was achieved by *Pseudomonas fluorescens* GPR3 and PRS9 [27]. When 4 commercial composts were added to stimulate PGPR and subsequent tomato growth, 66.7%–73.8% increases of the dry weight of tomato plant were observed [5]. Maudinas *et al.* [18] reported that dry weights of rice plants treated with the purple nonsulfur bacterium *Rhodopseudomonas capsulata* and *Azotobacter vinelandii* increased up to 257%. When compared withe the above results, in spite of the different experimental conditions, *Rhodopseudomonas* BL6 and KL9 are certainly efficient plant growth promoting bacteria.

The lengths of the root and shoot of germinated seedling were measured after inoculation of tomato seeds treated with tryptophan, a precursor of IAA, and glycine and succinate, precursors of ALA, to confirm the plant growth

Table 2. Lengths of germinated tomatoes inoculated with *Rhodopseudomonas* KL9 and BL6 and treated with 3 mM tryptophan as a precursor of IAA or 15 mM glycine and succinate as precursors of ALA. Plant length was determined after 7 days of incubation at 25°C.

Chemical treatment	Inoculation	Length (mm)[a]	
		Root	Shoot
3 mM tryptophan	CTL[b]	31.5±1.2	10.4±2.3
	Killed BL6	36.5±1.8	20.5±1.8
	BL6	51.8±2.0	17.0±1.4
	Killed KL9	33.7±1.4	19.0±1.1
	KL9	64.4±2.0	24.6±1.4
15 mM glycine and succinate	CTL[#]	29.8±1.8	17.8±1.6
	Killed BL6	31.7±2.0	22.0±1.1
	BL6	47.0±1.8	19.8±1.3
	Killed KL9	26.1±2.4	21.0±2.5
	KL9	67.0±3.4	26.8±2.2

Data were analyzed by Student *t*-test (n=5).
[a]CTL: uninoculated control.
[b]Lengths of germinated tomato seedlings were measured 7 days after inoculation of bacteria.

promoting effects of *Rhodopseudomonas* BL6 and KL9, which can produce both phytohormones. Root and shoot lengths of germinated tomato seedlings treated with tryptophan and strain KL9 increased by 104.4% and 136.5%, respectively, compared with the control, which was treated with tryptophan but uninoculated, and 91.1% and 29.5%, respectively, compared with the tryptophan-treated killed KL9 control (Table 2). The elongation efficiency of tomato seedling by strain BL6 was lower than that of KL9. The lengths of the root and shoot of germinated seedling from KL9-inoculated seeds with the addition of glycine and succinate increased by 124.8% and 50.6%, respectively, compared with the uninoculated control. In the case of strain BL6, 57.7% and 11.2% of enhancement of root and shoot lengths, respectively, were observed (Table 2). It seems that the difference of IAA and ALA production between KL9 and BL6 resulted in a difference of plant growth promoting capability of tomato with or without precursors of IAA and ALA.

Germination and growth promoting effect on tomato were demonstrated by purple nonsulfur bacteria, and it might be caused mainly by IAA and ALA production and partly by P solubilization, all of which are direct modes of the plant growth promotion mechanism. *Rhodopseudomonas* strain KL6 can be utilized as an efficient and environmentally friendly biofertilizer for agricultural crops including tomato. IAA production could be further enhanced by additional carbon substrates [23], and tomato growth promotion by PGPR could be significantly increased by organic and inorganic fertilizers [27]. Therefore, it is expected that *Rhodopseudomonas* strain KL9 supplied with various nutrients in agricultural environments can greatly enhance plant growth. The contents of IAA and ALA in the grown tomato plants should be determined, and other direct and indirect mechanisms of plant growth promotion by this bacterium should be examined in a further study. Molecular biological techniques, such as denaturing gradient gel electrophoresis or terminal restriction fragment-length polymorphism, are necessary to investigate the survival and fate of inoculated PNSB and their influence on the indigenous bacterial community for their application in agriculture.

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