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Article

In vitro Evaluation of the Mechanism of Antagonism and Phosphate Solubilization by the Insect Gut Bacteria *Pseudomonas* sp. PRGB06 that Exhibits Plant Growth Promotion and Bio-Fertilizing Traits

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***Pseudomonas* sp. PRGB06, a bacterial strain isolated from diamondback moth (*Plutella xylostella*) gut, was examined for its plant growth promotion and biofertilizing traits. The bacteria growth was observed under various conditions of carbon sources, temperature, pH and salt concentrations. In addition, the mechanisms of antagonism and phosphate solubilization were investigated. The bacterial strain PRGB06, grew well using most of the tested carbon sources. The best growth was observed at 30℃ and pH 7. The inhibition of the pathogenic fungi was likely due to the volatile antifungal metabolite and ammonia gas produced by the bacteria. A significant positive relationship was found between the phosphate solubilization and acid production. When inoculated with PRGB06 *in vitro* and in gnotobiotic condition, red pepper and maize showed increase in root length, seedling vigor and dry bio-mass.**

Key words: Diamondback moth, *Pseudomonas*, Volatile antifungal metabolite, Ammonia, P solubilization, Red pepper, Maize

Introduction

Pseudomonads are known to occur in a variety of habitats, including insect gut (Indiragandhi et al., 2007). Fluorescent species of *Pseudomonas*, viz., *P. fluorescens* and *P. putida* were identified from soil microorthropod collembola (Thimm et al., 1998) and a *Pseudomonas* sp. from soil macrofauna (e.g. earthworm and snail) has been reported (Hameeda et al., 2006). These microbes contain unique enzymes and secrete secondary metabolites, thus several biotechnological applications of those *Pseudomonas* sp. have been suggested. For instance, several *Pseudomonas* species fall in the category of promising plant growth-promoting bacteria, and a number of them have been studied for their antagonistic properties and phosphate solubilizing functions (Bano and Mussarat, 2003; Pandey et al., 2006). One of the practical applications of the microbial communities isolated from the insect gut is to use them as potential bio-inoculants in crop production aiming to reduce the input of synthetic fertilizers.

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Plant growth promoting bacteria (PGPB) plays a critical role in sustainable agriculture by serving as biofertilizers - an effective alternative for chemical fertilizers that have raised economical, social, and environmental concerns during the last decades. So far, the introduced PGPR have been mostly isolated from rhizosphere, phyllosphere, soil belongings, cattle manure, and milk (Swain and Ray et al., 2006; Nautiyal et al., 2005). However the number of available PGPR must be increased to meet the projected future population increase and food security. Possibly, the number of new PGPB may be increased by the exploration of new environmental niches. Use of more potent bacterial strain from other sources including insect gut can broaden the spectrum of PGPB, which hopefully increase the overall survival and performance of the microbes in the soil. The majority of known PGPB were screened based on their phytohormone production, bio-control potential and nutrient solubilization abilities (Hameeda et al., 2006).

In the present study, a gut bacterial strain, *Pseudomonas* sp. PRGB06, which produces a greenish-yellow pigment, was isolated from the diamondback moth - *Plutella xylostella* and examined for its antagonistic properties

against phytopathogenic fungi and for nutrient solubilization. In addition, the efficacy of bacterial inoculation in augmenting overall growth was evaluated using plant based bioassay under gnotobiotic conditions.

Materials and Methods

Microorganism and seeds The *Pseudomonas* sp. PRGB06 based on its non hemolytic activity (which distinguish the clinical and environmental isolates), was selected for the present study. The bacterial culture was maintained as described earlier (Indira Gandhi et al., 2007). Plant pathogenic fungus *Botrytis cinerea, Sclerotinia sclerotiorum* (Laboratory collection) and *Colletotrichum acutatum* KACC41832, *C. gloeosporioides* KACC40690, *C. coccodes* KACC40008, *C. capsici* KACC40978, *Fusarium oxysporum* f. sp. *niveum* KACC40902, *Phytophthora capsici* KACC40483, *Rhizoctonia solani* AG-1(IA) KACC40106, obtained from Department of Plant Medicine and Korean Agricultural Culture Collection (KACC), respectively were maintained in potato dextrose agar (PDA). Plant pathogenic fungi, such as *Pseudomonas syringae* pv. *tomato, Xanthomonas campestris* cv. *vesicatoria* (Laboratory collection), and the plant growth promoting bacteria, such as *Methylobacterium oryzae* CBMB20^T (AY683045), *M. oryzae* CBMB110 (AY683046), *Azospirillum lipoferum* CW1501 (AY518779) and *A. brasilense* CW903 (AY518777) strains were used in this study. Red Pepper (*Capsicum annuum* L. cv Barodda, New Seoul Seed Company, Kongju) and maize (*Zea mays*, Jeil Seed and Agricultural products Company Ltd, Jeonbuk) seeds were used as the test plant species.

Utilization of different carbon sources, growth at different temperature and pH Growth of bacterial strains using the different carbon sources was tested on agar mineral media contained (g l^{-1}): NH_4Cl 1.0, KH_2PO_4 1.5, K_2HPO_4 4.0, $MgSO_4 \cdot 7H_2O$ 0.5, trace element solution 5.0 ml (pH 7.2), which was then supplemented with different carbon sources including carbohydrates (2 g l^{-1}), amino acids and organic acids (1 g l^{-1}) (listed in table 1). The plates were incubated at 30℃ for 2 d and bacterial growth was scored visually. The ability of the bacterial strains to grow in different temperature and salt tolerance was tested in nutrient medium at 5, 20, 25, 30 and 40℃ and upto 6.5% NaCl, respectively. The effect of different pH on bacterial growth was tested in nutrient broth by adjusting the pH of the medium with NaOH or HCl to 0.5 unit intervals ranging from between pH 4.0 to 8.5.

Mechanism of antagonism (due to volatile antifungal metabolites) The antagonism caused by volatile antifungal metabolites was evaluated by streaking a bacterial cell on King's B agar plates. After incubation for 48 h, the lid was replaced by a plate containing an agar block (5mm diameter) of the test fungus grown on PDA. The two plates were sealed together with parafilm. Control sets were prepared in a similar manner, without bacteria in the bottom plate. Such sealed sets of Petri dishes were incubated at 30℃, and the observations were recorded from 24 h to 96 h. Growth inhibition of the test fungus was calculated in % using the formula: (Cr-Tr /Cr)*100, where Cr and Tr represent the radius of fungal grown in control plate and the radial growth of the fungus grown in agar plates (of which the lid was replaced with bacteria inoculated plate), respectively.

Quantitative estimation of phosphate solubilization Quantitative estimation of TCP solubilization in broth was carried out at 24, 48, 72, 96 and 120 h using Erlenmeyer flasks (250 ml) containing 100 ml of Pikovskaya's broth inoculated with 1 ml of bacterial suspension (10^8 CFU/ml); uninoculated control were also used in each case. The growth medium was withdrawn aseptically at 24 hr intervals from each flask and centrifuged (10,000 rpm, 20 min). The supernatant was analyzed for phosphate content by standard method (Murphy and Riley, 1962), the pH of the supernatant was measured in each case. All the data are an average of three replicates.

Gnotobiotic root elongation assay Gnotobiotic root elongation assay is considered to be an effective tool for screening the plant growth promoting bacterial strains. Hence, this assay was selected to study the effects of plant growth promoting potential of the bacterial strain PRGB06 on red pepper and maize. The seed treatment and the gnotobiotic growth pouch assay were performed according to Penrose and Glick (2003). Bacterial strain PRGB06 cultivated in 10 ml of nutrient broth for 12 h were harvested by centrifugation, washed and resuspended in 10 ml of sterile 0.03 M $MgSO_4$ and

Table 1. Phenotypic and physiological properties of *Pseudomons* sp. PRGB06.

Properties		*Pseudomonas* sp. PRGB06		References
Gram reaction		Negative		Indiragandhi et al., 2007
Motility		Negative		
Shape		Rod		
Haemolysis		Negative		
6.5% NaCl		Positive		This study
Growth at	4℃	20℃	40℃	
	Negative	Positive	Positive	
Carbon source utilization				
Cellobiose		+		This study
Cystein		-		
Fructose		-		
Glucose		+++		
Glutamic acid		++		
Glutamine		+++		
Glycerol		++		
Lactose		+		
Mannitol		+++		
Mannose		++		
Malic acid		+++		
Methanol		-		
Raffinose		-		
Sucrose		+		
Starch		+		
Sorbitol		-		
Sodium acetate		+		
Sodium citrate		++		
Sodium gluconate		++		
Sodium succinate		++		
Xylose		-		
Physiological traits related to plant growth promotion				
ACC deaminase activity		-		Indiragandhi et al., 2008
IAA production		10.04 μg/ml		
Siderophore production		3.6 cm (zone dia)		
HCN, Pectinase, Cellulose, Chitinase production		-		
SA production		6.77 μg/ml		
β-1,3 glucanase production		7.9 μg/min/mg prt.		
Nutrient solubilization				
'P' solubilization (Tricalcium phosphate)		249.88 μg/ml		Indiragandhi et al., 2008
Zn solubilization efficiency		171.43		
Sulfur oxidation		10 μg/ml		
Nitrogen fixing ability		3 nmol C_2H_4/h/mg protein		
Antagonism against				
Pseudomonas syringae pv. *tomato*		-		This study
Xanthomonas campestris cv. *vesicatoria*		-		
Methylobacterium oryzae CBMB20^T		-		
M. oryzae CBMB110		-		
Azospirillum lipoferum CW1501		-		
A. brasilense CW903		-		

-, indicates the absence of growth/trait; +, indicates the presence of the traits and the number of '+' indicates the magnitude of the bacterial growth.

diluted with the same to adjust the absorbance of the bacterial suspension (A_{600nm} = 0.15). Seeds were surface sterilized with 70% ethanol for 1 min and 2% sodium hypochlorite (NaOCl) for 10 min then the disinfectant was removed by rinsing with sterile distilled water for at least 5 times. Each crop seeds were incubated at room temperature for 4 h with sterile 0.03 M $MgSO_4$ (used as the negative control) or bacterial suspension. Bacterial

coated seeds were transferred aseptically to growth pouches (CYG seed germination pouch, Mega International Manufacturer, U.S.A) and incubated in a growth chamber maintained at 20 ± 1℃ with a cycle beginning with 12 h of dark followed by 12 h of light. Six and four seeds of red pepper and maize, respectively, were placed on each pouch and ten pouches were used for each treatment with three replications. Seeds that failed to germinate on day 2 after sowing were marked and exempted while observing for root elongation test. Three sets of experiments were performed simultaneously to measure the root length, seedling vigor and dry bio-mass. The primary root lengths of red pepper and maize were measured on day 10, and seedling vigor was calculated according to ISTA (1993). Dry mass was calculated after getting constant weight at 70℃.

Results and Discussion

Table 1 lists the results of the bacterial strain PRGB06's growth examined in various carbons sources, temperature, pH, and salinity conditions as well as its physiological traits related to plant growth promotion. *Pseudomonas* sp. strain PRGB06 grew well in glucose, glutamine, mannitol, malic acid, sodium citrate, gluconate and succinate. It indicates clearly that the strain PRGB06 could colonize the crop rhizosphere by using the root exudates, which contains glucose, succinic acid, malic acid that serve as carbon source for bacterial growth. In turn, the products of bacterial metabolism (e.g. phytohormones) and other enzymatic activity would accelerate the plant growth. The *Pseudomonas* sp strain PRGB06 has been reported to possess bio-control and plant growth promoting traits such as (1) production of chitinases, β-1,3-glucanases, salicylic acid, siderophores and IAA, (2) solubilization of phosphates and zinc, (3) oxidation of sulfur, and (4) inhibition of growth of phytopathogenic fungi (Indiragandhi et al., 2008).

We further investigated the mechanism of antagonism and 'P' solubilization ability of the bacteria strain. PGPB-produced volatile antifungal metabolites such as ammonia and hydrogen cyanide play an important role in biological control (Nagarajkumar, 2004). To examine functional significance of the antagonism, pathogenic bacteria and PRPB strains were treated. While the strain PRGB06 did not inhibit PGPB growth *in vitro,* it showed antifungal activity against phytopathogenic fungi (Fig. 1b and Fig. 2). The strain PRGB06 is known as HCN negative. Therefore, the mechanism of antagonism was likely to be associated either with the volatile antifungal metabolites such as ammonia or the production of salicylic acid. The inhibitory effect increased with time. The maximum inhibition of fungal growth was 38% for *R. solani* (observed after 48 h of incubation), whereas it amounted to 58% for *B. cinerea* and *C. acutata* (observed after 48-hours of incubation). The normal colors of *C. acutata* (orange) and *F. oxysporun* (Pink) were changed to white. The production of volatile antifungal compounds by *P. corrugata* and fluorescent Pseudomonads has been reported by Trivedi et al. (2007) and Tripathi and Johri (2002).

Similar to the present investigation, Trivedi et al. (2007) found that the volatile metabolite(s) produced by *P. corrugata* (HCN, cellulose, pectinase negative and ammonia positive) had a predominant inhibitory role in the antagonism of the test fungus *F. oxysporum.* The role of ammonia in antagonism has been well documented and it has been reported that it is the only gas present in sufficient concentrations in soil to inhibit soil fungi (Pavlica et al., 1978). Inhibition of pathogenic fungi

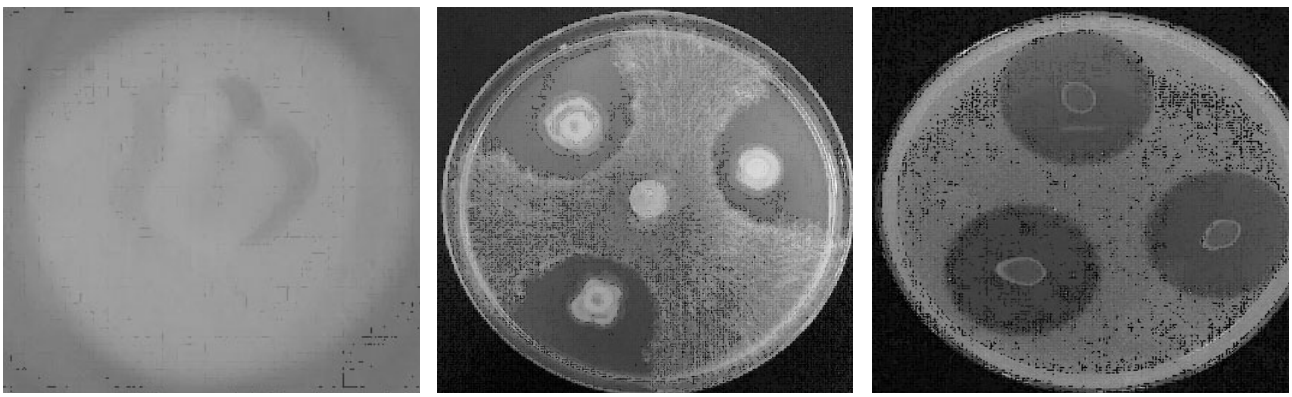

Fig 1. a) Siderophore production on chrome azurole S agar. b) Inhibition of *Rhizoctonia solani* on Potato dextrose agar. c) Zn solubilization on Pikovaskya's medium supplemented with 0.1% ZnO, respectivey by *Pseudomonas* sp. PRGB06.

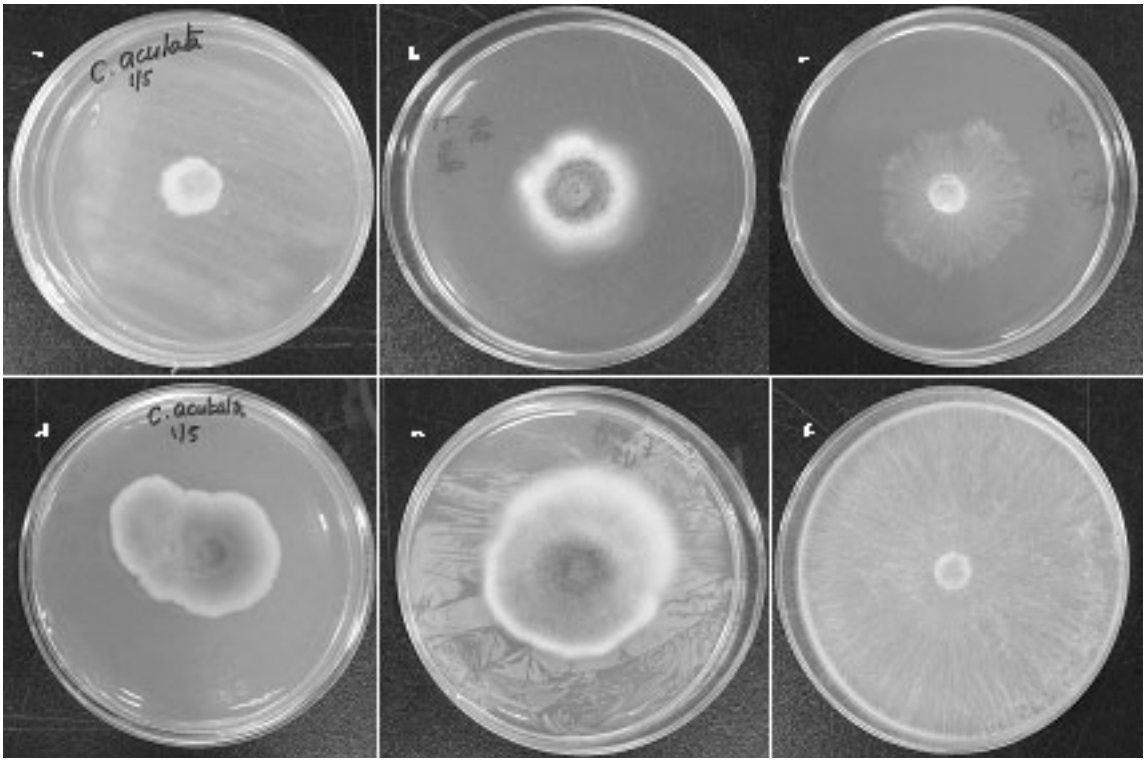

Fig 2. Antagonism of *Pseudomonas* sp. PRGB06. Inhibition of *C. acutata, F. oxysporum, R. solani* (a, b, c), respectively, due to diffusible metabolites produced by PRGB06 (top panel). Bottom panel shows the respective control (d, e, f).

growth was further confirmed by reduction in fungal biomass when treated with bacterially produced salicylic acid (data not shown). Therefore, the observed inhibition of more than one pathogenic fungi by *Pseudomonas* sp. PRGB06 implies that the broad spectrum antifungal activity implicated by an array of mechanisms. In addition to direct antagonism, bacterially produced salicylic acid would mediate an induced systemic resistance that confers protection against multiple pathogens in plants. Salicylic acid also acts as precursor for the production of siderophore, which preempt the iron in an environment and nutritionally challenge the invading pathogens (De Meyer et al., 1999). *Pseudomonas* sp. PRGB06 possessed the ability to produce siderophore (Fig.1a). A number of plants are capable of binding with the bacteria iron-siderophore complex, which is transported into the plant and then reductively releases iron (Vandana and Goel, 2004). The siderophores producing *Pseudomonas* sp. CDB35, isolated from macrofauna showed bio-control potential against *Sclerotium rolfsii*, *Macrophomina phaseolina, Fusarium solani,* and *Fusarium oxysporum* (Hameeda et al., 2006). Therefore, the application of siderophores producing bio-inoculants to crop plants would give multiple benefits.

The results of phosphate solubilization that was estimated from incubation from 24h to 120h are presented in Table 2. Compared with other *Pseudomonas* spp., PRGB06 had the highest potential for phosphate solubilization, as observed by Pandey et al. (2006). pH of the broth was found to decline with time (7.2 to 3.4) due to bacterial activity. And this declining pH was well correlated with increasing phosphate solubilization. The amount of soluble P was proportionally increased as pH decreased. The mechanism(s) behind the microbial solubilization of insoluble phosphate has been mainly attributed to production of variety of organic acids by bacterial strains (Anandham et al., 2007). Phosphorus (P), which is an essential mineral for plant growth, is the

Table 2. Assessment of phosphate solubilizing activity of *Pseudomonas* sp. PRGB06.

Time of incubation (h)	Soluble P (μg/ml)	pH
0	0	7.2
24	87.31	5.15
48	207.70	4.22
72	235.08	4.01
96	294.40	3.86
120	352.10	3.42

The amount of soluble phosphate was determined from the absorbance data using the calibration curve with KH_2PO_4 at 600nm. Values are mean of three replications.

worlds second largest agricultural chemical. Soluble P is often the limiting mineral nutrient for biomass production in natural ecosystems as well. There is evidence that some *Pseudomonas* species increase plant absorption of N, P and Fe, Zn (as shown in Fig. 1c) in addition to their bio-control effects against phytopathogenic fungi. In addition, these species are known to produce phytohormones in the rhizosphere. Therefore, all these factors can interact together to promote plant growth (Hameeda et al., 2006; Pandey et al., 2006; Vandana and Goel, 2006).

The importance of fluorescent *Pseudomonas* in soil nutrient cycling and their ability to colonize aggressively make them the preferred choice for eco-friendly studies. Gnotobiotic root elongation assay was performed to see the effect of the bacterial strain PRGB06 on plant growth (Table 3). By comparing the growth of the inoculated plants (red pepper and maize) with that of untreated control, this assay was almost free from other interfering variables in the soil-plant system. The results revealed that the PRGB06 strain was markedly effective in promoting the growth of both the test plants. Red pepper showed 28, 20, and 33% increases in root length, seedling vigor, and dry biomass, respectively. Similarly, maize showed 23, 56 and 22% increase in root length, seedling vigor, and dry biomass, respectively. It is highly plausible that such an enhancement in plant growth is also associated with phytohormones such as salicylic acid and indole derivatives (e.g. IAA). The similar growth promoting effects were reported for canola and tomato inoculated with PRGB06 (Indiragandhi et al., 2008).

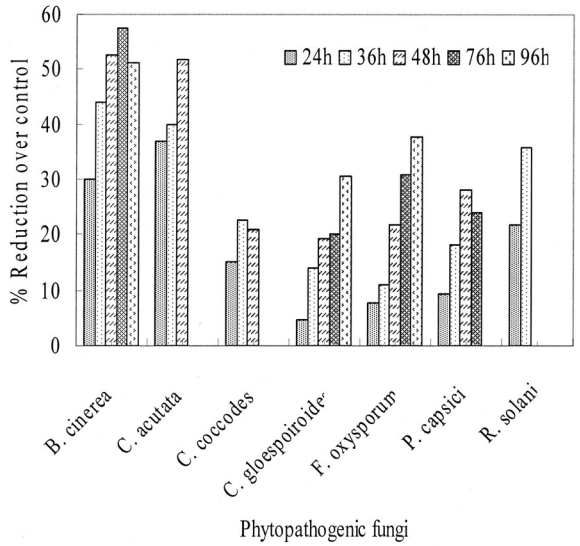

Fig 3. *In vitro* fungal growth reduction due to volatile compound produced by *Pseudomonas* sp PRGB06. Inhibition of fungal growth was measured in terms of reduction in radial growth over control as a function of time. For *C. acutata, C. coccodes* data on 76 h were not taken. *P. capsici* and *R. solani* completed their growth in control plate on 76 h and within 48 h respectively.

Conclusion

A significant increase in P availability to plant through inoculation with the bacterial strain PRGB06 was observed in both control and field conditions. In addition, the microorganism acts also as an efficient scavenger of Fe through siderophore, can facilitate biological nitrogen fixation and enhance the availability of plant growth promoting substances as well as other trace elements including Zn. Definitely, a microorganism capable of phytohormone production, disease control, and phosphate solubilization is considered to be an ideal bio-inoculants. Such a bacterial strain with multiple beneficial traits may greatly improve the growth and development of crop plants. However, the prevailing soil and/or the climatic conditions would influence the microbial performance.

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Table 3. Maize and red pepper bioassay for plant growth promotion effect of *Pseudomonas* sp. PRGB06.

Treatment	Root length (cm)		Seedling vigor		Dry mass (mg)	
	Red pepper	Maize	Red pepper	Maize	Red pepper	Maize
Pseudomonas sp. RGB06	7.3±0.2a (28)	6.3±0.5a (23)	306.0±4.1a (20)	1399.8±7.2a (56)	227.0± 8.4b (33)	3336.3±17.6a (22)
Control	5.7±0.9b	5.0±0.9b	254.2±2.6b	899.2±6.6b	214.6±10.5a	2728.6±143.2b
LSD ($P \leq 0.05$)	2.4	1.0	2.4	4.6	22.1	156.0

Each value represents the mean of six replicates per treatment. In the same column, significant differences according to the LSD at 0.05% levels are indicated by different letters.

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배추좀나방 내장에서 분리한 식물생장촉진미생물 *Pseudomonas* sp. PRGB06의 길항기작과 인산가용화의 기내 평가

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배추좀나방 유충의 내장으로부터 분리한 *Pseudomons* sp. PRGB06의 식물생장촉진능과 생물비료로서의 특성을 연구하였다. 또한 탄소원, 온도, pH, 염류농도 등의 다양한 배양 상태에서 균주의 생장을 확인하였다. 이에 더하여 *Pseudomons* sp. PRGB06의 진균에 대한 길항기작과 인산가용화능을 연구하였다. PRGB06 균주는 실험에 사용한 대부분의 탄소원에서 잘 자랐으며, 균주 최적 배양조건은 30℃, pH7이었다. 식물병원성 진균의 억제는 균주로부터 생성된 휘발성 항진균 대사산물과 암모니아 가스로 인한 것으로 사료된다. 또한 균주는 인산가용화와 산의 생성에 크게 관여하였으며, 뿐만 아니라 공중질소고정, 식물호르몬생성, siderophore생성, 아연가용화 등 다양한 식물생장촉진 특성을 가지고 있는 것으로 확인되었다. 이러한 PRGB06을 고추와 옥수수에 접종한 결과 뿌리길이, 유묘의 생육, 건물량이 증가하였다.