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Article

Solubilization of Inorganic Phosphates and Plant Growth Promotion by *Pantoea* Strains

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Two phosphate solubilizing *Pantoea* strains (*P. agglomerans* and *P. rodasii*) were employed in elucidating their phosphate solubilizing potential under different carbon and nitrogen sources, pH, temperature and salt conditions. Plant growth promoting characteristics such as ACC deaminase activity, indole acetic acid (IAA), HCN, ammonia, and siderophore production of the two strains were assessed *in vitro*. Potential applicability of the strains as bio-inoculants was also evaluated in pot experiments conducted under green house conditions. Phosphate solubilization measured as the amount of phosphorous released into the medium was recorded as 810 and 788 µg ml^{-1} respectively by *P. agglomerans* and *P. rodasii*. Glucose at the rate of 2% was found be the best carbon source, while $(NH_4)_2SO_4$ was the best nitrogen source for both strains. Despite a slight decrease in phosphate solubilization observed at higher temperature, pH and salt concentrations, both strains could withstand against a range of temperature (30-35°C), pH (7-9) and the presence of NaCl (up to 5%) without much compromising the phosphate solubilization. Different plant growth promoting traits (ACC deaminase activity, IAA, HCN, ammonia, and siderophore production) of the strains and their ability to promote the growth of green gram seedlings indicate that both strains possess high potential to be used as bio-inoculants.

Key words: *Pantoea agglomerans*, *Pantoea rodasii*, phosphate solubilization

Plant growth promoting characteristics and results of the pot experiment.

Plant growth promoting characteristics			
Ammonia production	Positive		
HCN production	Positive		
ACC deaminase activity	Positive		
	Inoculated		
	P. agglomerans	*P. rodasii*	Un-inoculated
Shoot length (cm)	24.52	25.22	15.34
Root length (cm)	16.83	18.56	10.64

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Introduction

Phosphate solubilizing bacteria are known to be effectively involved in the transformation of insoluble phosphate to soluble forms enhancing the nutrient status of the soil (Lugo et al., 2008; Son et al., 2006; Souchie et al., 2006). This process not only compensates high cost of phosphatic fertilizer production but also minimizes environmental contamination.

Positive impacts of phosphate solubilizing bacteria on growth and phosphorous uptake by plants have been reported consistantly (Reyes et al., 2002; Zaidi et al., 2003; Nezarat and Gholami, 2009; Collavino et al., 2010). However, the performance of these bacteria varies highly with the prevailing environmental conditions such as temperature, pH and salt concentration of the soils. Furthermore, bacterial population generally decreases upon inoculation, thus target strain is needed to be inoculated at higher concentrations (Jain et al., 2010).

The degree of phosphate solubilization depends on various physico-chemical factors viz. nature and amount of phosphate sources, pH, temperature, salt and acid concentration. Apart from them, carbon and nitrogen sources and their concentrations also have strong influence on phosphate solubilization (Relwani et al., 2008; Dave and Patel, 2003). It has been reported that microbial-mediated production of desired products (enzymes, organic molecules) can be augmented through the optimization of culture conditions (Scervino et al., 2010). Therefore, it is apparent that phosphate solubilizing bacteria should be provided with optimum combination of physico-chemical factors along with the energy sources in order to achieve greater phosphate solubilization.

As revealed by the previous studies, *Pantoea* spp. are considered being efficient phosphate solubilizers (Son et al., 2006; Dastager et al., 2009; Malboodi et al., 2009). Despite the fact that these species can be cultured competently, their phosphate solubilizing potential under varying culture conditions are worth investigating, because such information would be vital in developing successful inoculum production programmes.

The present study was under taken to evaluate phosphate solubilizing potential of two *Pantoea* species under different carbon and nitrogen sources, pH, temperature and salt conditions. In addition, their other plant growth promoting characteristics such as production of indole acetic acid (IAA), HCN, ammonia, and siderophore, and ACC deaminase activity were also assayed. The effectiveness of the strains as bio-inoculants was assessed in pot experiments conducted under green house conditions.

Materials and Methods

Isolation of phosphate solubilizing bacterial strains Soils collected from the button mushroom media at Chungchugnam-do province, Buyeo-Gun area in South Korea were used in isolating bacterial strains. The mushroom media were shacked into a sterile polythene bag in order to collect the soil adhering to the media. They were immediately transported to the laboratory and 10 g of moist soil from each mushroom samples was weighed and transferred to 250 ml Erlenmeyer flask containing sterilized 90 ml of 0.85% NaCl solution. The mixture was then shaken for 30 minutes at approximately 150 rpm. Immediately after shaking, a series of tenfold dilutions of the suspension was made by pipetting 1 ml aliquot into sterilized 9 ml of 0.85% NaCl solution. Aliquots of 0.1 ml of the sample from each of these dilutions were spread on to a petri dish with National Botanical Research Institute Phosphorus (NBRIP) medium containing 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25 g of Mg $SO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999). The pH of the media was adjusted to 7 using HCl. The plates were incubated for 7 days in an incubator at 30°C. The colonies with clear halos were considered to be phosphate solubilizing colonies. Predominant colonies were further purified by re-streaking on the fresh NBRIP agar plates at 30°C.

16S rDNA gene sequencing and Phylogenetic analysis of the isolated bacteria The partial sequencing of 16S rRNA for the bacterial strains was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F (5'-AGAGTTTGATCCTGGCT CAG -3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The online program BLAST was used in identifying the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A Phylogenetic tree was constructed using CLUSTAL X program (Thompson et al., 1997), which involved sequence alignment by neighbor joining method (Saitou and Nei, 1987) and maximum parsimony using the MEGA4 program (Kumar et al., 2001). Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Optimum culture condition for phosphate solubilization Bacterial strains were grown in sterilized liquid NBRIP medium (20 ml) at 30°C for 2 days with continuous shaking at 150 rpm. Aliquots of culture (1 ml) having diluted to 10^6 CFU ml^{1-} was then transferred to a 500 ml flask (n=3 per strain) containing sterilized liquid NBRIP medium (200 ml) and incubated for 7 days with continuous

shaking at 30°C. The same non-inoculated medium served as control in each case. A sample (10 ml) of each cultured and control were taken daily and centrifuged at 8000 rpm for 15 min. The clear supernatant was used in determining the amount of phosphorous released into the medium. The pH of the culture medium was also recorded with the pH meter equipped with glass electrode. The phosphorus availability was determined using phospho-molybdate blue color method (Murphy and Riley, 1962).

Phosphate solubilizing ability of bacterial strains was tested under different carbon and nitrogen sources. Effect of carbon source on phosphate solubilization was tested by using monosaccharides (D-Galactose, D-fructose, D- Xylose), disaccharides (lactose, maltose, sucrose) and derivatives sugar (D-sorbitol, D-mannitol) instead of glucose in the NBRIP medium. To test the effect of the nitrogen source, $(NH_4)_2SO_4$ in the NBRIP medium was replaced by NH_4Cl, NH_4NO_3, KNO_3, $NaNO_3$ and $Ca(NO_3)_2$.

The effect of salt on phosphate solubilization was tested by growing the strains on NBRIP containing various concentrations of NaCl (0, 2.5, 5 and 10%). Further, the effect of pH on phosphate solubilization was tested by adjusting the pH of NBRIP medium using HCl or NaOH to different pH levels (7-10). For estimation of high temperature induced phosphate solubilization, NBRIP medium inoculated with strains was incubated at different temperature conditions (30-40°C). In all cases, phosphate solubilization and pH of the culture medium were measured as described earlier and experiments were performed in triplicate. The absolute value of the control refers to the amount of phosphorus solubilized (μg ml^{1-}) by each strain when individually grown for 3 days in NBRIP medium under the 30°C temperature, pH 7 and in the presence of 0% salt (NaCl).

Assay of plant growth promoting characteristics ACC deaminase (1-aminocyclopropane-1-carboxylic acid) activity was assayed according to Penrose and Glick (2003). Solid DF minimal medium containing ACC was inoculated with 10 μl of starter culture (grown overnight at 30°C). Plates were then incubated at 30°C in dark and colony emergence was checked daily for consecutive 3 days.

IAA (Indole acetic acid) production was determined using the method described by Gutierrez et al. (2009). The strain grown in sterilized liquid NBRIP medium (100 ml) containing 1 ml of 0.2% tryptopan was incubated for 36 hrs with continuous shaking at 30°C. IAA production was measured every 12 hours. For that centrifuged clear supernatant of 1 ml was mixed with 4 ml of the Salkowski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.05 M $FeCl_3$ solution). Development of pink color indicated the IAA production, which was then quantified with optical density measurements taken at 530 nm using UV spectrophotometer (Shimadzu UV-VIS).

Ammonia production was tested using peptone water. Fresh cultures were inoculated into 10 ml peptone water and incubated for 48 hrs at 30°C. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour indicated the production of ammonia (Cappucino and Sherman, 1992).

HCN production was assessed by growing the bacteria in 10% tryptic soy agar (TSA) supplemented with glycine (4.4 g L^{-1}). Filter paper soaked in picric acid and Na_2CO_3 (0.5% and 2% respectively) was fixed to the underside of the lids of plates and incubated for 5 days at 30°C. A change in filter paper color from yellow to orange-brown was considered to be the indication of HCN production (Donate-Correa et al., 2005).

For quanttiative determination of siderophore production, modified succinate medium (1 L) containing 4 g succinic acid, 6 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$. $7H_2O$ was used to inoculate 24 hrs old cultures of *Pantoea* strains. It was incubated for 72 hrs with continuous shaking at 30°C. A sterilized uninoculated medium was served as the control. Each sample was taken into centrifugation tube for every 24 hrs and centrifuged for 10 min at 10000 rpm. The clear supernatant was subjected to detection of siderophore production as described by Schwyn and Neilands (1987). Quantitative estimation of siderophores was done by CAS-shuttle assay (Payne, 1994). Culture supernatant (0.5 ml) was mixed with same amount of CAS reagent (0.5 ml) and absorbance was measured at 630 nm against a reference Siderophore content in the aliquots were calculated using following formula.

$$\% \text{ Siderophore units} = \frac{Ar - As}{As} \times 100$$

Ar − Absorbance of reference
As − Absorbance of sample

Plant growth promotion bioassay with green gram (*Vigna radiata*) Green gram (*Vigna radiata* var. paiyur 1) seeds were surface sterilized by immersing in 0.1% sodium hypochlorite solution for 10 minutes followed by washing with distilled water. Seeds were soaked in bacterial suspensions at the concentration of 10^8 CFU ml^{-1} about 30 min prior to planting. Inoculated seeds were placed in earthen pots (six seeds/pot) and covered with a 15 mm thick uniform soil layer. Control plants received seeds soaked in diluted nutrient broth with no bacteria. Pots were watered daily to maintain moisture at field capacity during the study period. After one week of germination, plants were thinned out allowing 3 plants per pot to remain. Growth promotion effects of bacterial treatments were assessed by measuring

shoot and root length of green gram plants after 4 weeks of planting.

Statistical analysis The data were subjected to analysis of variance (ANOVA) using SAS package (SAS, 1999). The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at P $\leq$ 0.05.

Results and discussion

Isolation and identification of phosphate solubilizing bacterial strains Selected bacterial strains showed a marked solubilizing ability of inorganic phosphate as visualized by the clear zone developed around the colony after 3 days of incubation. According to 16S rRNA sequence analysis, the strains showed close proximity (> 99%) with *Pantoea agglomerans* DSM3493 (Accession number AJ233423.1) and *Pantoea rodasii LG*M 26273 (Accession number JF295053.1). Phylogenetic tree (Fig. 1) shows the position of isolated phosphate solubilizing bacterial strain with respect to related species. Different *Pantoea* stains have previously been identified as efficient phosphate solubilizers (Son et al., 2006; Malboodi et al., 2009; Khalim et al., 2012; Collavino et al., 2010; Viruel et al., 2011; Silini-Cherif et al., 2012).

Optimum culture conditions for phosphate solubilization Fig. 2 and 3 represents the results of inorganic phosphate solubilization by isolated *Pantoea* strains and the associated pH changes in the NBRIP medium during the 7-day incubation period. Both strains exhibited more or less similar capacity to solubilize inorganic phosphate (810 µg ml^{1-} in *P. agglomerans* and 788 µg ml^{1-} in *P. rodasii* at the end of 7-day incubation). Phospahate solubilization in NBRIP medium inoculated with *P. agglomerans*, occurred predominantly during the first 1-2 days of the incubation,

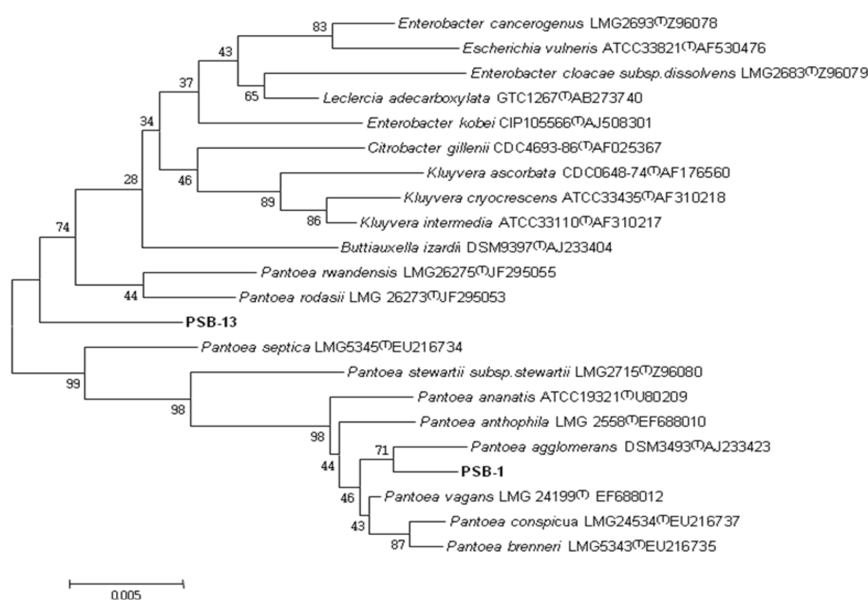

Fig 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Pantoea agglomerans* and *Panotea rodasii* strains with respect to related species. The scale bar indicates 0.005 substitutions per nucleotide position and accession numbers are given in parenthesis.

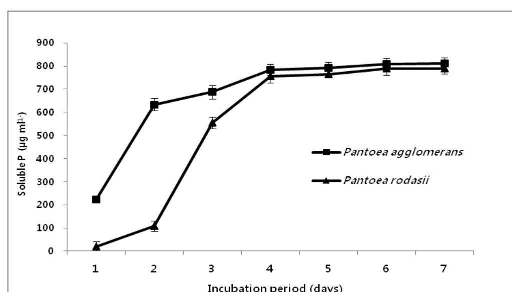

Fig 2. Phosphate solubilization by *Pantoea agglomerans* and *Panotea rodasii* strains grown in NBRIP medium at 30oC. Values are the means (n = 3) ± standard deviation.

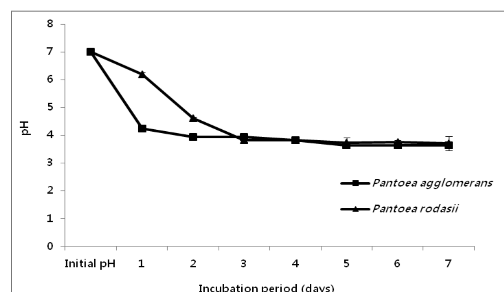

Fig 3. Changes of pH in NBRIP culture medium containing *Pantoea agglomerans* and *Panotea rodasii* strains grown in NBRIP medium at 30oC. Values are the means (n = 3) ± standard deviation.

Table 1. Effect of various carbon and nitrogen sources on phosphate solubilization by *Pantoea agglomerans* and *Pantoea rodasii*.

	Phosphate solubilization compared to control (%)	
	Pantoea agglomerans	*Pantoea rodasii*
Control	100 (3.93)	100 (3.62)
Absolute value	810.4 ± 11.45	788.2 ± 9.25
Carbon source		
Fructose	35.6 (4.55)	49.1 (4.10)
Galactose	59.8 (4.33)	50.9 (4.13)
Sorbitol	19.1 (5.13)	3.0 (6.67)
Mannitol	92.2 (4.05)	98.6 (3.93)
Xylose	94.1 (3.95)	59.4 (4.26)
Sucrose	31.2 (4.43)	3.6 (6.79)
Maltose	22.3 (4.82)	16.0 (4.58)
Lactose	50.2 (4.32)	17.7 (5.27)
Nitrogen source		
NH_4Cl	90.8 (4.23)	93.2 (3.84)
NH_4NO_3	97.7 (4.11)	98.8 (3.80)
KNO_3	72.8 (4.29)	72.2 (3.96)
$NaNO_3$	31.4 (4.86)	21.8 (4.62)
$Ca_3(NO_3)_2$	91.9 (4.04)	95.5 (3.80)

Control strains were grown for 3 days in NBRIP medium Final pH of the growth medium is given within the parentheses.
Absolute value of phosphate solubilization (μg/ml) of control corresponding to 100%.

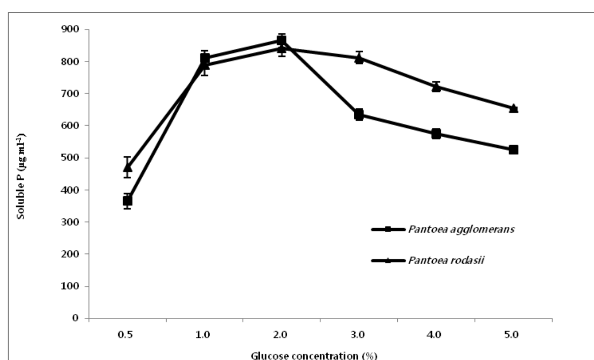

Fig 4. Effect of glucose concentration on phosphate solubilization by *Pantoea agglomerans* and *Panotea rodasii* grown in NBRIP medium at 30oC. Values are the means (n = 3) ± standard deviation.

whereas for *P rodasii,* rapid solubilization was recorded during first 2-3 days. However, as incubation progressed, soluble phosphorus content in the medium was found to be decreased. Moreover, both strains lowered the pH of the medium, and the relative time course coincided with the increase in the phosphate solubilization. At the end of 7-day incubation, the pH drop by *P. agglomerans* and *P. rodasii* were 3.63 and 3.71 respectively.

Strains response to different carbon and nitrogen sources is depicted in Table 1. Phosphate solubilization by *P. agglomerans* was recorded to be high when glucose, mannitol and xylose were used as the sole source of carbon, while glucose and mannitol were found to be better carbon sources for *P. rodasii*. When the medium was supplemented with sorbitol, maltose or sucrose, poor phosphate solubilization was recorded from *P. agglomerans.* In addition to them, lactose was also recorded to be poor carbon source for *P. rodasii*.

Out of the eight carbon source, glucose was found to be the best source for phosphate solubilization, thus the effect of glucose concentration on phosphate solubilization was assessed by adding different glucose concentrations (ranging from 0.5 to 5%) to the NBRIP medium. As shown in Fig. 4, both strains exhibited the maximum phosphate solubilization at the 2% glucose concentration. Further increase in glucose concentration caused reduction in phosphate solubilization by both strains. Son et al., (2006) isolated phosphate solubilizing bacteria, *P. agglomerans* R-42, which recorded the maximum phosphate solubilization at 3% glucose concentration.

As shown in Table 1, both strains exhibited higher phosphate solubilization with all the nitrogen sources except $NaNO_3$, where 31 and 32% reductions respectively for *P. agglomerans* and *P. rodasii* were observed compared to the control. However, the maximum solubilization was recorded with $(NH_4)_2SO_4$.

As depicted in Table 2, the optimum pH for phosphate solubilization was recorded to be 7. However, *P. rodasii* showed considerably high phosphate solubilization even at higher pH values such as pH 8 and 9 (98 and 96% increments compared to the control respectively at pH 8 and 9). In the case of *P. agglomerans,* phosphate solubilization was over 50% higher than the control even at pH 10. Therefore, both strains were shown to be good phosphate solubilizers at wide range of pH conditions. As reported by Silini-Cherif et al. (2012) and Costa et al. (2002), *P. agglomerans* Ima2 and *P. agglomerans* CPA-2 could grow both in alkaline and acidic conditions.

Temperature is considered to be the most important factor affecting physiology and growth of microorganisms (Rahman et al., 2006). As shown in Table 2, present strains recorded the maximum phosphate solubilization at 30°C. Generally performance of bacteria reduces at high temperature conditions (Malboodi et al., 2009), which was further confirmed by the present results of dramatically decreased phosphate solubilization by *P. rodasii* at 35 ^{o}C (37% reduction compared with the control). However,

relatively higher phosphate solubilization was recorded by *P. agglomerans* even when temperature increased up to 35°C.

Bacterial strains that survive in high salt conditions may also successfully colonize with the roots of plants grown under the same conditions (Saleena et al., 2002). Therefore, tolerance to salt is considered to be an important feature, which ensures the growth and survival of microorganisms in soil. The impact of salt concentrations on phosphate solubilization was assessed by adding NaCl to culture medium and the amount of soluble phosphorous released into the medium was decreased with increasing salt concentrations. However, *P. rodasii* was able to record more than 80% phosphate solubilization when compared to the control at 5% salt concentrations. Therefore *P. rodasii* could be considered as a halotolerant phosphate solubilizer which could be applied to such soils.

Table 2. Effect of various pH, temperature and salt concentration (NaCl %) on phosphate solubilization by *Pantoea agglomerans* and *Pantoea rodasii*.

	Phosphate solubilization compared to control (%)	
	Pantoea agglomerans	*Pantoea rodasii*
Control	100 (3.93)	100 (3.62)
Absolute value	810.4 ± 11.45	788.2 ± 9.25
Initial pH		
8	79.5 (3.98)	98.1 (4.07)
9	60.9 (4.16)	96.1 (4.15)
10	54.1 (4.22)	72.8 (5.12)
Temperature (oC)		
35	85.1 (3.95)	37.4 (4.32)
40	12.3 (4.92)	8.7 (5.48)
NaCl (%)		
2.5	67.4 (4.14)	98.4 (4.08)
5	50.6 (4.34)	80.4 (4.15)
7.5	13.5 (5.12)	6.1 (5..92)
10	2.1 (6.33)	1.4 (6.49)

Control strains were grown for 3 days in NBRIP medium Final pH of the growth medium is given within the parentheses.
Absolute value of phosphate solubilization (µg/ml) of control corresponding to 100%.

Table 3. Plant growth promoting characteristics and results of the pot experiment.

Plant growth promoting characteristics			
Ammonia production	Positive		
HCN production	Positive		
ACC deaminase activity	Positive		
	Inoculated		
	P. agglomerans	*P. rodasii*	Un-inoculated
Shoot length (cm)	24.52	25.22	15.34
Root length (cm)	16.83	18.56	10.64

Assay of other plant growth promoting characteristics and Plant growth promotion bioassay Results of the assays on ACC deaminase activity, and production of ammonia, HCN, IAA and siderophore are given in Fig. 5, 6 and Table 3. The strains showed positive responses for all the tested plant growth promotion traits. The maximum IAA production (229 µg ml^{-1}) was recorded by *P. rodasii* and the highest siderophore production was recorded by *P. agglomerans*. Similar to the present findings, *Pantoea* strains with pronounced ACC deaminase activity (Kausar and hahzad 2006; Viveros et al., 2010) and IAA production (Cimmino et al., 2006; Feng et al., 2006) have been reported previously.

Pantoea strains inoculated green gram seedlings showed

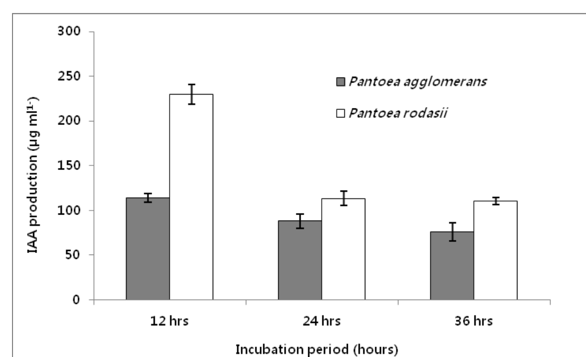

Fig. 5. IAA production (µg/ml) by *Pantoea agglomerans* and *Panotea rodasii*. Values are the means (n = 3) ± standard deviation.

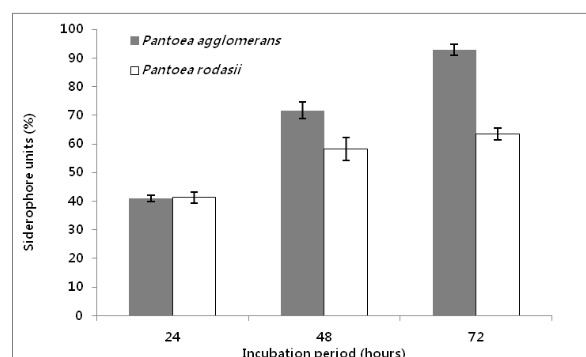

Fig. 6. Siderophore production (%) by *Pantoea agglomerans* and *Panotea rodasii*. Values are the means (n = 3) ± standard deviation.

significantly higher shoot and root length when compared with un-inoculated plants. Moreover, plants inoculated with *P. rodasii* which demonstrated comparatively lower phosphorus release showed higher shoot and root growth compared to plants inoculated with *P. agglomerans*. Therefore, it is evident that in addition to providing available phosphorus to plants, the isolated strains can enhance the growth of plant through other mechanisms as well. As shown in Table 3, inoculated seedlings recorded higher shoot and root lengths compared to un-inoculated control. Khalim et al. (2012) reported that inoculation of *P. agglomerans* strains PaJ and BS2 as a mixture or as single strain effectively increase the growth and yield of rice.

Growth promotion of green gram could be attributed to bacteria-induced absorption of nutrients, particularly P, as well as other plant growth promoting activities of the strains. Based on the results, both strains could be identified as being effective plant growth promoters, thus could be recommended for the production of bio-inoculants.

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