CHAPTER 3

Characterization of MPS abilities of *E. asburiae* PSI3 containing Gluconate dehydrogenase operon (*gad*) from *Pseudomonas putida* KT2440.

Chapter 3 ***Characterization of MPS abilities of E. asburiae PSI3 containing Gluconate dehydrogenase operon (gad) from Pseudomonas putida KT2440.***

3.1: INTRODUCTION

Phosphorus is a major macronutrient after nitrogen required for plant growth (Theodorou et al.,1991). P is abundant in the soil in the form of organic and inorganic metal complexes (Ca, Al, Fe) depending on nature of soil but not available to plants. Soluble P forms, $H_2PO_4^-$ and HPO_4^{--}, are used by plants (Ae et al.,1991). Application of chemical fertilizers in the field is not very efficient due to high P refixing capacity of the soil (Goldstein et al.,1995). Rhizobacteria promote plant growth by different mechanism and one of them is mineral phosphate solubilization (Kucey et al.,1989). A large number of bacteria belong to different genera and many fungi possess mineral phosphate solubilization ability which has been attributed to secretion of organic acid *viz*, D- gluconic acid (GA), citric acid, oxalic acid and 2-keto-D-gluconic acid (2-KG) etc. (Goldstein 1995; Khan et al. 2010; Archana et al. 2012).

Solubilization of mineral phosphate has been extensively studied in Gram negative bacteria using glucose as a carbon source (Goldstein 1995; Gyaneshwar et al. 1999; 2002). GA is prominent organic acid produced via direct oxidation pathway by membrane bound quinoprotein dependent glucose dehydrogenase (GDH). Pyrroloquinoline quinine (PQQ) is the cofactor for GDH enzyme and its biosynthesis requires many genes which vary in bacteria (Choi et al., 2008). Genes conferring mineral phosphate solubilization ability were cloned from phosphate solubilizing microorganisms (PSMs) like *Erwinia herbicola, Pseudomonas cepacia, Enterobacter intermedium, Serratia marcescens, Rahnella aquatilis* and non-PSMs *Synechocystis* PCC 6803 (Zaidi et al. 2009; Gyaneshwar et al.,1998a). Incorporation of *pqqE* gene from these PSMs has enabled to develop mineral phosphate solubilization ability in

Escherichia coli, *Burkholderia cepacia* IS-16, *Pseudomonas* sp. PSS and *Azospirillum* sp. Field studies of plant inoculations with PSMs had inconsistent effect on plant growth and crop yields. This has been attributed to variations in soil, crop and environmental factors influencing the survival and colonization of the rhizosphere. Buffering capacity of Alkaline vertisol, refixation of acidic alfisols, availability of carbon sources, nature of nitrogen source, nature of mineral phosphates and catabolite repression of organic acid secretion have been demonstrated to determine the efficacy of PSMs in field conditions (Kucey et al.,1989; Gyaneshwar et al.,1998b; Srivastava et al. 2007; Patel et al.,2011).

Enterobacter asburiae PSI3, an isolate of pigeon pea (*Cajanus cajan*) rhizosphere, solubilizes RP in buffered conditions by secretion of GA mediated by phosphate starvation inducible GDH (Gyaneshwar et al. 1999). In addition to glucose, *E. asburiae* PSI3 can utilize various mono- and di-saccharides for solubilizing RP due to the broad substrate specificity of GDH and it requires a mixture of seven aldosugars at 15mM concentrations for solubilizing RP in buffered medium (Sharma et al., 2005). 2-ketogluconic acid (2-KG) is much stronger than GA and also efficiently chelates calcium in soils (Moghimi et al.,1978; Moghimi and Tate,1978). Many bacteria are known to secrete 2-KG (Misenheimer et al.,1965; Webley and Duff, 1965; Yum et al.,1997; Hwangbo et al.,2003; Saichana et al.,2009; Vyas and Gulati, 2009; Gulati et al., 2010; Park et al.,2010). GA gets converted to 2-KG in the periplasm by gluconate dehydrogenase (GAD) enzyme encoded within *gad* operon (Yum et al., 1997; Toyama et al., 2007; Saichana et al., 2009). GAD has been characterized and purified from *Pseudomonas*, *Kleibsiella pneumoniae*, *Serratia*

marcescens, acetic acid bacteria (*Gluconobacter* sp.) and *Erwinia cypripedii* ATCC 29267. *gad* operon encodes for three subunits namely, FAD dependent gluconate dehydrogenase, cytochrome c and smallest third subunit of unknown function (Matsushita et al.,1982; Yum et al.,1997; Toyama et al.,2007; Saichana et al.,2009). In present study, *Pseudomonas putida* KT 2440 *gad* operon was expressed in *E. asburiae* PSI3 which led to secretion of 2-KG and solubilized rock phosphate more efficiently in buffered condition.

3.2 Material and Methods

3.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmid used in this study are listed in **Table 3.1**. Routine DNA manipulation was done in *E. coli* DH10B (Invitrogen) using standard molecular biology protocols (Sambrook and Russel, 2001). pTTQ18 and pGM160 were generously gifted by Dr. Michael J.R. Stark (Leicester Biocentre, University of Leicester) and Dr. Günther Muth (University of Beilfeld, Germany). *E. coli* DH10B, *E. asburiae* PSI3 (*Cajanus cajan* rhizosphere isolate) and its plasmid derivatives were grown on Luria-Bertanni (LB) medium (Hi Media, India) containing 20 µg/ml streptomycin, 50 µg/ ml ampicillin, erythromycin (20µg/ml) and 10 µg/ml gentamycin as and when required.

Table 3.1 Bacterial strains and plasmids used in this study

Plasmid/Strains	Relevant characteristics	Source/Reference
Plasmids		
pTTQ18	Cloning vector-ColE1 origin, P_{tac}, Ap^r	Stark *et al.* 1987
pGM160	Cloning vector, Gm^r	Muth *et al.*, 1989
pTTQGm^r	pTTQ18 with *acc*, Ap^r Gm^r	This study
pJET 2.1	Blunt end PCR cloning vector	Fermantas
pCNK12	pJET2.1 with *gad* operon	This study
pCNK14	pTTQGm^r with *acc* gene, *gad* operon, Ap^r Gm^r	This study
Bacterial Strains		
E. coli DH10B	Host strain for routine DNA manipulation experiments and plasmid maintenance	Invitrogen
E. asburiae PSI3	*Cajanus cajan* rhizosphere isolate	Gyaneshwar *et al.*, 1998b
E. a. PSI3(pCNK14)	*E. asburiae* PSI3 containing pCNK14	This study
E. a. PSI3(pTTQGm^r)	*E. asburiae* PSI3 containing pTTQGm^r	This study

3.2.2: Cloning of gluconate dehydrogenase (*gad*) operon in blunt end cloning vector pJET2.1

Genomic DNA isolation from *Pseudomonas putida* KT2440 was done as described by Sambrook and Russel (2001). Sequence of *gad* operon was obtained from *P. putida* KT2440 genome database (Accession No. AE015451). Gene specific primer pair 5'CGGATCCCG*AGGAGG*AATGTCATGCCTGAGCATGCCCC3' and 'GCTCTAGAGCTCAGCGAAGCGACTTTACATC 3'were used for obtaining *gad* amplicon. Underline sequence showed *Bam*HI and *Xba*I restriction enzyme site used for cloning. Italic sequence indicates RBS. PCR amplification was done using XT-20 polymerase (Banglore Genei, India) from 10 ng template DNA of *Pseudomonas putida* KT2440. Amplified PCR product was ligated into blunt end PCR cloning vector pJET2.1 (MBI Fermantas, India) according to manufacturer instruction. Positive clones were confirmed by restriction digestion and designated as pCNK12.

3.2.3: Cloning of *aacC3* gene confer for gentamycin in pTTQ18

E. asburiae PSI3 is naturally resistant to ampicillin. Hence, aminoglycoside-(3)-N-acetyltransferase III (*aacC3*) gene from pGM160 (1.6kb *Hind*III fragment) was cloned in *Hind*III site of pTTQ18 to obtain pTTQGm^r. Recombinant plasmid was confirmed by growing them on gentamycin containing LA plates and restriction digestion. Plasmid was designated to pTTQGm^r.

3.2.4: Construction of pCNK14 containing gluconate dehydrogenase (*gad*) operon of *Pseudomonas putida* KT2440 under control of *tac* promoter

pCNK12 was digested with *Bam*HI and *Xba*I which gives 3.8 kb release of GAD operon. 3.8 kb fragment was gel eluted and subcloned in gel purified pTTQGm^r digested with *Bam*HI and *Xba*I to obtained pCNK14. Clone was confirmed by restriction digestion. Functionality of the operon was confirmed by GAD enzyme assay.

3.2.5: Physiological experiments

Bacterial cells were prepared by growing the cells overnight at 37 °C in LB. Batch culture studies were performed under aerobic conditions in Orbitek rotary shaker with temperature maintained at 37°C and agitation speed was kept constant at 200 rpm. The composition of minimal medium used for the physiological studies is as follows: TRP minimal medium- 100mM Tris-Cl (pH 8.0), (45mM, 50 mM and 60mM) glucose, 10mM NH_4Cl, 10mM KCl, 2mM $MgSO_4$, 0.1mM $CaCl_2$ and micronutrient cocktail (Gyaneshwar et al., 1998b) with 1mg per ml Senegal Rock phosphate (RP) as sole phosphorus source. Erythromycin and gentamycin was added to a final concentration of 7.5 μg ml^{-1} and 2.5μg ml^{-1}, respectively.

3.2.6: Monitoring of MPS ability of recombinant *E. asburiae* PSI3

MPS ability of recombinant *E. asburiae* PSI3 was determined on TRP minimal medium plates (100mM tris buffer pH-8.0, RP 1mg/ml, methyl red, 1.8% agar and varying concentration of glucose *viz* 60 mM, 50mM and 45 mM). Same combination was used for shake flask experiments without agar. Absorbance at 600

nm was used for cell growth and pH drop was used as acidification of medium. Supernatant of medium was used for Pi estimation using KH_2PO_4 as standard (Ames, 1964).

3.2.7: Measurement of GDH and GAD activities

Wild type and recombinant *E. asburiae* PSI3 were grown on TRP containing minimal medium containing 75mM glucose as carbon source. After pH dropped below 5.5, cells were harvested (10000 g for 5 min) and washed with sterile saline and resuspended in 50mM Tris buffer pH 8.75. Whole cells were used for GDH and GAD enzyme assay and the assay was performed according to Matsushita and Ameyama, (1982) and Matsushita et al., (1982). GDH (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of DCIP at 600nm. The molar absorbance of DCIP was taken as 15.1 $mM^{-1}cm^{-1}$ at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16mM; DCIP, sodium salt, 0.05mM; PMS, 0.66mM; sodium azide, 4mM; D-glucose, 66mM; whole cells and water to 3.0 ml. For GAD activity, the protocol was similar to GDH assay except the 0.01M potassium phosphate buffer pH 6.0 containing 66mM D-gluconate. The molar absorbance of DCIP was taken as 10.0 $mM^{-1}cm^{-1}$ at pH 6.0.

Total whole-cell protein was estimated using a modified Lowry's method (Peterson, 1979). Units of activity of GDH and GAD are defined as nmol of 2, 6-dichlorophenolindo-phenol (DPIP) reduced per minute using glucose or gluconate as substrates, respectively. Specific activity is defined as units per mg total protein.

3.2.8: Analytical methods

All the physiological experiments used an initial cell density of ~0.025 OD at 600nm as monitored spectrophotometrically (path length of 1cm; Shimadzu UV-1700 spectrophotometer). Growth was monitored as increase in absorbance at 600nm and pH of the medium was monitored at 12h time intervals. When pH of the medium reached below 5 sample (2 ml) were taken from flask were centrifuged at 9,200 *g* for 10 min at 4 °C and the culture supernatants were used to estimate organic acid and P release in the medium. For HPLC analysis, the culture supernatant was passed through 0.2μm nylon membranes (Pall Life Sciences, India) and the secreted organic acids were quantified using Luna C-18 column (Phenomenex, India). The column was operated at room temperature using mobile phase of 5mM H_2SO_4 at a flow rate of 1.0 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm. Standards of organic acids were prepared in double distilled water, filtered using 0.2μm nylon membranes and were subjected to chromatography under similar conditions for determining the individual retention time. Comparison of peak area with external standards was used for quantification. The statistical analysis of all the parameters has been done using Graph Pad Prism (version 5.0) software.

3.2.9: Plant inoculation study

For plant inoculation study was carried out in pots of sterile local alkaline vertisol. Mung bean (*Vigna radiata*) seeds were surface sterilized with 0.1% $HgCl_2$ solution, followed by 70% ethanol washed thoroughly and germinated in sterile distilled water. Overnight 50 ml LB grown wild type and genetic transformants of *E. asburiae* PSI3 were washed thrice with normal saline and dissolved in 5 ml saline.

Mung bean seeds were soaked in bacterial culture for 30 min. Uninoculated control seeds were treated with saline. 15 to 20 grown seeds were inoculated in a single pot. Three sets of 5-6 pots each of uninoculated control, *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) were grown under natural light at ambient temperature for 15 days. Uprooted plant shoot and root were separated and dried at 65°C for constant dry weight measurement. Dried plant shoots (n=6) were ashed in muffle furnace at 550°C for 15 h and ashes were dissolved in 0.9M H_2SO_4 at 10 mg dry weight per ml acid (Richardson et al., 2001). Subsequently P contents of H_2SO_4 extract was estimated by molybdate- blue method (Murphy and Reilay, 1962).

3.2.10: Data analysis

Physiological experiments were done in three independent triplicates. Data are expressed in mean and standard deviation. In plant experiments, three independent duplicate studies were performed. Differences in mean values were determined using general analysis of varience (ANOVA) and linear regression analysis was done using GraphPad Prism5.0.

3.3 Results

3.3.1 Cloning and heterologous overexpression of *gad* operon of *P. putida* KT 2440 in *E. asburiae* PSI3.

Cloning strategy of gad operon schematically represented in Figure 2. *gad* operon was cloned from genomic DNA of *P. putida* KT 2440 in blunt end cloning vector pJET 2.1. Clone was confirmed by RE digestion with *Bam*HI/*Xba*I, *Bgl*III and *Bam*HI/*Hind*III. *Bam*HI/*Xba*I digestion gives release of 3.8kb gad operon and 2.6kb vector. BglII present in both side of MCS so digestion will give 3.8 gad operon release and vector backbone. *Bam*HI/*Hind*III digestion gives 4.2kb due to presence of *Hind*III in vector, 400bp downstream of MCS. 3.8 kb *gad* operon was subcloned in *Bam*HI/*Xba*I RE sites of expression vector pTTQGm^r under the control of *tac* promoter to obtain pCNK14. pCNK14 was confirmed by RE digestion with *Bam*HI/*Hind*III. *Bam*HI /*Hind*III digestion give 3.8 kb *gad* operon, 1.6kb gentamycin and 4.5 kb vector backbone.

pTTQGm and pCNK14 were transformed into *E. asburiae* PSI3. *E. asburiae* PSI3 *gad* operon transformant showed similar GAD activity in the presence and absence of IPTG indicative of constitutive expression. In LB, GAD enzyme activity was found to be 985±44 which was enhanced by ~1.7 fold in presence of 2% D-gluconate. In TRP medium containing 75mM glucose, GAD and GDH specific activities of the *E. asburiae* PSI3 (pCNK14) were found to be 438.3± 0.113 U and 884.2±0.182 U, respectively. There was no significant difference in GDH activity was observed as compared to wild type and plasmid control (**Table 3.2**).

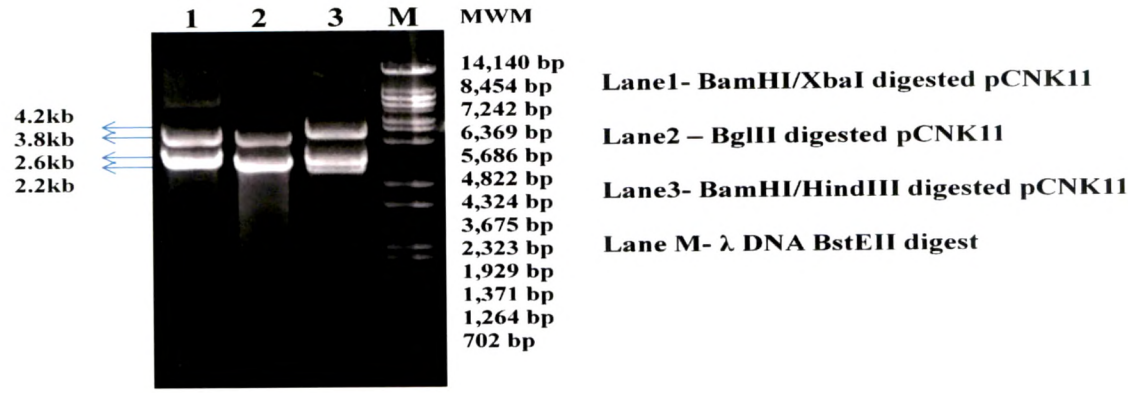

Fig.3.1 Restriction digestion pattern of pCNK11

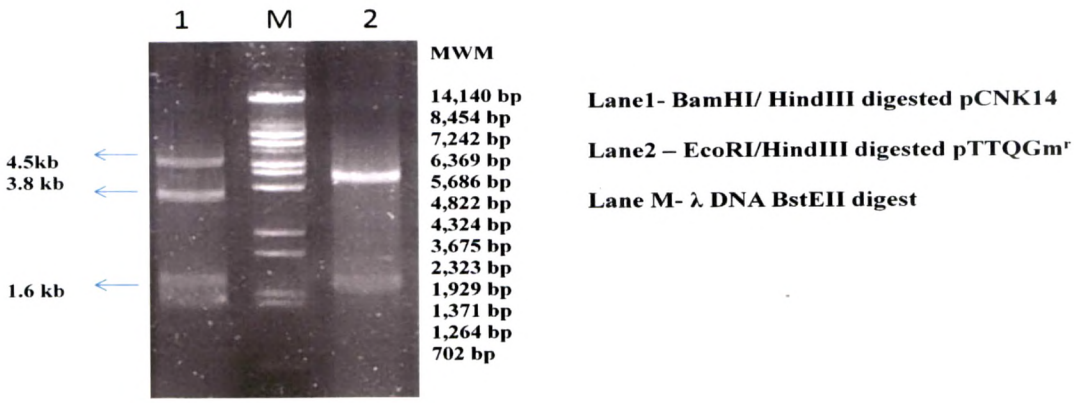

Fig.3.2 Restriction digestion pattern of pCNK14

3.3.2 Effect of *gad* overexpression on mineral phosphate solubilization ability of *E. asburiae* PSI3

E. asburiae PSI3 (pCNK14) showed acidification and mineral phosphate solubilization ability on TRP plates containing 45mM glucose while *E. asburiae* PSI3 and *E. asburiae* PSI3 (pTTQGm^r) did not show acidification and mineral phosphate solubilization ability when the glucose concentration was less than 75 mM (**Fig.3.3**).

In liquid TRP medium, *E. asburiae* PSI3 (pCNK14) grew upto 0.3 O.D. and acidified the medium to pH 3.9 in 96 h (Fig.2). Pi release was found to be 0.84mM. *E. asburiae* PSI3 secreted ~ 50 mM GA in presence of 75 mM glucose in TRP medium whereas *gad* transformant secreted 11.5 mM 2-KGA and 21.6 mM GA when grown in TRP medium containing 45mM glucose as a carbon source (**Table 3.3**). Thus, the efficiency of GA conversion to 2KGA by *E. asburiae* PSI3 (pCNK14) is ~30%.

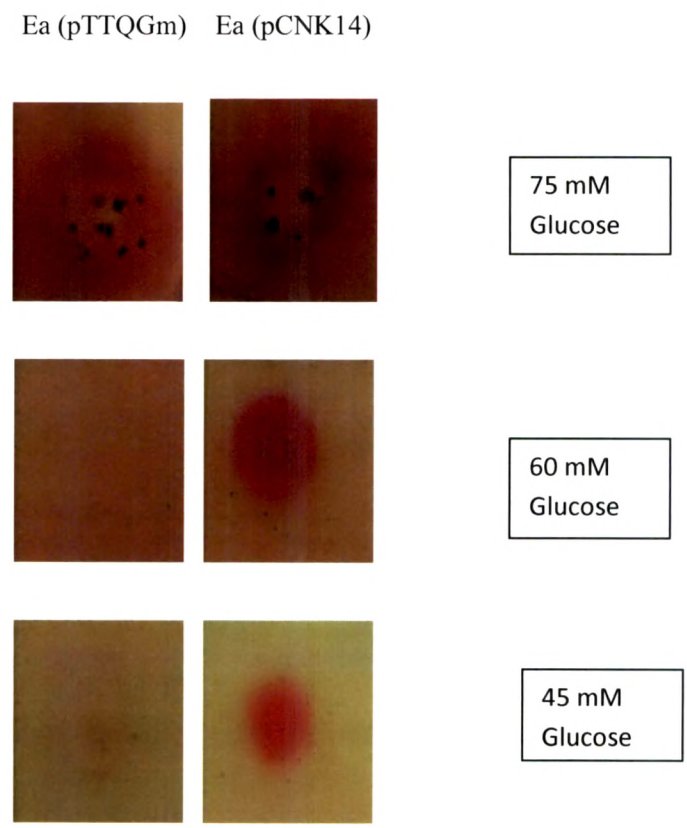

Fig.3.3 MPS ability of *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) in TRP agar medium with glucose as carbon source.

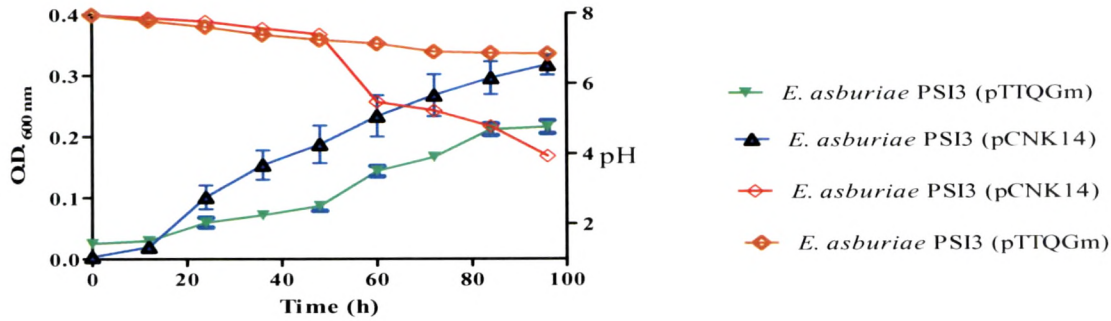

Fig. 3.4: Growth and pH profile of *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) in TRP medium containing 45 mM glucose.

Table 3.2 Activities of GAD and GDH enzymes.

Strain	GAD activity (U)	GDH activity (U)
P. putida KT2440	328± 0.048	ND
E. asburiae PSI3 (pTTQGm)	UD	991±0.13
E. asburiae PSI3 (pCNK14)	438.3±0.032	884±0.052

E. asburiae PSI3 was grown in 75mM glucose and 100mM Tris bufferd medium. *P. putida* KT2440 was grown in 75mM glucose and 50mM Tris buffered condition. UD- Undetected, ND- Not Done

Table 3.3 Amount of Organic acid secretion and P release by *E. asburiae* PSI3 (pTTQGm) and *E. asburiae* PSI3 (pCNK14) in TRP medium containing 45 mM glucose.

Strain	Initial O.D.	Final O.D	Gluconic acid	2-ketogluconic acid	Pi (mM)
E. asburiae PSI3 (pTTQGm)	0.02±0.002	0.21±0.01	33.74 ±1.78	UD	UD
E. asburiae PSI3 (pCNK14)	0.03±0.01	0.31±0.03	21.65±0.94	11.63±0.93	0.84±0.04

UD- Undetected

3.3.3 Effect of *gad* overexpression *E. asburiae* PSI3 transformant on *Vigna radiata.*

Mung bean (*Vigna radiata*) growth was monitored in pot experiments in sterile alkaline vertisol soil. Dry shoot weight, dry shoot/root weight and shoot P content were significantly improved in *E. asburiae* PSI3 pCNK14 as compared to vector control as as well as uninoculated control while dry shoot weight and dry shoot/root weight were improved in vector control compared to uninoculated control but no significant P content was observed (**Table 3.4**).

Table 3.4 Effect of *E. asburiae* PSI3 (pCNK14) inoculation on the mung bean (*Vigna radiata)* dry shoot weight, dry shoot /root weight ratio and shoot P content after 15 days.

	Growth Parameters					
Bacterial strain	Dry shoot weight (mg)		Dry shoot/root weight ratio of plant		Shoot P concentration of plants (mg/g dry weight)	
Uninoculated control	22.65		3.654		5.98	
E. asburiae PSI3	pTTQGm	pCNK14	pTTQGm	pCNK14	pTTQGm	pCNK14
	25.49*	29.39**†	5.02*	8.67**††	6.69ns	7.75**††

Plants were grown (12 plants, 5 replicates) for 15 days and data are means of individual experiments (for dry weight and shoot/root ratio of plants, n=12, and for shoot P content, n=6). ns: comparison with uninoculated control (Fisher LSD test, $p<0.05$); *: significance in comparison with uninoculated control; †: significance in comparison with the corresponding vector control (single sign: $p<0.05$; double sign: $p<0.001$).

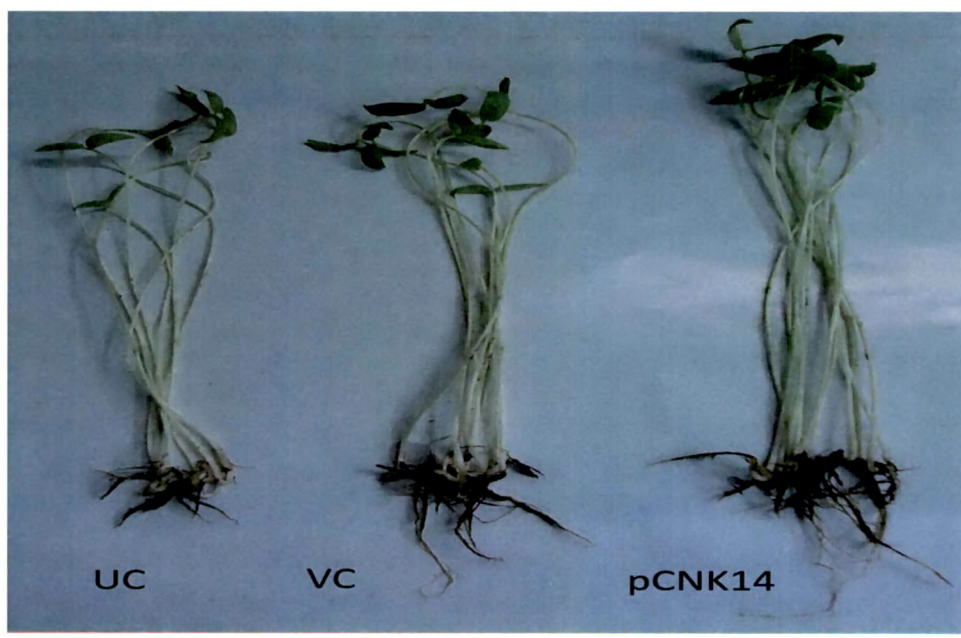

Fig. 3.5: Effect of *E. asburiae* PSI3 (pCNK14) on the growth of mung bean (*Vigna radiata*) after 15 days in alkaline vertisol soil. UC- Uninnoculated control, VC- *E. asburiae* PSI3 (pTTQGm),

3.4 Discussion

Nature and amount of organic acid determine the efficacy of phosphate solubilization by rhizobacteria. Most of the PSMs secrete GA but not 2KGA which is much stronger acid. Hence, incorporation of *gad* in GA producing PSM could significantly improve their plant growth ability. In the present study, incorporation of *P. putida* KT 2440 *gad* operon in *E. asburiae* PSI3 showed ~ 438 U and 985 U of enzyme activity in TRP and LB media respectively. Earlier reports of incorporation of the *gad* operon from *E. cypripedii* ATCC 29267 with ~ 1360 U enzyme activity in *E. coli* resulted in ~410 U GAD activity in LB medium which was increased to 2150 U in presence of gluconate (Yum et al., 1997). Similar increase in GAD activity (1738 U) was found in *E. asburiae* PSI3 *gad* transformant in presence of gluconate. *E. asburiae* PSI3 requires 75mM glucose to solubilize rock phosphate in TRP medium. However, *gad* transformant could solubilize rock phosphate in presence of 45 mM glucose due to secretion of ~21 mM GA and ~11mM 2KGA. In contrast, *E. coli* containing *E. cypripedii* ATCC 29267 *gad* operon could secrete ~ 13mM 2KGA in LB containing 100mM glucose (Yum et al., 1997).

Recent plant growth studies with PSMs have demonstrated improvement in P status upon supplementation with tricalcium phosphate (Gulati et al., 2010; Vyas and Gulati, 2009). *Acinetobacter rhizosphaerae* strain, which produced very high (upto ~ 87mM) GA, enhanced maize plant growth upon supplementation with tricalcium phosphate as compared to the plants supplemented with super phosphate (Gulati et al., 2010). On the other hand, phosphate solubilizing fluorescent pseudomonads strains improved maize plant growth as well as P content on

tricalcium phosphate more than that of plants supplemented with superphosphate (Vyas and Gulati, 2009). *E. asburiae* PSI3 improved mung bean growth in alkaline vertisol soil even without supplementation of any mineral phosphate probably due to the broad substrate specificity of GDH enzyme (Sharma et al., 2005). *E. asburiae* PSI3 (pCNK14) further improved plant dry shoot weight, shoot/root dry weight and P content as compared to *E. asburiae* PSI3 (pTTQGm^r). Improved efficacy of *gad* transformant can be attributed to the ~11mM 2KGA and ~21mM GA. 10mM 2KGA is sufficient for the release of P from alkaline soils (Moghimi et al., 1978). Genetic modifications have resulted in the improvement of mineral phosphate solubilization ability in a wide variety of bacteria (Chapter 1, **Table 1.6**). However, most of these studies have been directed towards increasing the secretion of GA. 2-KGA secretion by overexpression of *gad* gene cluster is unique and could improve the efficacy of bacteria with GA secretion capacity. PSMs with an ability to utilize soil P has gained more prominence as the existing cultivated soils have accumulated high P due to application of chemical fertilizers (Goldstein, 1995) and to the rapid decrease in the phosphate reserves in world (Elser and Bennet, 2011).