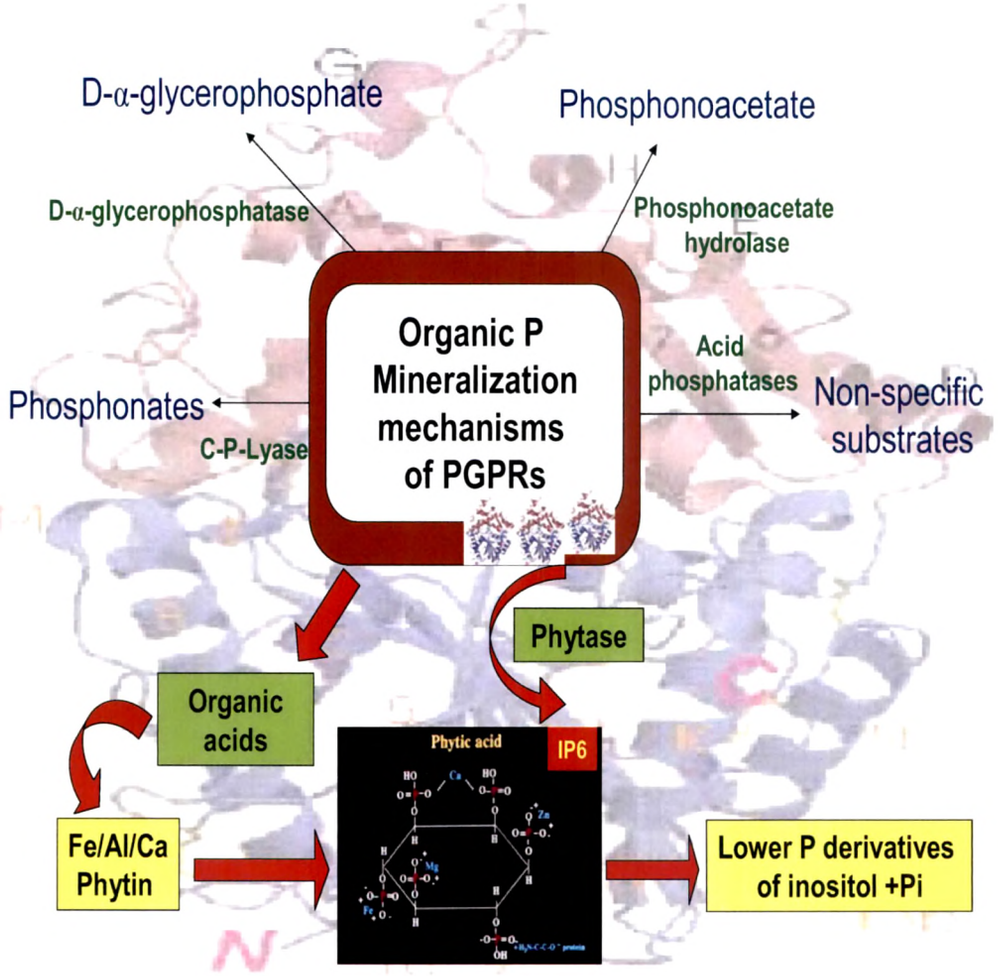
# CHAPTER 4

## *Overexpression of Escherchia coli appA gene in different rhizobacteria - Effect of acidification on phytate utilization and growth promotion of maize*



## 4.1 INTRODUCTION

### 4.1.1 Interaction of inositol phosphates in soils

IP6 has a high charge density due to six phosphate groups and therefore undergoes strong interaction with the soil, either being adsorbed to clays (Anderson and Arlidge, 1962; Celi et al., 1999), or precipitated as insoluble salts such as sesquioxides of Fe and Al in acid soils and insoluble calcium salts in alkaline soils (Jackman and Black, 1951). This strong reaction prevents phytate to interact with hydrolytic enzymes and results in inositol phosphates accumulating to form the dominant fraction of organic P in most soils (Harrison, 1987). Since the number of P groups associated with inositol decreases from hexakisphosphate to monophosphate, adsorption to the soil reduces due to decreases in charge density of the molecule (Anderson and Arlidge, 1962; Anderson and Malcolm, 1974). pH of the soil exerts considerable control over the stabilization of inositol phosphates (Hawkes et al., 1984). Complexes of inositol phosphates with Fe, Al, Ca and clays show pH-dependent variations in solubility, being most stable at pH 5 and 7.5 (Jackman and Black 1951; Emsley and Niazi, 1981), which is further regulated by the type of cations (Celi et al., 2001).

Phytate gets adsorbed to soil particles or forms insoluble precipitates with Ca, Fe and Al, therefore cannot be directly taken up by plants (Sanyal and Datta, 1991). Non-specific enzymes such as phosphatases secreted by soil microorganisms and plant roots are unable to hydrolyse the phosphomonoester bonds of these phytate (Turner et al., 2002; Mudge et al., 2003). However, phytases hydrolyse phytate to their lower forms which can be acted upon non-specific phosphatses such as acid or alkaline phosphatases (Wodzinski and Ullah, 1996). Despite the existence of phytase-positive soil organisms, which may account for 30-50% of total soil microbial flora (Greaves and Webley, 1965; Richardson and Hadobas, 1997), phytate remains the most recalcitrant pool of organic phosphorus in soils (Ognalaga et al., 1994) because of the inability of the phytase to act upon adsorbed and insoluble form.

### 4.1.2 Organic acid and phytate in soil

Plants in natural environments may rely heavily on their ability to access recalcitrant IP6 and that root-induced changes in pH or organic acid excretion play a

major role in this process. By desorbing Pi adsorbed to soil constituents, organic acids were shown to elevate by as much as 10–1000 fold soil solution Pi concentrations (Earl et al., 1979; Traina et al., 1986; Gyaneshwar et al., 1998; Srivastava et al., 2006). Organic acids either a direct exchange with Pi adsorbed to soil constituents such as crystalline $Al(OH)_3$ or $Fe(OH)_3$, or by chelation of metal ions in cation-Pi complexes (Jones, 1998). Under P stress, plant roots elevate the secretion of citrate and malate (Lipton et al., 1987; Grierson, 1992; Hoffland et al., 1992; Johnson et al., 1996; Keerthisinghe et al., 1998). Citrate may also increase the availability to plants of soil Po, by solubilising a fraction that can be hydrolysed by enzymes. The enzyme-labile component of citrate-extractable Po at low pH was equivalent to, or exceeded, the quantity of extractable Pi (Hayes, 2000). Citrate is an effective chelator of trivalent metal ions such as $Fe^{3+}$ and $Al^{3+}$ (Jones and Darrah, 1994).

### 4.1.3 Hydrolysis in the presence of organic acids in vitro

Phytase can hydrolyze phytate complexed with $Ca^{+2}$ or $Mg^{+2}$ ions, but cannot when complexed with $Al^{+3}$, $Cu^{+2}$, $Fe^{+2}$, $Fe^{+3}$ or $Zn^{+2}$ (Tang et al., 2006). Stabilities of these cations when complexed with phytate is in the order of $Fe^{+2}$- $Zn^{+2}$-$Fe^{+2}$ > $Mn^{+2}$ > $Ca^{+2}$ > $Mg^{+2}$ (Maenz et al., 1999). Citrate is effective in solubilizing $Al^{+3}$, $Fe^{+2}$, $Fe^{+3}$ and $Zn^{+2}$ phytate for phytase hydrolysis, whereas malate and oxalate are not effective. Hydrolysis of phytate adsorbed to Al (III) precipitates enhanced by organic acids in the descending order citrate >oxalate >malate presumably by releasing free phytate from the precipitates (Jones, 1998; Tang et al., 2006). The mechanism for desorbing free phytate could be ligand exchange in the case of oxalate while metal complexation for citrate.

### 4.1.4 Factors affecting the properties of extracellular phytase in soil

Factors affecting the stability and efficacy of extracellular enzymes in soils include: (i) proteinase and microbially-mediated degradation, (ii) partial or total deactivation by adsorption onto soil particles and (iii) deactivation via interaction with polyvalent anions, metal ions and microbial metabolites (Quiquampoix and Mousain, 2005). These factors differentially affect phytase activity depending on both the characteristics of the soil environment (Rao et al., 1994; George et al., 2005a) and the source and biochemical properties of the discrete phytase (George et al., 2006).

### 4.1.5 Properties of *E. coli* phytase

Phytase from E. coli belongs to Histidine acid phosphatase group whose three-dimensional structure doesnot hydrolyze a metal–phytate complex because the binding of IHP to the enzyme involves the interaction of all six deprotonated phosphate groups on the inositol ring with the side chains of several basic amino acid residues(Lim et al., 2000; Liu et al., 2004). $Ca^{+2}$:IP6 at (6:6) molar ratios significantly reduced enzymatic dephosphorylation of Na-IHP even at pH 4.5 (Dao, 2003) supported by the fact that chelating agents such as EDTA, phthalate and presence of organic acids enhances enzymatic dephosphorylation of insoluble phytates *in vitro* (Maenz et al., 1999; Wyss et al., 1999; Tang et al., 2006). The *appA* gene of *E. coli* encodes a bifunctional enzyme exhibiting both acid phosphatase and phytase activities that have identical pH optima of 4.5, and are active at broad pH values from 2.0 to 6.0 (Golovan et al., 2000). *E. coli* phytase, AppA has been recognized as one of the ideal candidate in commercial application because of its higher catalytic efficiency for phytic acid than other phytases, acidic pH optimum is similar to the physiological pH of stomach and is resistant to pepsin (Lei and Stahl, 2001; Miksch et al., 2002; Lee et al., 2005; Kim and Lei, 2008).

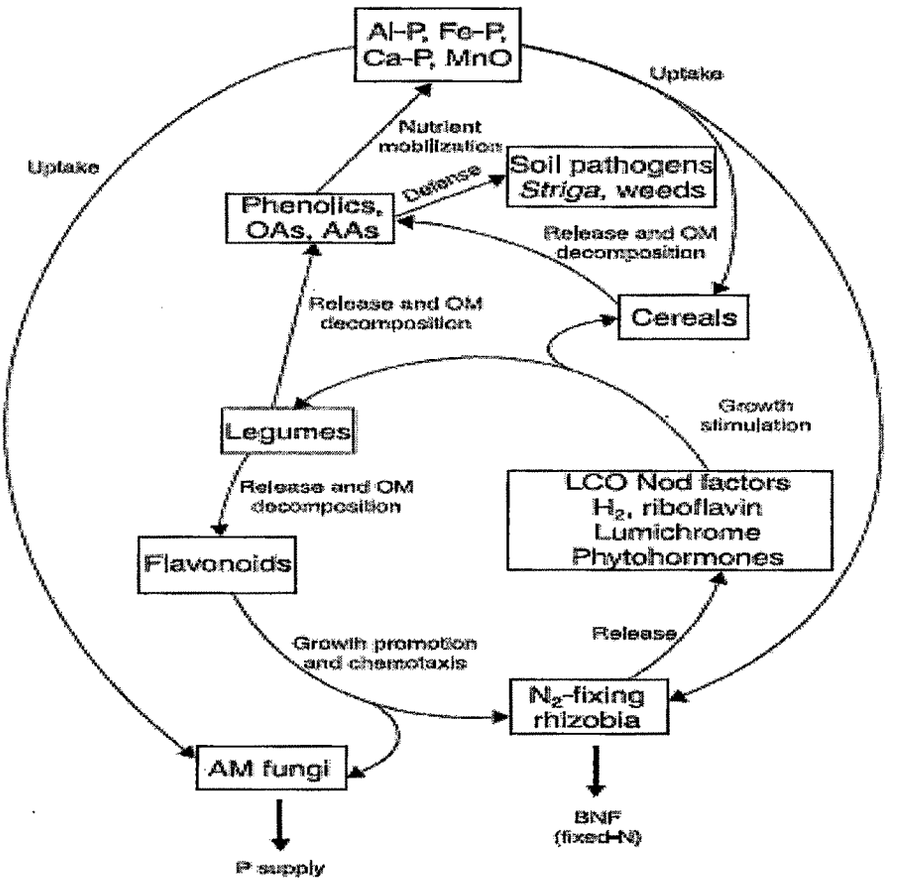
### 4.1.6 Expression in other microorganisms

*E. coli* phytase has been expressed in microorganisms such as *Pseudomonas putida* (Dharmsthiti et al., 2005), *Streptomyces lividans* (Stahl et al., 2003), *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* (Rodriguez et al., 2000; Chen et al., 2004; Lee et al., 2005), and in various plants *i.e.* soybean seeds (Bilyeu et al., 2008), rice (Hong et al., 2004) and in mice (Golovan et al., 2001). All these studies have resulted in reducing the phytate P in the manure and increasing the availability of P and other micronutrient to the animals.

### 4.1.7 Why Rhizobium?

Rhizobia fix nitrogen efficiently in legume nodules and promote plant growth in multiple ways (**Fig. 4.1**).

**Fig. 4.1: Model of plant growth promotion by rhizobia in legume–cereal cropping systems.**




OM=organic matter; OAs=organic acid anions; AAs=amino acids; LCO=lipo-chito oligosaccharide; AM=arbuscular mycorrhizae; BNF= biological $N_2$ fixation.

### 4.1.7.1 Rhizobial molecules as mediators of nutrient supply in plant cultures

Most rhizobial strains produce siderophores, vitamins, such as riboflavins, and organic acids in culture media which enhance mineral nutrition for the plants growing in low nutrient environments (Antoun et al., 1998; Dakora and Phillips, 2002). Phytohormones such as indole acetic acid (IAA), gibberellins, cytokinins released by these rhizobia help plant enhance the root's absorptive capacity and nutrient uptake by massive proliferation of root hair in both legumes and non legumes (Phillips and Torrey, 1972; Law and Strijdom, 1989; Yanni et al., 2001). *Rhizobium* and *Sinorhizobium* produce aspartic acid, glutamic acid, serine, glycine, histidine,

threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine when are cultured in defined media lacking growth factor using mannitol as sole carbon source (Gonzalez-Lopez et al., 2005; Salmeron-Lopez et al., 2004). Siderophores are used to mobilize iron (Fe) (Plessner et al., 1993) whereas organic acids solubilize phosphorus (P) and manganese (Mn) (Loper and Buyer, 1991). Field studies have shown that inoculating groundnut cv. Florunner with different rhizobial strains stimulated greater accumulation of calcium (Ca), P, magnesium (Mg), potassium (K), zinc (Zn) and other nutrient elements in seeds and nodules relative to that in uninoculated controls in both legumes and non legumes (Howell, 1987; Dakora, 2003).

### 4.1.7.2 Plant growth promotion by rhizobia

A number of studies have explored the use of rhizobia as $N_2$-fixing bacteria and as plant growth-promoting rhizobacteria for nonleguminous crop species such as wheat, maize, rice, potato, radish, oilseed rape and canola. (Al-Mallah et al., 1989, 1990; Spencer et al., 1994; Chabot et al., 1996; Noel et al., 1996; Reddy et al., 1997; Schloter et al., 1997; Yanni et al., 1997; Antoun et al., 1998).

During nodule formation, rhizobia produce complex lipochito-oligosaccharide, Nod factors as signals to the legume host, and other compounds which promote plant growth (**Table 4.1**) (Dakora, 2003). Some Nod factors directly promote plant growth such as seed germination and seedling development or indirectly induce the expression of genes involved in flavonoid biosythesis i.e. phenylalanine ammonia-lyase, chalcone synthase and isoflavone reductase, which in turn lead to increased phytoalexin accumulation and protection of plants against pathogens. Riboflavin easily gets converted enzymatically or photochemically into lumichrome. Lumichrome-induced $CO_2$ release by the plant probably stimulates increased populations of rhizobia and mycorrhizal fungi in sole and mixed cropping systems.

Rhizobial inoculation of plants has also been suggested to alleviate water stress in symbiotic legumes. Although the mechanisms underlying this observation is still unknown, it is possible that a rhizobial products such as abscisic acid or lumichrome decrease leaf stomatal conductance and reduce water loss via transpiration in the leaves.

**Table 4.1: Rhizobial molecules with potential for plant growth promotion (Dakora, 2003)**

| Rhizobial compounds | Functional role | Plant for study |
|---|---|---|
| Nod factors | Stimulate seed germination Promote seedling development | Maize, cotton |
| | Increase foliar photosynthetic rates | soybean, maize, common bean, rice, canola, apple and grapes |
| | Induce expression of flavonoid genes | soybean |
| | Stimulate root colonization by arbuscular mycorrhizal fungi | |
| | Cause cell division and embryogenesis | |
| Lumichrome | Stimulates seedling growth | Cell culture |
| | Stimulates root $CO_2$ production and therefore plant growth | Alfalfa, maize, soybean, cowpea, sorghum |
| Riboflavin | Serves as vitamin for plants and bacteria | |
| Nitrogenase-linked $H_2$ | Promotes soil microbial populations; Stimulates diversity of soil microorganisms and stimulate plant growth; Increases deposition of C in soil | soybean, barley, canola, spring wheat |

### 4.1.7.3 Rhizobial control of plant pathogens

Rhizobia are major biocontrol agents in natural and agricultural ecosystems. *Bradyrhizobium japonicum* strains can decrease in sporulation up to 75% in *Phytophthora megasperma*, 65% in *Pythium ultimum*, 47% in *Fusarium oxysporum* and 35% in *Ascochyta imperfecta* (Tu, 1978; 1979). This suggests that a single strain of a root-nodule bacterium can have a suppressive effect on the soil population of a wide range of pathogens. 49 strains of *Sinorhizobium meliloti* inhibited growth of *F. oxysporum* by up to 50% (Dakora, 2003). Rhizobia isolated from root nodules of *Acacia pulchella* similarly decreased the survival of the zoospores of *Phytophthora cinnamoni in vitro* (Malajczuk et al., 1984), thus providing bioprotection for the host plant. Plants with their respective microsymbionts significantly decreased the severity of *Phytophthora* and *Fusarium* root rot in soybean and common bean (Tu, 1979;

Buonassis et al., 1986). Different rhizobial strains successfully protected field-grown soybean, mungbean, sunflower and okra plants from infection by the root-borne pathogens *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Fusarium* species (Ehteshamul-Haque and Ghaffar, 1993) by parasitizing the hyphal tips of the fungal pathogens and decreasing the contact with the host plant cells (Tu, 1979).

#### 4.1.7.4 Rhizobium in bioremediation

Rhizospheric microorganisms with intrinsic ability to reduce/detoxify the heavy metal stress by several mechanisms are diverse and may involve energy-dependent efflux of metal (Nies and Silver, 1995; Nies, 1999), precipitation of metals as insoluble salts (Blake et al., 1993), alteration in membrane permeability to metal (Bruins et al., 2000), metal immobilization in the cell wall (Beveridge and Murray, 1980; Mullen et al., 1989; Scott and Palmer, 1990), production of chelating agents (Silver and Phung 1996), or biochemical transformation of metal ions (Bruins et al., 2000). There are only few reports of rhizobial spp. where plant growth promoting was checked under the metal stress (Wani et al., 2008). Chromium (VI) is more toxic than Cr (III) and produces mutagenic and carcinogenic effects (McLean and Beveridge, 2001). Reduction of Cr (VI) to Cr (III) leads to its detoxification possibly through the soluble chromate reductase by *Mesorhizobium* (Wani et al., 2008). It increased the dry matter accumulation, number of nodules, seed yield and grain protein compared to uninoculated plants. *Mesorhizobium* also decreased the uptake of chromium in roots, shoots and grains. *Mesorhizobium* not only provides tolerance to green gram and pea under different metal stress of Cd, Cr and Cu but also promotes the growth by secretion of growth promoting molecules (Wani et al., 2007 b, c).

#### 4.1.7.5 Rhizobium as P solubiliser

Solubilisation of P by root nodulating bacteria such as *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium* which solubilise rock phosphate, hydroxyapatite or tricalcium phosphate and utilize organic phosphate has been reported (Halder and Chakrabartty, 1993; Abd-Alla, 1994; Peix et al., 2001; Daimon et al., 2006; Alikhani et al., 2006). 2-keto-gluconic acid secreted by *Rhizobium meliloti* solubilized mineral phosphates (Halder and Chakrabartty, 1993). The increase of dry matter of maize by the inoculation with the P-solubilizing *Rhizobium leguminosarum bv. Phaseoli* (R1Lux+) in the rich soil and its higher root colonization ability compared to control

support the relationship between P-solubilization, root colonization and growth promotion (Chabot et al., 1996).

### 4.1.8 Expression of phytase gene in different plants

Plants have limited capacity to obtain P directly from phytate when grown under controlled conditions as availability of phytate in soils is low due to sorption and precipitation reactions (Shang et al., 1992; Hayes et al., 2000; Richardson et al., 2001) and plants do not efficiently exude phytase to the rhizosphere (Hayes et al., 1999; Richardson et al., 2000). Phytase from different bacteria and fungi has been expressed in various plants (**Table 4.2**).

**Table 4.2: Transgenic plants express different phytase gene for utilizing phytate P**

| Organisms | Phytase gene | Plant used for study | Reference |
|---|---|---|---|
| *Aspergillus niger* | *PhyA* | Tobacco | George et al., 2005 |
| | | *Trifolium subterraneum* L. | George et al., 2004 |
| | | *Arabidopsis* | Mudge et al., 2003 |
| | *phyA2* | Maize | Chen et al., 2007 |
| *A. awamori* 3.324 | *PhyA* | soybean | Gao et al., 2007 |
| *E. coli* | *appA* | soybean | Bilyeu et al., 2008 |
| *E. coli* | *appA* | Potato | Hong et al., 2008 |
| *Bacillus subtilis* | *168phyA* | Tobacco | Yip et al., 2003 |
| | | *Arabidopsis* | Lung et al., 2005 |
| Synthetic phytase gene | *PHY* | Potato | Zimmermann et al., 2003 |

Phytase activity was increased to levels sufficient to replace additional supplementation of feed and food with microbial phytases. Transgenic plants could be used as bioreactors for the production of phytase as a supplement (Haefner et al., 2005). Phytase was expressed in seeds to reduce the cost associated with production costs for the enzyme and requirement of special cares in feed processing and diet

formulation (Chen et al., 2007). Overexpression of *E. coli* phytase *appA* in transgenic potato offered an ideal feed additive for improving phytate-P digestibility in monogastric animals, improved tuber yield, enhanced P acquisition from organic fertilizers, and had a potential for phytoremediation (Hong et al., 2008).

### 4.1.9 Effect on phytase on phytate utilization

Irrespective of the presence of soil microorganisms, utilization of phytates by plants in soil environments is poor (Adams and Pate, 1992; Hübel and Beck, 1993) which is due to precipitation by cations such as Ca, Fe and Al and adsorption on soil particles (Tang et al., 2006). Most of the reports on phytate utilization and their effect on plant growth were carried out either in soils amended with phytate or in synthetic media containing sodium phytate as sole P source (Richardson et al., 2001; Idriss et al., 2002; George et al., 2005; Unno et al., 2005) **(Table 4.3).**

**Table 4.3: Organisms producing phytase used for P availability to different plants from Phytate**

| Organisms used as source of phytase | Plant used for study | Reference |
|---|---|---|
| *Pseudomonas spp.* | Pasture grass utilize IHP | Richardson et al., 2001 |
| *Bacillus amyloliquefaciens* FZB45 | Maize seedlings | Idriss et al., 2002 |
| *Burkholderia spp* | *Lupin albus* | Unno et al., 2005 |

The objective of the present study is to determine (i) the effect of organic acid secretion by rhizobacteria on the utilization and hydrolysis of phytate, and (ii) the phytate utilization by *Sinorhizobium meliloti* overexpressing *E. coli appA* gene and its effect on maize, a non leguminous plant, growth promotion. Hence, the *E. coli appA* gene was cloned and expressed under the strong constitutive *lac* promoter in three different rhizobacteria *E. asburiae* PSI3, *Pseudomonas putida* KT2440 and *S. meliloti* 1021.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Bacterial strains and plasmids used

**Table 4.4: Bacterial strains and plasmids used in this study**

| Bacterial strains or plasmids | Genotype or relevant characteristics | Reference |
|---|---|---|
| ***E. coli* strains** | | |
| DH5α | *F' Phi80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+) phoA supE44 lambda- thi-1* | Sambrook and Russell, 2001 |
| S17.1 | *thi pro hsdR recA RP4-2* (Tet::Mu) (Km::Tn7); $Tmp^r$ | Simon et al., 1983 |
| ***P. putida* strains** | | |
| KT2440 | Wild type, prototroph; $Cm^r$, $Rif^r$ | Franklin et al., 1981 |
| *P. p* (pBBRMCS-2) | *P. putida* KT2440 containing pBBRMCS-2 plasmid, $Km^r$ | This study |
| *P. p* (pVA1) | *P. putida* KT2440 expressing *appA* gene of *E. coli*, $Km^r$ | This study |
| ***Sinorhizobium* strains** | | |
| *S. meliloti* 1021 | Wild type of alfalfa group | Young et al., 2006 |
| *S. m* (pBBRMCS-2) | *S. meliloti* 1021 containing pBBRMCS-2 plasmid, $Km^r$ | This study |
| *S. m* (pVA1) | *S. meliloti* 1021 expressing *appA* gene of *E. coli*, $Km^r$ | This study |
| ***E. asburiae* strains** | | |
| *E. asburiae* PSI3 | Rhizosphere isolate from *Cajanus cajan* | Gyaneshwar et al., 1999 |
| *E. a* (pBBRMCS-2) | *E. asburiae* PSI3 containing pBBRMCS-2 plasmid, | This study |
| *E. a* (pVA1) | *E. asburiae* PSI3 expressing *appA* gene of *E. coli*, $Km^r$ | This study |
| **Plasmids used** | | |
| pTZ57R/T | PCR cloning T/A vector, $Ap^r$ | MBI Fermantas |
| pBBRMCS-2 | Broad-Host-Range vector, $Km^r$ | Kovach et al., 1995 |
| pVA1 | pBBR1MCS-2 with *appA* gene of *E. coli* under $P_{lac}$, $Km^r$ | This study |

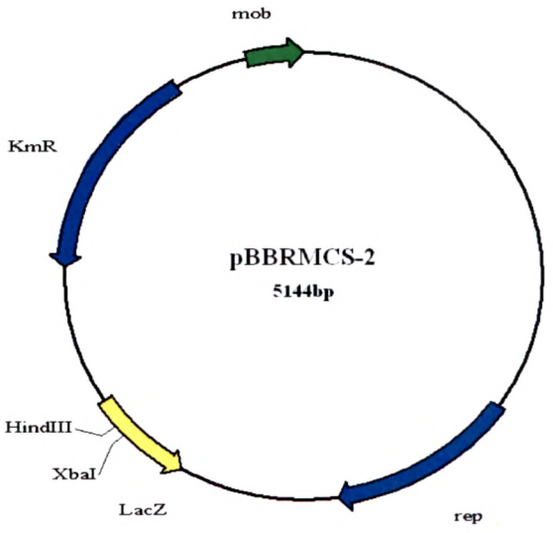
Ap=Ampicillin; Km=Kanamycin; Tmp =Trimethoprim; r=resistance.

*P. putida* KT2440 was a generous gift from Dr. **Soren molin**, Department of Microbiology, The Technical University of Denmark, Denmark. *S. meliloti* 1021 was a generous gift from **Dr. P.S. Poole,** Department of Molecular Microbiology, John Innes Centre, U.K. pBBRMCS-2 was generous gift from **Dr. M. E. Kovach,** Louisiana State University Medical Center, U.S.A

### 4.2.2 Description of plasmids used

**pBBR1MCS-2** (GenBank accession number: U23751) plasmid conferring kanamycin resistance is originally derived from pBBR1 plasmid of *Bordetella bronchiseptica* (Kovach et al., 1995). This plasmid facilitates cloning due to availability of unique and broad restriction sites and direct selection of recombinant plasmids based on blue-white selection strategy. pBBR1MCS-2 is broad host range mobilizable vector. It is compatible with IncP, IncQ and IncW group plasmids, as well as with ColE1 and P15A based replicons. It can replicate stably in a variety of gram negative hosts including *E. coli*, *P. fluorescens*, *P. putida*, *S. meliloti* and *R. leguminosarum* for more than 4 weeks in absence of antibiotic selection (Kovach et al., 1995) **(Fig. 4.2).**

**Fig 4.2: Restriction map of pBBRMCS-2**

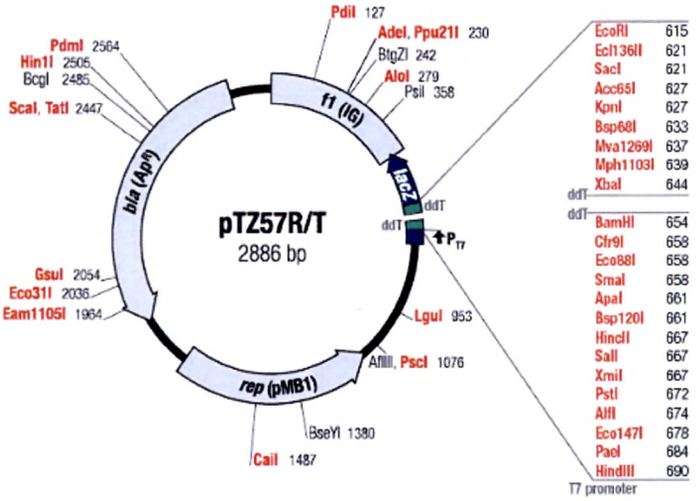



(Generous gift from **Dr. Kovach M. E.,** Louisiana State University Medical Center, U.S.A)

**pTZ57R/T**

pTZ57R/T is 2886 bp in length containing unique and broad MCS. The vector is digested with Eco321 to give blunt end which was then added with 3'-ddT overhangs. The 3'-ddT overhangs at both ends of the cloning site prevent recircularization of the vector during ligation, therefore resulting in high cloning yields and low background. This vector is used for cloning the PCR product amplified with non proof reading polymerases such as *Taq* DNA polymerase. The cloning vector confers ampicillin resistance marker and T7 promoter (**Fig. 4.3**).

**Fig 4.3: Restriction map of pTZ57R/T**




**4.2.3 Media and Culture Conditions**

*E. coli, E. asburiae* PSI3 and *P. putida* KT2440 were routinely grown on Luria-Bertani (LB) broth whereas *S. meliloti* 1021 was grown on TYE (Trypton yeast extract) broth. *P. putida* KT2440 was maintained on pseudomonas agar where as *S. meliloti* was maintained on Yeast extract mannitol agar. For plasmid bearing strains appropriate rich media were supplemented with kanamycin at 25 µg $ml^{-1}$, trimethoprim (60 µg $ml^{-1}$) or ampicillin (50 µg $ml^{-1}$) (Sambrook and Russell, 2001) which was reduced to $1/4^{th}$ concentration when grown in minimal media.

Media composition for Trypton yeast extract (for1L): Trypton 5 g, Yeast extract 3g and $CaCl_2$. $2H_2O$ 0.89 g.

Yeast Mannitol medium (for1L): Yeast extract 1g, Mannitol 10g, $K_2HPO_4$ 0.5g, $MgSO_4$ 0.2 g, NaCl 0.1g and Congo red 0.025 g

### 4.2.4 Phytate utilisation and growth profile Studies in liquid medium

P release and growth profile studies were carried out in 100mM Tris-phytate minimal broth containing (for 1 L) $NH_4Cl$ 5 g, KCl 5g, NaCl 2.5 g, and $CaCl_2$ 15 mg, supplemented with micronutrients consisting of (for 1 L) $FeSO_4 \cdot 7H_2O$ 3.5 mg, $ZnSO_4 \cdot 7H_2O$ 0.16 mg, $CuSO_4 \cdot 5H_2O$ 0.08 mg, $H_3BO_3$ 0.5 mg, $CaCl_2 \cdot 2H_2O$ 0.03 mg, and $MnSO_4 \cdot 4H_2O$ 0.4 mg with arabinose 15 g or glucose 15 g as a sole carbon source and Ca-phytate or Na-phytate 4 g as a phosphorous source. Supplementation of IPTG 0.5mM in case of *E. asburiae* PSI3 and sodium glutamate 2 g, in case of *S. meliloti* was provided.

*E. coli* and *E. asburiae* PSI3 were grown at 37°C while *P. putida* KT2440 and *S. meliloti* 1021 were grown at 30°C at 200 rpm. Aliquots were taken at regular interval till the pH drops below 5.0. Absorbance at 600 nm and pH drop were used as parameters for growth pattern and acidification. Cultures were centrifuged at 9200 x g for 5 min. and culture supernatant was taken for P estimation (Ames, 1964). Results are means of six independent experiments.

### 4.2.5 Molecular Biology tools and techniques

Plasmid extraction using the CTAB method, agarose gel electrophoresis, SDS PAGE, preparation of competent cells of *E. coli* and transformation were carried out as described by Sambrook and Russell (2001).

#### 4.2.5.1 PCR amplification of *E. coli appA*

The DNA corresponding to the coding region of *E. coli appA* was amplified by PCR from genomic DNA of DH5α. The primer used were appAEcF1 (5' CGG AAT TCC GTA AGG AGG AAC ATA TCG ATG AAA GC 3') and appAR1 (5' AGG ATC CTT ACA AAC TGC ACG CCG GTA TG 3'). The forward primer contained an artificial ribosome binding site ATAGGAGG in frame with the ATG and an EcoRI site, reverse primer contained a BamHI site. Temperature profile of the PCR reaction was- 94°C for 5 min, (94°C/30 s, 58°C/30 s, and 72°C/ 90 s) for 40 cycles and 72°C

for 10 min. final extension. Final concentration of all the ingredients per reaction was: 200µM dNTPs, 30ng genomic DNA, 10 pmoles each primer, 1X of Taq buffer and 1.25U of Taq DNA polymerase. The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C were decided with the help of online primer designing software Primer 3 (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 2000).

### 4.2.6 Construction of pVA1 containing *appA* gene from *E. coli*

1338 bp PCR product was purified from agarose gel using Bangalore Genei gel purification kit and subcloned into 2886 bp T/A cloning vector pTZ57R/T (MBI fermantas) according to manufacturer's instructions. The ligation mixture was then transformed into *E. coli* DH5α with blue white selection on ampicillin X-gal plate. The 1338bp EcoRI/BamHI fragment containing *appA* was isolated and further cloned at the corresponding sites into 5144bp broad host range pBBRMCS-2 vector for heterologous expression under *lac* promoter to generate pVA1. The clones were confirmed by release of 1.3 kb fragment with EcoRI/BamHI digestion. pVA1 when digested with BamHI gave 6.5kb band. The final size of the plasmid was 6464 bp.

### 4.2.7 Conjugative transfer of plasmid pVA1

pVA1 plasmid was transferred from *E. coli* S17.1 to *P. putida* KT2440, *S. meliloti and E. asburiae* PSI3 by a biparental mating (Franklin et al., 1981). *P. putida* KT2440, *S. meliloti* and *E. asburiae* PSI3 were grown overnight at 30°C and 37°C, respectively. Both the donor and recipient cultures were mixed in 1:1 proportion in case of *P. putida* KT2440, 1:10 in case of *S. meliloti* 1021 and 1:5 in case of *E. asburiae* PSI3, collected on 0.22 µm pore size nylon-66 membrane filter disc which was then placed on an LB agar plate, incubated at 30°C and 37°C, respectively, for 16 h. Cells were then scraped off the disc and suspended into normal saline. The transconjugants of *P. putida* and *E. asburiae* PSI3 were obtained by plating on luria agar containing kanamycin (25 µg $ml^{-1}$) and erythromycin (30 µg $ml^{-1}$) since *P. putida* KT2440 and *E. asburiae* PSI3 are inherently resistant to erythromycin. The transconjugants of *S. meliloti* were plated on YEM agar containing kanamycin (25 µg $ml^{-1}$). Mucoid colonies were purified by repeated plating or streaking. The

transconjugants were further confirmed with plasmid isolation and restriction endonuclease analysis.

### 4.2.8 Western blot analysis

The cultures were grown overnight on luria broth and TYE media, centrifuged at 9200 x g for 3 min at 4°C. The cells were then washed twice with PBS buffer. The *S. meliloti* cells were washed with 70% ethanol to remove EPS before washing with PBS buffer. The cells were centrifuged again and pellet was resuspended in periplasmic protein extraction buffer containing (Miksch et al., 2002) Protein concentration was estimated by modified Folin lowry method (Peterson, 1979). Protein extracts from transformants harboring plasmid backbone was used as negative control. Samples were heated for 10 min. at 85°C and heat-labile denatured proteins were removed by centrifugation at 12000 x *g* for 10 min. The protein samples were electrophoresed on 10% SDS-polyacrylamide gels, followed by electrotransfer to nitrocellulose membrane (Hybond ECL, Amersham, Buckinghamshire, England) at 100V for 30 min. All lanes were loaded with equal concentration (150 µg) of protein. The membrane was incubated in the blocking solution (3 % BSA, 0.02 % $NaN_3$, 0.2 % Tween 20 and 1x PBS) for 2 h. The blocking solution was discarded and the membrane was incubated with primary antibody (Rabbit anti-appA antibody) and diluted to 1: 500 in blocking solution, for 2 h and then washed 5 times (20 min each) with the blocking solution. Then, the membrane was incubated with the alkaline phosphatase labeled secondary antibody (Goat anti-rabbit IgG antibody; Bangalore Genei, Bangalore) diluted to 1: 1000 in secondary solution (0.15 M NaCl; 0.05 M Tris; 0.2% Tween 20, 0.02 % $NaN_3$ and 5 % skim milk powder), for 1 h and the blot was then washed 4-5 times (20 min each) in secondary solution. The immunoblots were developed by adding alkaline phosphatase buffer (0.5 M NaCl; 5 mM $MgCl_2$; 0.1 M Tris, pH 9.5) containing 66 µl of (10 mg $ml^{-1}$) nitroblue tetrazolium (NBT) and 33 µl of (10 mg $ml^{-1}$) 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

### 4.2.9 Phytase assay

Phytase assay was carried out according to Richardson and Hadobas (1997) with modifications. The cells were grown overnight in their respective media. Bacterial cell pellets obtained by centrifugation at 9200 x g for 3 min. The cells were washed

thrice with saline and final resuspended in 990 µl of saline. The cell suspensions were then treated with 10 µl of lysozyme solution (50mg $ml^{-1}$ in double distilled $H_2O$) for 30 min. After 30 min., 4 ml of acetate buffer, pH 4.5 containing 2mM Na-IHP (1.6 mM final concentration) was added and incubated for 1 hour at 37°C. 750µl of the aliquote was removed and to it 750µl of 5 % trichloroacetic acid was added to terminate the reaction. The samples were then centrifuged at 9800 x g rpm for 5min prior to incubation in ice for 10 min. Free Pi concentration was determined qualitatively using Ames method (1964). One unit of phytase activity was defined as the amount of enzyme that released 1µmol of inorganic phosphate in 1min. specific activity was calculated as Units (U) of phytase activity $mg^{-1}$ of protein under the assay condition.

### 4.2.10 Acid phosphatase assay

Acid phosphatase activity was determined as described by Rodriguez et al. (2000) with modifications. The cells were grown overnight in their respective media. Bacterial cell pellets obtained by centrifugation at 9200 x g for 3 min. The cells were washed thrice with saline and final resuspended in 990 µl of saline. The cell suspensions were then treated with 10 µl of lysozyme solution (50mg $ml^{-1}$ in double distilled $H_2O$) for 30 min. Different aliquots were taken and phosphatase assay was determined by using *p*-nitro phenyl phosphate (pNPP) as a substrate. Sample were incubated with 0.1ml of 0.5M Na-acetate buffer pH 4.5, 0.1ml of 0.5 M pNPP along with 0.1 ml of 0.1M $MgCl_2$. Total system was made up to 2 ml by using distilled water and incubated in dark at 30°C for 30 min. The reaction was stopped by addition of 2 ml of 2N NaOH. The reaction mixture was vortexed and the amount of pNP released was estimated by measuring absorbance at 405 nm. One unit of phosphatase activity is defined as the amount enzyme catalyzing the formation of 1 µmol of p-nitro-phenol per minute.

### 4.2.11 Plant experiments

#### 4.2.11.1 Media used

Plants were grown in semi solid semi synthetic media containing (for 1L)

**Macro element composition (10X) :** $CaCl_2$ .$2H_2O$ 4400 mg, $KNO_3$ 19000 mg, $MgSO_4$. $7H_2O$ 3700 mg, $NH_4NO_3$ 16500 mg, $KH_2PO_4$ 1700 mg.

**Micro element composition (10X) :** $CoCl_2$. $6H_2O$ 0.25 mg, $CuSO_4$. $5H_2O$ 0.25 mg, Na-EDTA 373 mg, $FeSO_4.7H_2O$ 278 mg, $H_3BO_3$ 62 mg, KI 8.3 mg, $MnSO_4.H_2O$ 169 mg, $Na_2MoO_4.2H_2O$ 2.6 mg, $ZnSO_4.7H_2O$ 86 mg.

Stock solutions were prepared for $CuSO_4$. $5H_2O$ (0.1ml of 25mg 10 $ml^{-1}$), $CoCl_2$. $6H_2O$ (0.2ml of 25mg 20$ml^{-1}$) and $Na_2MoO_4.2H_2O$ (2ml of 25mg 20$ml^{-1}$)

Glucose 20 g and sucrose 20 g were added for bacterial and plant growth respectively. Agar-agar 10 g was added as solidifying agent. pH was adjusted to 5.5 and the above media is made and autoclaved at 121°C at 15 psi for 15 min. Na-Phytate was filter sterilized and added to the cooled agar (approximately 42°C). The media contained either no added P (NoP), P as $KH_2PO_4$ (Pi) or P supplied as Na-phytate (NaP).

### 4.2.11.2 Surface sterilization of the seeds

The seeds of *Zea mays* (AHM 1001 from Akash ganga seeds ltd.) were surface sterilized with 0.1% $HgCl_2$ for 5 min. It was discarded, washed several times with distilled water and 70% ethanol was added and again kept for 5 min. Seeds were then finally washed six to seven times with sterile distilled water before kept for germination.

### 4.2.11.3 Inoculum preparation of *S. meliloti*

*S. meliloti* transconjugants were used for inoculation studies. The cells were grown overnight at 30°C in TYE broth shaken at 200 rpm and then centrifuged at 9200 x g for 3min. The pellet obtained was washed thrice with physiological saline before resuspending the cells in double distilled water. The count of the bacteria was adjusted to ~ $10^3$ by serially diluting the bacteria in sterile saline and spreading them on YEM agar containing kanamycin (25mg $ml^{-1}$).

### 4.2.11.4 Plant inoculation

The seeds were transferred to sterile Petri plates containing moist filter paper and kept at 30°C for germination. Paper was kept moist with sterile distilled water

containing 60 μg $ml^{-1}$ cyclohexamide. After one to two weeks seedlings were transferred aseptically to 100ml test tubes containing 50ml of modified, semisolid Murashige and Skoog medium (M.S.) (Murashige and Skoog, 1962), in which $KH_2PO_4$ was replaced by 1 mM Na-IHP. 50 μl of *S. m* (pVA1) and *S. m* (pBBRMCS-2) suspensions (suspended in distilled water) were added to the cooled agar tubes (approximately 42°C), and the tubes were kept at 16 h light/8 h dark conditions at 30°C under gnotobiotic conditions. *S. m* (pBBRMCS-2) was taken as plasmid control and M.S. media without any phosphate was taken as media control. M.S. medium containing soluble phosphate acted as positive control. Each treatment had 10 tubes.

#### 4.2.11.5 Plant parameters

After 20 days of growth the plants were harvested and their shoot and root length were measured using a metric scale. These plants were washed and weighed for Fresh weight (FW) determination, they were oven dried in pre weighed borosilicate glass tubes at 75°C for 48h for dry weight (DW) determinations.

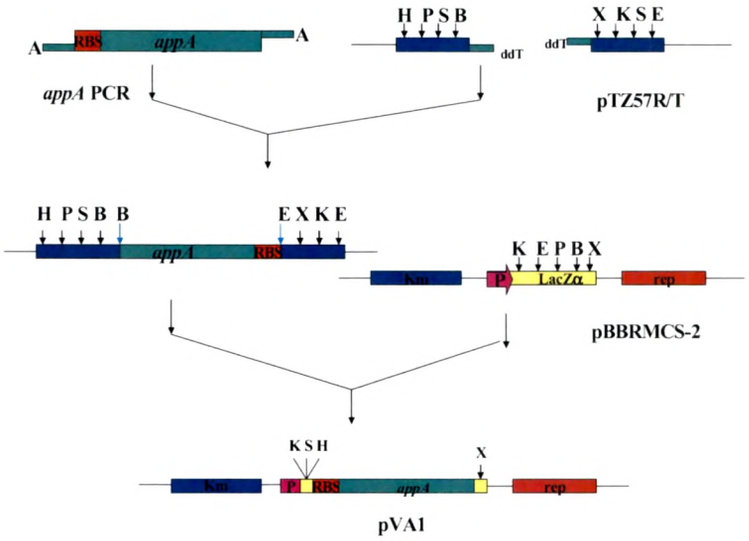
Total P and N were determined on material ashed in a muffle furnace at 550°C for 16h.Ashed sample were dissolved in 0.9M $H_2SO_4$ at approximately 10mg DW $ml^{-1}$ acid . Concentration of P in the extracts was subsequently determined by the molybdate-blue method (Murphy and Riley, 1962). Total N was determined by micro Kjeldahl method (Keys, 1940). Total leaf area was measured.

### 4.3 RESULTS

#### 4.3.1 Construction of pVA1 containing *appA* gene from *E. coli*

pVA1 was constructed as mentioned in Materials and methods. The schematic representation of the cloning procedures is depicted in **Fig. 4.4, with Fig. 4.6** showing map of pVA1. Plasmid was confirmed based on restriction digestion pattern shown in **Fig. 4.5.** Restriction digestion of T-vector of *appA* with EcoRI/ BamHI showed 1.3 Kb release of *appA* suggesting the opposite orientation with respect to the promoter. Similar release of 1.3Kb was also seen with pVA1 digested with EcoRI/ BamHI.

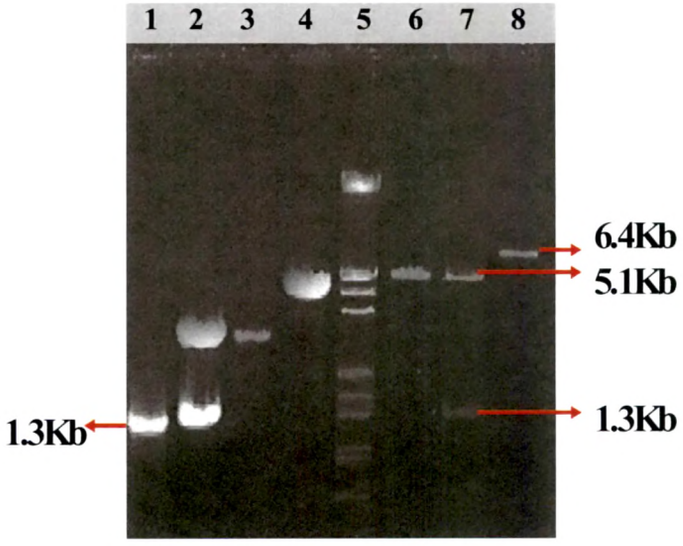
**Fig 4.4: Schematic representation of construction of plasmid pVA1**




Key: E=EcoRI; B=BamHI; K=KpnI; H=HindIII; S=SalI; P=PstI; X=XbaI

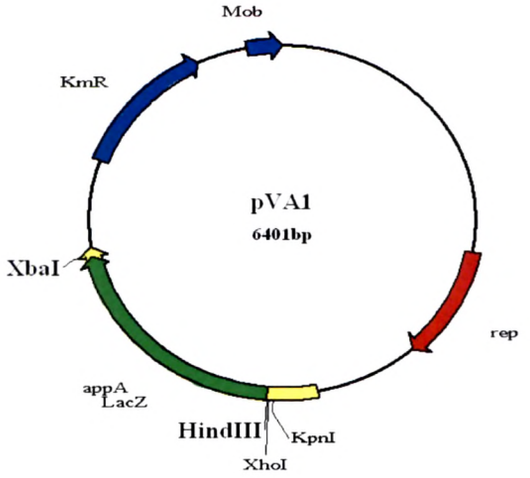
P= promoter; RBS =Ribosomal binding site; Km=Kanamycin resistance gene; Rep= origin of Replication

**Fig 4.5: Restriction endonuclease analysis of pVA1**




Lane 1: PCR product of *appA* gene; Lane 2: T-vector digested with EcoRI/ BamHI; Lane 3: T-vector digested with EcoRI/ BamHI; Lane 4: T-Vector clone digested with ApaI; Lane 5: Molecular Weight Marker (MWM)-Lambda DNA cut with HindIII/EcoRI; Lane 6: pBBRMCS-2 with EcoRI/ BamHI; Lane 7: pVA1 digested with EcoRI/ BamHI; Lane 8: pVA1 digested with BamHI

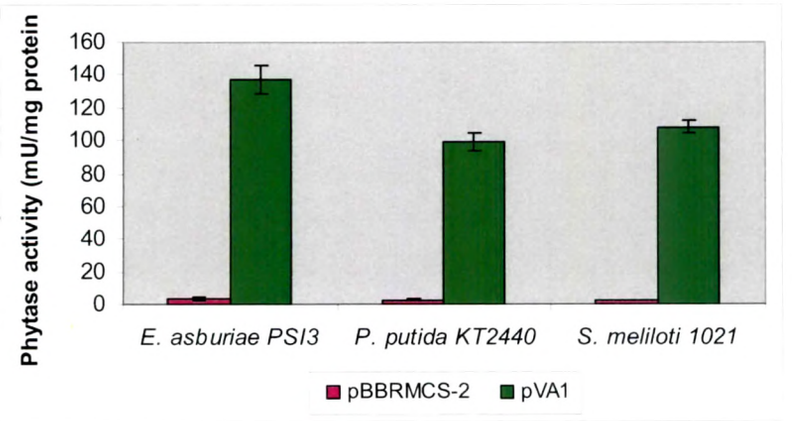
**Fig 4.6: Restriction map of pVA1 plasmid**




### 4.3.2 Phytase activity of *E. asburiae* PSI3, *P. putida* KT2440 and *S. meliloti* 1021 harboring *appA*

*E. a* (pVA1) showed 137. 2 ± 8.3 mU of specific phytase activity, which was ~45 fold higher than the control *E. a* (pBBRMCS-2) having 3.2 ± 0.9 mU of phytase activity. Similarly *P. p* (pVA1) showed 99.2 ± 5.8 mU of activity and was ~ 40 fold higher than the control *P. p* (pBBRMCS-2) which showed 2.4 ± 0.5 mU of specific phytase activity. In *S. m* (pVA1) showed 108 ± 3.6 mU compared to control *S. m* (pBBRMCS-2) having 2.1 ± 0.2 mU, therefore showed ~ 50 fold increase in activity **(Fig. 4.7).**

**Fig. 4.7: Phytase activity of transconjugants**

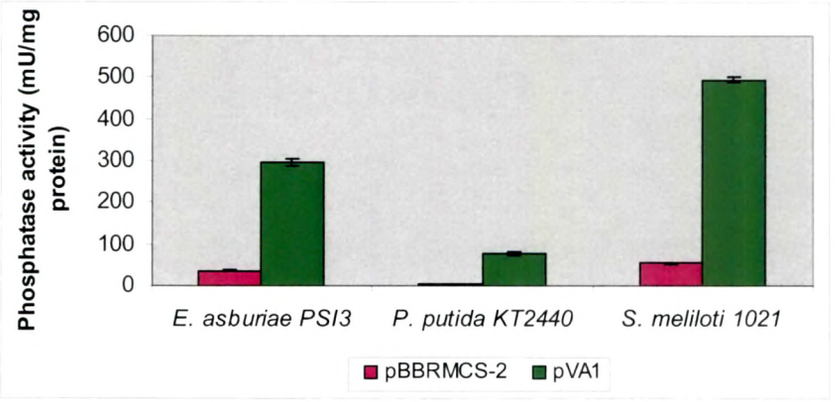



Results are expressed as Mean ± S.D. of six independent experiments.

### 4.3.3 Acid phosphatase activity of *E. asburiae* PSI3, *P. putida* KT2440 and *S. meliloti* 1021 harboring *appA*

*E. a* (pVA1) showed 296.8 ± 8.4 mU of specific acid phosphatase activity and was ~9 fold higher than the control *E. a* (pBBRMCS-2) which had 35.6 ± 1.9 mU of acid phosphatase activity. Similarly *P. p* (pVA1) had 76.9 ± 3.8 mU which was ~ 35 fold higher than the control *P. p* (pBBRMCS-2) showing 2.2 ± 0.6 mU of specific acid phosphatase activity. *S. m* (pVA1) showed 494.5 ± 5.6 mU compared to control which showed 54.3 ± 2.2 mU, and ~ 9 fold increase in specific acid phosphatase activity was observed (**Fig. 4.8**).

**Fig. 4.8**: **Acid phosphatase activity of transconjugants**




Results are expressed as Mean ± S.D. of six independent experiments.

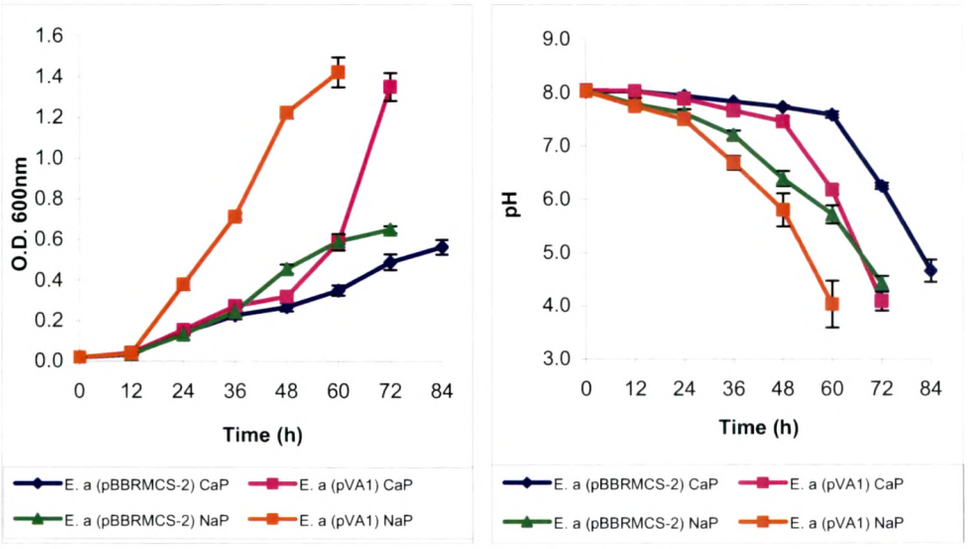
### 4.3.4 Growth profile and acidification pattern of *E. asburiae* PSI3 pVA1 transconjugant on buffered and unbuffered minimal media containing sodium or calcium phytate as sole P source.

*E. asburiae* PSI3, when grown on NaP and CaP as the sole P source and 100 mM L-arabinose as sole carbon source acidified the unbuffered medium in 15 and 18h respectively. All *E. asburiae* PSI3 transconjugants also acidified the buffered (100mM Tris, pH 8.0) NaP and CaP media to pH less than 5.0 in 72 and 84h respectively (**Fig. 4.9A**). Growth ranged from 0.58 $O.D._{600}$ in the case of *E. a* (pBBRMCS-2) to 1.35 $O.D._{600}$ with *E. a* (pVA1) in 84h on CaP buffered medium. In case of NaP buffered medium the growth ranged from 0.65 $O.D._{600}$ in case of control when compared to *E. a* (pVA1) which showed 1.4 $O.D._{600}$ in 72h (**Fig 4.9 A**). In unbuffered CaP medium *E. a* (pBBRMCS-2) and *E. a* (pVA1) showed 0.37 and 0.45

O.D $_{600}$ respectively, whereas on unbuffered NaP growth was found to be 0.26 and 0.26 respectively **(Fig. 4.9 B).** The amount of P released on CaP and NaP buffered media was 3.48 ± 0.35mM and 3.89 ± 0.36mM, respectively. In unbuffered medium, the amount of P release from NaP and CaP was 4.32 ± 0.23mM and 4.01 ± 0.33mM, respectively. Organism with control plasmid did not release P in both buffered and unbuffered media (**Table 4.5**).

**Fig. 4.9: Growth and acidification profile of *E. a* (pVA1) and *E. a* (pBBRMCS-2) on buffered (A) and unbuffered (B) minimal media containing NaP and CaP.**

**(A)**




**(B)**

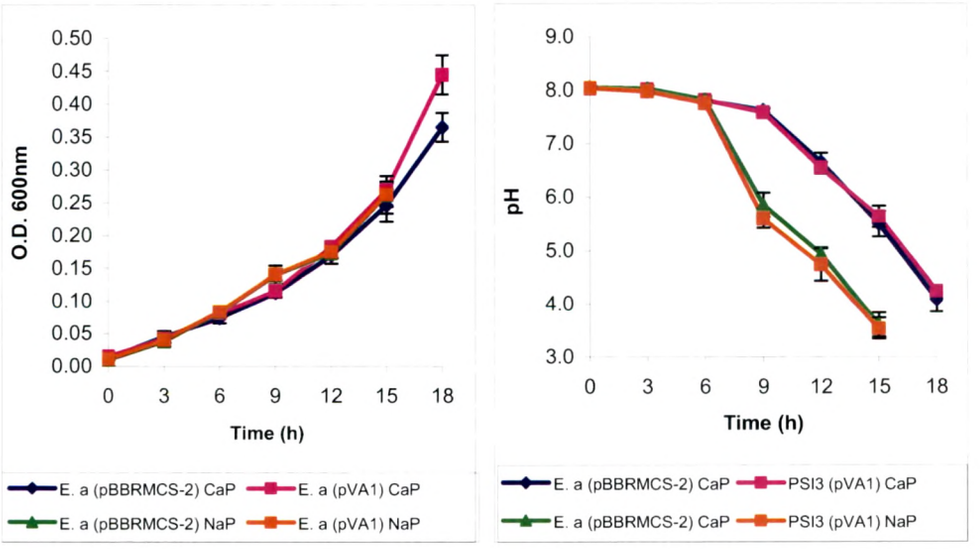
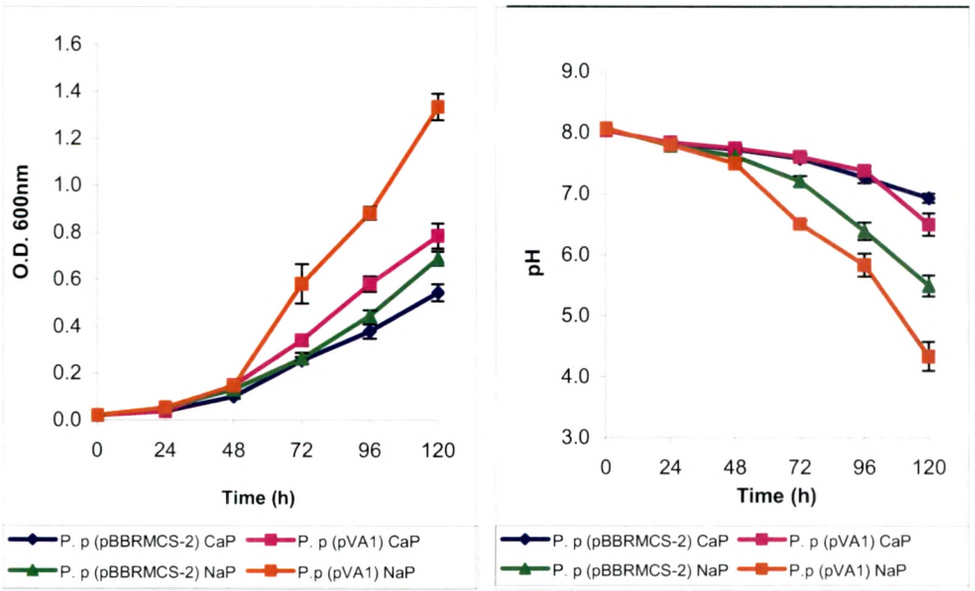
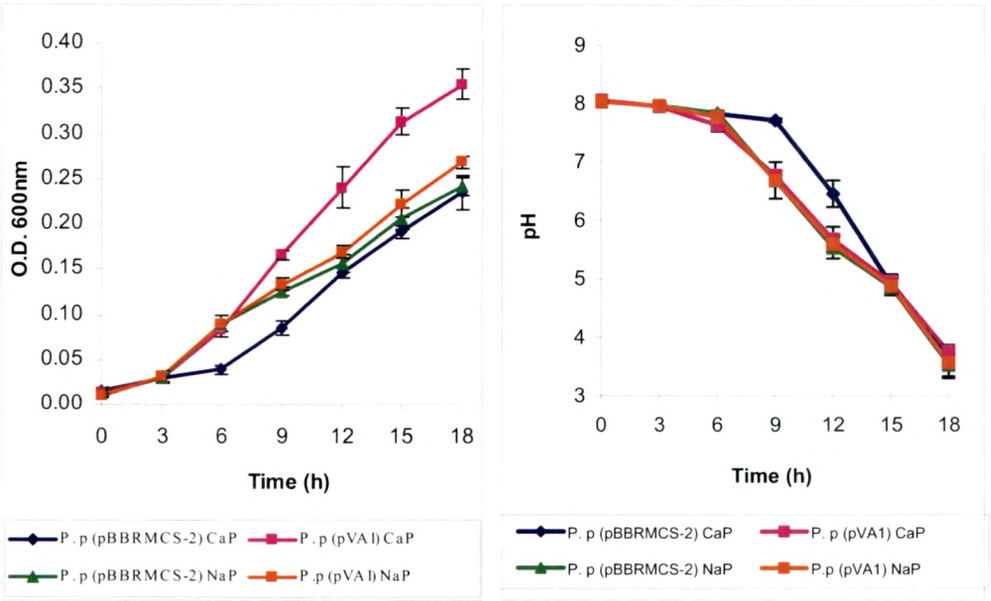


**Fig. 4.10: Growth and acidification profile of *P. p* (pVA1) and *P. p* (pBBRMCS-2) on buffered (A) and unbuffered (B) minimal media containing NaP and CaP.**

**(A)**




**(B)**



### 4.3.5 Growth profile and acidification pattern of *P. putida* KT2440 pVA1 transconjugant on buffered and unbuffered minimal media containing sodium or calcium phytate as sole P source.

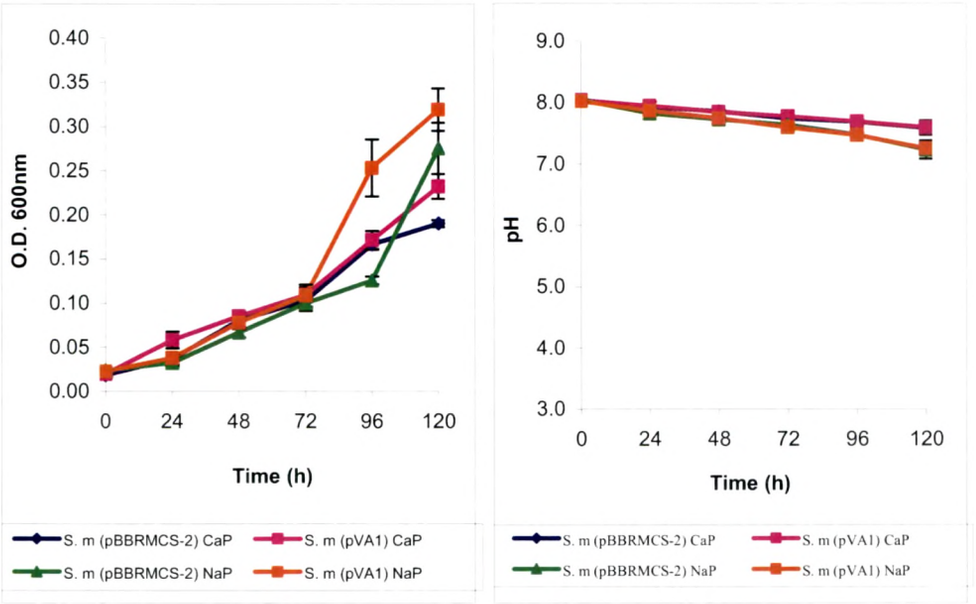
*P. putida* KT2440 transconjugants, *P. p* (pBBRMCS-2) and *P. p* (pVA1), when grown on NaP and CaP as the sole P source and 100 mM glucose as sole carbon source could acidified the unbuffered medium in 18h with growth of 0.24, 0.23 and 0.27, 0.35 $O.D._{600}$, respectively. However only *P. p* (pVA1) showed acidification on NaP buffered (100mM Tris, pH 8.0) medium to pH less than 5.0 after 120h with growth $O.D._{600}$ of 1.33. *P. p* (pVA1) on CaP buffered with growth at $O.D._{600}$ 0.79 and *P. p* (pBBRMCS-2) on NaP and CaP did not acidified the medium below 5.0 even after 120h with growth of $O.D._{600}$ 0.54 and 0.69 respectively (**Fig 4.10 A and B**). The amount of P released in case of NaP buffered, NaP and CaP unbuffered media was found to be 3.21 ± 0.22mM, 3.46 ± 0.13 mM and 3.43 ± 0.17, mM, respectively. However, other transconjugants did not release P (**Table 4.5**).

### 4.3.6 Growth profile and acidification pattern of *S. meliloti* 1021 pVA1 transconjugant on buffered and unbuffered minimal media containing sodium or calcium phytate as sole P source.

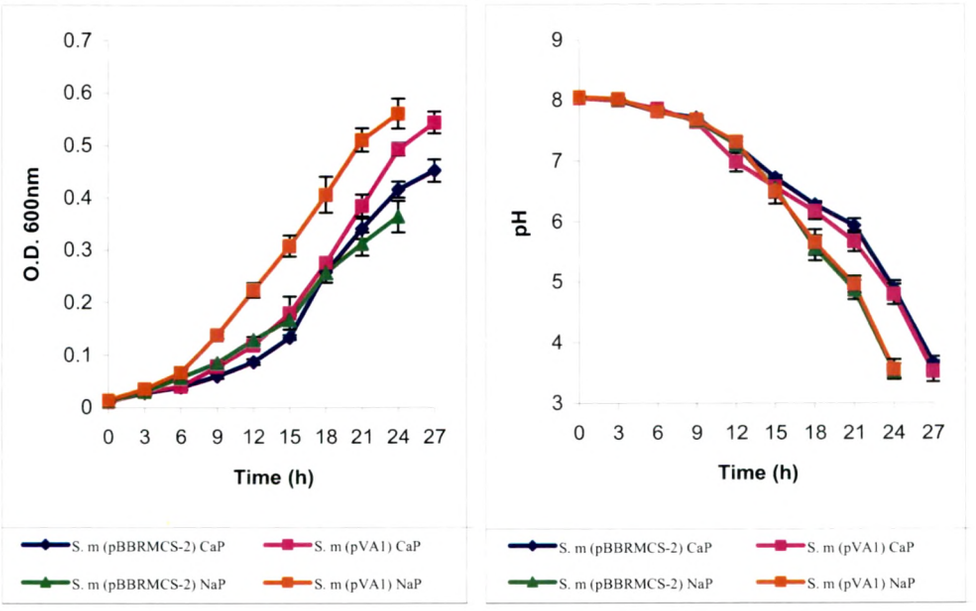
None of the transconjugants of *S. meliloti* 1021 i.e *S. m* (pVA1) and *S. m* (pBBRMCS-2) growth on buffered NaP and CaP media after 120h was 0.32, 0.28 and 0.23, 0.19, respectively (**Fig 4.11 A and B**), but could neither acidify the media nor release P (**Table 4.5**). However all the transconjugants, *S. m* (pVA1) and *S. m* (pBBRMCS-2) could drop the pH of unbuffered NaP and CaP media below 5.0. Growth of *S. m* (pVA1) on NaP and CaP was found to be 0.56 and 0.54 $O.D._{600}$ after 24 and 27h, respectively, while growth with control plasmid was found to be 0.36 and 0.45 $O.D._{600}$ after 24 and 27h respectively. Transconjugants, however, failed to release P in buffered NaP and CaP medium, whereas P release was found to be 3.30 ± 0.30 and 3.06 ± 0.25 in unbuffered NaP and CaP media, respectively. There was no P release in unbuffered medium in case of control.

**Fig. 4.11: Growth and acidification profile of *S. m* (pVA1) and *S. m* (pBBRMCS-2) on buffered (A) and unbuffered (B) minimal media containing Nap and CaP.**

**(A)**




**(B)**



### 4.3.7 P- release by *E. asburiae* PSI3, *P. putida* KT2440 and *S. meliloti* 1021, pVA1 transconjugant on buffered and unbuffered minimal media containing sodium or calcium phytate as sole P source.

**Table 4.5: P-release by transconjugants on buffered and unbuffered NaP and CaP media.**

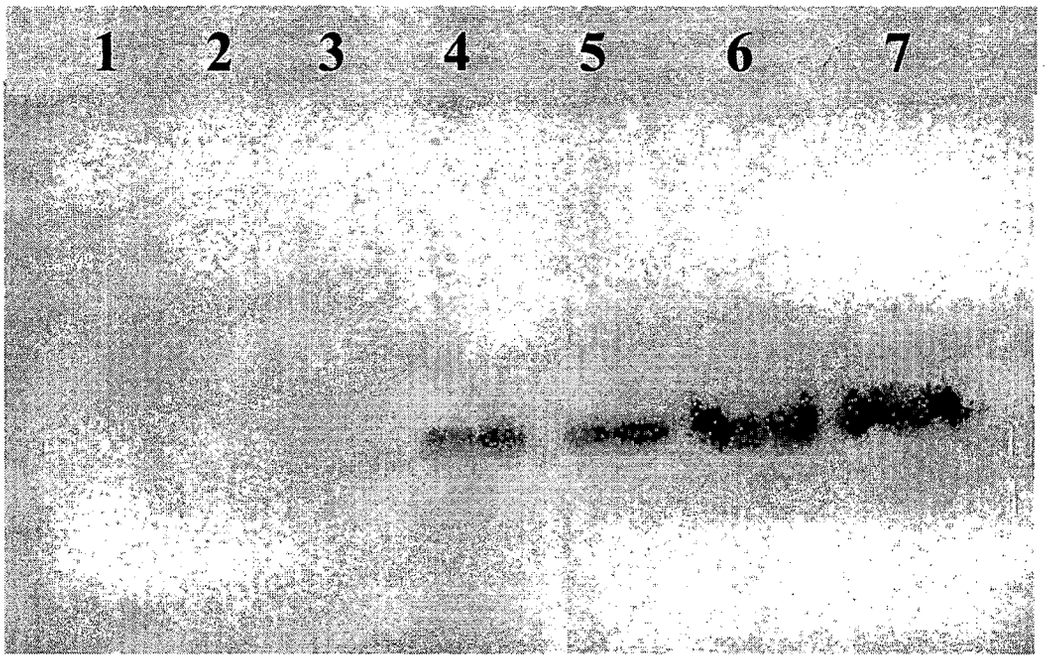
| Organism | Phytate source | Buffered | | Unbuffered | |
|---|---|---|---|---|---|
| | | Time (h) | Pi (mM) | Time (h) | Pi (mM) |
| *E. a* (pBBRMCS-2) | CaP | 84 | UD | 18 | UD |
| | NaP | 72 | UD | 15 | UD |
| *E. a* (pVA1) | CaP | 72 | 3.48 ± 0.35 | 18 | 4.01 ± 0.33 |
| | NaP | 60 | 3.89 ± 0.36 | 15 | 4.32 ± 0.23 |
| *P. p* (pBBRMCS-2) | CaP | 120 | UD | 18 | UD |
| | NaP | 120 | UD | 18 | UD |
| *P. p* (pVA1) | CaP | 120 | UD | 18 | 3.46 ± 0.13 |
| | NaP | 120 | 3.21 ± 0.22 | 18 | 3.43 ± 0.17 |
| *S. m* (pBBRMCS-2) | CaP | 120 | UD | 27 | UD |
| | NaP | 120 | UD | 27 | UD |
| *S. m* (pVA1) | CaP | 120 | UD | 24 | 3.06 ± 0.30 |
| | NaP | 120 | UD | 24 | 3.30 ± 0.25 |

UD- Undetectable; Initial Pi in Sodium phytate media was found to be less then 117 µM. Initial $O.D._{600}$ was less then 0.025. Initial pH was maintained at 8.00 ± 0.10; Results are expressed as Mean ± S.D. of six independent experiments.

### 4.3.8 Detection of phytase protein

Western blot analysis of *E. asburiae* PSI3, *P. putida* KT2440, *S. meliloti* and *E. coli* DH5α containing *appA* gene of *E. coli* showed the presence of phytase protein (**Fig. 4.12**). The transcojugants with the plasmid backbone served as control.

**Fig. 4.12: Western blot of AppA from different strains harboring *E. coli appA***




Lane 1; 2 and 3 are periplasmic protein from *P. p* (pBBRMCS-2), *S. m* (pBBRMCS-2) and *E. a* (pBBRMCS-2) respectively. Lane 4-7 are periplasmic protein from *P. p* (pVA1), *S. meliloti* 1021(pVA1), *E. a* (pVA1) and *E. coli* DH5α (pVA1).

### 4.3.9 Plant Inoculation studies

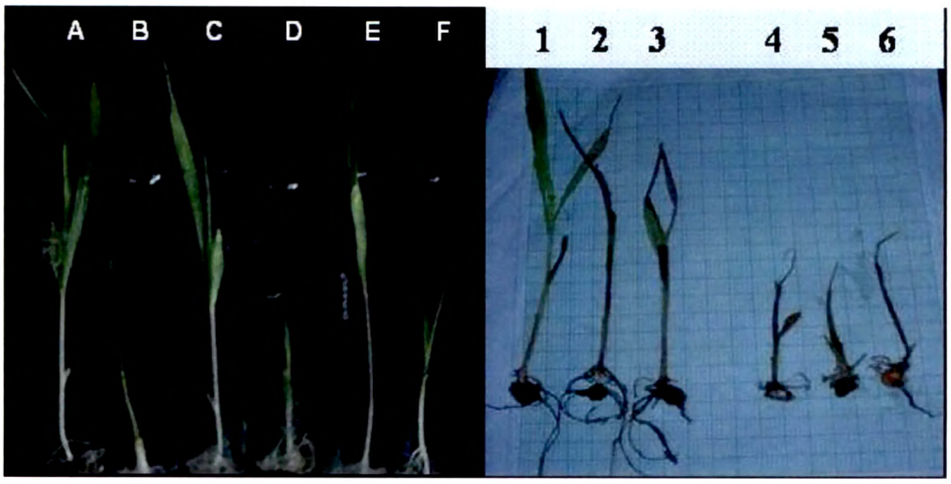
Plant parameters in case of *S. m* (pVA1) were found to be equivalent to the parameters of plants grown in presence of $KH_2PO_4$ (soluble Pi). Plants grown with no phosphate source in the medium even in the presence of *S. m* (pVA1) showed least growth which was equivalent to plant grown on NaP without organism. Plants grown on media containing NaP in the presence of control organism showed less growth compared to *S .m* (pVA1) and significantly better growth compared to the plants without P source. Substantiating the growth the total P content showed the similar pattern ranging from 0.76 µg in case of *S.m* (pVA1) to 0.16 µg in case of NoP (**Table 4.6**; **Fig. 4.13**).

**Table 4.6: Effect of *S. meliloti appA* transconjugant inoculation on maize (*Zea mays*) growth parameters**

| Organism | P Source | Shoot length (cm) | Root length (cm) | Fresh weight (g) | Dry weight (g) | Leaf area ($cm^2$) | Total P (µg) | Total N (µg) |
|---|---|---|---|---|---|---|---|---|
| *S.m* (pVA1) | NaP | 28 ± 1.0 | 7 ± 0.6 | 5.4 ± 0.9 | 0.38 ± 0.1 | 14.5 ± 0.5 | 0.76 ± 0.1 | 7.6 ± 0.5 |
| *S.m* (pBBRMCS-2) | NaP | 10 ± 0.3 | 3 ± .01 | 1.3 ± 0.1 | 0.12 ± 0.1 | 3.2 ± 0.2 | 0.24 ± 0.0 | 2.4 ± 0.1 |
| No organism | $KH_2PO_4$ | 24 ± 0.8 | 8 ± 0.2 | 4.8 ± 0.2 | 0.34 ± 0.1 | 13.8 ± 0.6 | 0.68 ± 0.1 | 6.8 ± 0.4 |
| *S.m* (pVA1) | No P | 6 ± 0.2 | 2 ± 0.2 | 0.9 ± 0.1 | 0.08 ± 0.0 | 2.3 ± 0.1 | 0.16 ± 0.0 | 1.6 ± 0.0 |
| *S.m* (pBBRMCS-2) | $KH_2PO_4$ | 26 ± 0.7 | 8 ± 0.3 | 4.2 ± 0.6 | 0.36 ± 0.1 | 14.1 ± 0.5 | 0.72 ± 0.2 | 7.2 ± 0.2 |
| No organism | NaP | 8 ± 0.5 | 3 ± 0.5 | 1.0 ± 0.0 | 0.10 ± 0.0 | 2.9 ± 0.1 | 0.20 ± 0.0 | 2.0 ± 0.1 |

Results are expressed as Mean ± S.D. of ten plants; NoP- No Pi; NaP- Na-phytate; pH of the media was around 3.0 when plants were harvested. NaP phytate was in precipitated form.

**Fig. 4.13: Effect of *S. meliloti appA* transconjugant inoculation on maize (*Zea mays*) growth**




(A) Lane: A, C and E shows growth of *Zea mays* with *S.m* (pVA1) with NaP; No organism with $KH_2PO_4$ and *S.m* (pBBRMCS-2) with $KH_2PO_4$. Lane B, D, F shows growth *S.m* (pVA1) with NoP; No organism with NaP and *S.m* (pBBRMCS-2) with NaP. (B) Lane 1,2,3 shows the root and shoot length of *Zea mays* with *S.m* (pVA1) with NaP; No organism with $KH_2PO_4$ and *S.m* (pBBRMCS-2) with $KH_2PO_4$.Lane 4,5,6 shows shoot and root length of *Zea mays* with No organism with NaP; *S.m* (pVA1) with NoP and *S.m* (pBBRMCS-2) with NaP.

## 4.4 DISCUSSION

Cloning of *appA* gene from *E. coli* under the *lac* promoter was carried out in conjugable broad host range plasmid pBBRMCS-2. pVA1 plasmid containing *appA* gene was then conjugated to three different bacteria i.e *E. asburiae* PSI3, *P. putida* KT2440 and *S. meliloti* 1021, to impart phytate utilizing ability, which constitutes 20-50% of the total phosphate in the soil (Dalal, 1977). Though phytase has been characterised from various bacteria such as *Bacillus* sp. DS11, *B. subtilis, Pseudomonas syringae, Klebsiella* sp. (Haefner et al., 2005), *E. coli* phytase was chosen because of its higher catalytic activity for phytic acid than other phytases, encodes a bifunctional enzyme exhibiting both acid phosphatase and phytase activities having identical pH optima of 4.5, active at broad pH values from 2.0 to 6.0 and is resistant to pepsin (Golovan et al., 2000; Lei and Stahl, 2001; Miksch et al., 2002; Lee

et al., 2005; Kim and Lei, 2008). These bacteria were chosen for their different plant growth promoting properties. *E. asburiae* PSI3 has good phosphate solubilising ability (Gyaneshwar et al., 1999), *P. putida* KT2440 has plant growth promoting ability (Ramos-González et al., 2005) and *S. meliloti* 1021 has an efficient nitrogen fixing ability (Young et al., 2006).

Periplasmic nature of the protein was confirmed by western blot analysis of the periplasmic protein extract of the transconjugants **(Fig. 4.12)**. *P. p* (pVA1) and *S. m* (pVA1) showed ~40-50 fold higher expression than the controls which is in agreement with the strong and constitutive nature of *lac* promoter in *Pseudomonas* and *Rhizobium* (Labes et al., 1990; Santos et al., 2001). *E. coli appA* expression in *P. putida* PZ1 resulted in 67mU in luria broth (Dharmsthiti et al., 2005). However, over expression of *appA* in *P. putida* KT2440 resulted in ~1.5 fold increase in phytase level present study. *E. asburiae* PSI3 could not be used further, since *appA* of *E. coli* was cloned under the *lac* promoter which is inducible in *E. asburiae* PSI3. To the best of our knowledge, this is the first report of heterologous expression of phytase gene in *Rhizobium*. Expression of *appA* in P-solubilising *E. asburiae* PSI3 could be advantageous since the organism can release P both by solubilization of RP as well hydrolysis of phytate. Since nonspecific phosphoesterase such as acid phosphatases act upon lower phosphoesters from IHP, are important in releasing P from final stage, dual ability of *appA* for acid phosphatase could further enhance available P (Turner et al., 2002).

Microorganisms secreting low molecular weight organic acids play an important role in soil by solubilizing fixed cation complexes (Jones, 1998) particularly by increasing the P availability to plants by their mineral phosphate solubilizing properties (Goldstein, 1986; Kucey et al., 1989). Similarly availability of Po for phytase catalysis, from soil depends upon pH changes caused by the organic acid secretion by the plants (Grierson, 1992; Hoffland et al., 1992; Johnson et al., 1996) or reported *in vitro* mediated by either chelation property in case of AlP or FeP, acid hydrolysis in case of CaP or simply exchange of adsorbed phytate from soil particles (Jones, 1998; Tang et al., 2006). *Burkholderia* spp from rhizoplane and rhizosheath of *Lupinus albus* has been shown to hydrolyse Na-phytate and bring about acidification

of the medium (Unno et al., 2005). In our studies results show that acidification of the medium is important for the release of Po from media containing CaP or NaP.

Transconjugants of *E. asburiae* PSI3 containing *appA* was able to release Po from both buffered and unbuffered CaP and NaP media, due to acidification below 5.0 (**Fig. 4.9 A and B; Table 4.5**), whereas transconjugants of *S. meliloti* could not acidify the buffered CaP and NaP media and therefore could not release Po (**Fig. 4.10 A and B; Table 4.5**). This could be attributed to *S. meliloti* not being able to produce enough acid to overcome the buffering barrier. Optimum pH of *appA* is 4.5 therefore it is necessary to acidify the media below 5.0 to release Po even from soluble NaP. Buffering of the media with 100mM Tris pH 8.0 mimicks the alkaline vertisol (Gyaneshwar et al., 1998) and therefore is an important criteria for selection of the organism. Transconjugants of *P. putida* could acidify the buffered NaP media and release Po but did not acidify the buffered CaP media. It could be attributed to the growth of the bacteria, whose critical mass or number is important for the acidification as to overcome the buffering of the media (**Fig. 4.11 A and B; Table 4.5**). Since in buffered media containing NaP ~ 150µM of soluble P is found which was probably sufficient to sustain the high growth of the bacteria compared to CaP. All the transconjugants could release Po from unbuffered CaP and NaP media which could be attained with secretion of any acid even in small amount. Higher growth has resulted in less time taken in acidification of the media as seen in case of *E. asburiae* PSI3 buffered and unbuffered NaP media.

Since the *appA* of *E. coli* belongs to HAP class of phytase whose 3D structure justifies the binding of IHP to the enzyme involves the interaction of all six deprotonated phosphate groups on the inositol ring with the side chains of several basic amino acid residues (Lim et al., 2000; Liu et al., 2004). This therefore affects the action of enzyme on metal IHP complexes which could be overcome by adding chelating agents such as EDTA, and phthalate therefore, removing the divalent cation (Maenz et al., 1999; Wyss et al., 1999). Presence of organic acids also enhances enzymatic dephosphorylation of insoluble phytates *in vitro* (Tang et al., 2006). These studies were carried out at pH 6.0 at which the solubility of CaP is higher (Maenz et al., 1999) and with the purified enzymes which is totally different scenario comparing to rhizosphere. In transgenic plants secreting phytase, the assimilation of phytate P is

limited by the availability of phytate in the soluble phase (George et al., 2004; 2005; Lung and Lim, 2005), which could be extrapolated to phytase producing rhizobacteria under poorly soluble phytate conditions. Our studies are carried out with whole cells where accessibility of the phytate to the periplasmic protein needs soluble form of phytate.

It can be deduced from this study that phytase-producing rhizobacteria could prove beneficial to plants if phytase possessing bacteria also have the ability to acidify (P-solubilising) the surrounding under these conditions. Since most bacterial, fungal and plant phytases belong to HAP, these findings are highly relevant (Oh et al., 2004). *S.m* (pVA1) was able to release P from the NaP which maize plant is able to take as concluded by plant parameters compared to control. Total P and N also increased in the plants. Growth of the maize plants in treatment with NaP and without any inoculum showed slightly better than the treatment without phosphate, NoP could be because of low levels of P present in the medium. NaP gets precipitated in the media due to high Ca salt but extent of precipitation was not determined. There was no negative effect of the microbial growth on the plants was negated by *S.m* (pBBRMCS-2) with Pi treatment. *Sinorhizobium* does not have any other positive effect except releasing on Po was negated by *S. m* (pVA1) with no P source. Therefore *Sinorhizobium* heterologously expressing *appA* gene was able to release the Po by precipitated NaP by acidification of the media.