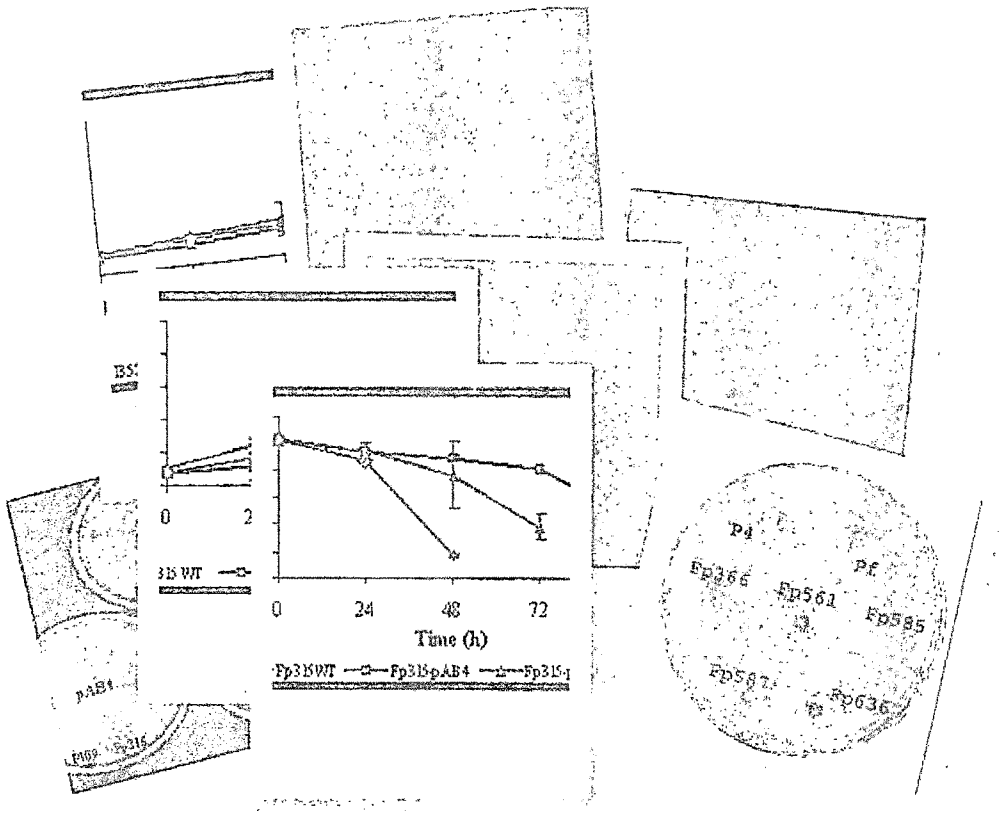



# CHAPTER 7

## Effect of *ppc* and *cs* overexpression on the mineral phosphate solubilizing (MPS) ability of fluorescent pseudomonads

*Education is a process of living and not a preparation for future living.*
*-John Dewey*

## 7.1: INTRODUCTION

Fluorescent pseudomonads emerged as most versatile plant growth promoting rhizobacteria (PGPR). Pseudomonads indirectly benefit the plant growth and development by potent biocontrol mechanisms through secretion of antibiotics, siderophores, hydrogen cyanide and other antifungal molecules (**Fig. 1.6;** Compant et al., 2005; Hass and Defago, 2005). Additionally, they act as efficient biofertilizers which directly enhance the plant growth by inducing systemic plant defense responses, influencing the levels of phytohormones (Lucy et al., 2004; Preston, 2004; Egamberdieva, D., 2008) and increasing phosphate (P) availability due to efficient mineral phosphate solubilizing (MPS) ability (Vessey, 2003, Vassilev et al., 2006). Several *P. fluorescens* isolates (Paul and Sarma, 2006), *Pseudomonas* sp. NBRI 4014 (Gupta et al, 2002) and *Pseudomonas putida* (B0) isolate (Pandey et al, 2006), *P. fluorescens* PGPR1 and *P. aeruginosa* PUPa3 (Dey et al., 2004; Kumar et al., 2005) exhibit efficient MPS ability as well as biocontrol properties.

The MPS ability is attributed to secretion of high amounts of acetic, lactic, malic, gluconic, 2-ketogluconic, citric, tartaric, oxalic and succinic acids (Khan et al., 2006). MPS pseudomonads secrete predominantly gluconic acid which is mediated by periplasmic PQQ-GDH activity (Babu-khan et al., 1995). GDH and PQQ biosynthetic genes have been demonstrated to be responsible for imparting MPS phenotype to non-MPS bacteria (Kim et al., 1998; Liu et al., 1992; Rodriguez and Fraga, 1999). Periplasmic gluconic acid production in pseudomonads via direct oxidation of glucose is an integral part of glucose catabolism (Lessie and Phibbs, 1984). Alternately the glucose catabolism also occurs by glucose uptake and intracellular phosphorylative oxidation.

Earlier Chapters dealt with effects of individual and combined overexpression of phosphoenolpyruvate carboxylase (*ppc*) and citrate synthase (*cs*) genes on glucose catabolism of *Pseudomonas fluorescens* 13525. Both these enzymes being involved at the junction of PEP-Pyruvate-OAA node and TCA cycle were targeted to manipulate the intracellular glucose catabolic pathway and were demonstrated to affect gluconic, pyruvic, acetic and citric acids secretion. Present study describes the effect of *ppc* and *cs* overexpression on MPS ability of *P. fluorescens* 13525 and a few native isolates of fluorescent pseudomonads.

## 7.2: EXPERIMENTAL DESIGN

The experimental plan of work includes the following-

### 7.2.1: Bacterial strains used in this study

| Strains | Relevant characteristics | Reference |
|---|---|---|
| *Pf*(pAB3) | *P. fluorescens* 13525 expressing *S. elongatus* PCC 6301 *ppc* gene from pAB3 plasmid; $Tc^r$ | Chapter 3 |
| *Pf*(pAB4) | *P. fluorescens* 13525 containing pAB4 plasmid, control; $Tc^r$ | Chapter 3 |
| *Pf*(pAB7) | *P. fluorescens*13525 expressing *E. coli cs* gene from pAB7 plasmid; $Km^r$ | Chapter 5 |
| *Pf*(pAB8) | *P. fluorescens* 13525 containing pAB8 plasmid, control; $Km^r$ | Chapter 5 |
| *Pf*(pAB9) | *P. fluorescens* 13525 expressing *E. coli cs* gene from pAB9 plasmid; $Km^r$ | Chapter 5 |
| *Pf*(pBBR1MCS-2) | *P. fluorescens* 13525 with pBBR1MCS-2 plasmid, control; $Km^r$ | Chapter 5 |
| *Pf*(pAB37) | *P. fluorescens* 13525 expressing *ppc* and *cs* genes from pAB3 and pAB7 plasmids respectively; $Km^r/Tc^r$ | Chapter 6 |
| *Pf*(pAB48) | *P. fluorescens* with pAB4 and pAB8 plasmids, control; $Km^r/Tc^r$ | Chapter 6 |
| *Pf*(pAB39) | *P. fluorescens* expressing *ppc* and *cs* genes from pAB3 and pAB9 plasmids respectively; $Km^r/Tc^r$ | Chapter 6 |
| *Pf* (pAB4BBR1MCS-2) | *P. fluorescens* with pAB4 and pBBR1MCS-2 plasmids, control; $Km^r/Tc^r$ | Chapter 6 |
| Native isolates | A48, P109, Fp315, Fp366, Fp636, Fp441, Fp561, Fp585, Fp587, Fp600 | Chapter 2, Table 2.2 |
| Fp585 (pAB4) | Fp585 with pAB4 plasmid; $Tc^r$ | This work |
| Fp585 (pAB3) | Fp585 with pAB3 plasmid; $Tc^r$ | This work |
| Fp315 (pAB4) | Fp315 with pAB4 plasmid; $Tc^r$ | This work |
| Fp315 (pAB3) | Fp315 with pAB3 plasmid; $Tc^r$ | This work |
| P109 (pAB4) | P109 with pAB4 plasmid; $Tc^r$ | This work |
| P109 (pAB3) | P109 with pAB3 plasmid; $Tc^r$ | This work |

**Table 7.1: List of *Pseudomonas* strains used in this study.** Rhizopsheric isolates of fluorescent pseudomonads were generously gifted by Prof. B. N. Johri, Pantnagar and were already characterized with respect to MPS (di-calcium phosphate solubilziation on Pikovaskya's (PVK) agar and biocontrol abilities (ACC deaminase activity). Transformants of *P. fluorescens* and native pseduomonads were grown 30°C with tetracycline and kanamycin (as and when required) at concentrations varying for rich and minimal media as described in Section 2.2.

### 7.2.2: Preliminary characterization of native rhizospheric isolates

All the 10 native isolates were tested for presence of native plasmids and antibiotic sensitivity in order to have convenient selection after the desired genetic modification (Section 2.3; 2.4.1.2). Based on this antibiotic sensitivity profile and the available information on preliminary P-solubilization profile three native isolates were selected for incorporating genetic manipulations.

### 7.2.3: Development of *P. fluorescens* 13525 and native pseudomonad isolates with genetic manipulations

The plasmids pAB3 expressing *S. elongatus* PCC 6301 *ppc* gene from *lac* promoter of pUCPM18 (Chapter 3; Section 3.2.3) with pAB4 as its control were transformed into *P. fluorescens* 13525 by direct transformation (Section 2.4.2) and into the native pseudomonads *via E. coli* S17.1 mediated conjugation (Section 2.4.3). *P. fluorescens* 13525 *ppc* transformants were selected on Pseudomonas Agar with tetracycline while the *ppc* transconjugants of native pseudomonads were selected on Pseudomonas Agar with tetracycline and spectinomycin. These transformants/ transconjugants were confirmed on Koser's medium (Section 2.2; 2.6). pAB7 and pAB9 plasmids with pAB8 and pBBR1MCS-2 as their respective controls, were transformed into *P. fluorescens* 13525 by direct transformation (Chapter 5; Section 5.2; 5.3.1; 5.3.2) and the individual transformants were selected on Pseudomonas agar containing kanamycin. Similarly, *Pf* (pAB3) and *Pf* (pAB4) were used to transform pAB7, pAB8, pAB9 and pBBR1MCS-2 to develop the corresponding double transformants *Pf* (pAB37), *Pf* (pAB48), *Pf* (pAB39) and *Pf* (pAB4BBR1MCS-2) which were selected on Pseudomonas agar containing tetracycline and kanamycin (Section 6.2.2).

### 7.2.4: Growth and MPS phenotype of transformant strains of *P. fluorescens* 13525 and native isolates of fluorescent pseudomonads

The MPS ability of transformants of *P. fluorescens* 13525 and native isolates was monitored on Pikovaskya's (PVK) agar (glucose and xylose as carbon source) and 100mM Tris buffered RP (TRP) agar as described in Section 2.2.4, 2.2.5 and 2.7. The growth and pH profile of all the transformants was checked on TRP liquid medium (Tris buffered rock phosphate broth; Section 2.9.2) and the culture supernatants of the samples withdrawn at regular intervals till the time of pH drop (media pH<5) were analyzed for $O.D._{600nm}$, pH and organic acid production (Section 2.9.3).

## 7.3: RESULTS

### 7.3.1: Morphological and phenotypic characterization of rhizospheric isolates of pseudomonads

10 bacterial isolates of wheat rhizosphere showed varied degree of fluorescence (**Fig. 7.1**). Preliminary antibiotic sensitivity profile (**Table 7.2**) showed that Fp441 was resistant to gentamycin and tetracycline, hence was not studied further. 7 strains were sensitive to tetracycline, kanamycin and gentamycin. The DNA isolated from Fp561 and Fp636 showed a distinct restriction digestion pattern indicating the presence of native plasmids (**Fig. 7.2**).

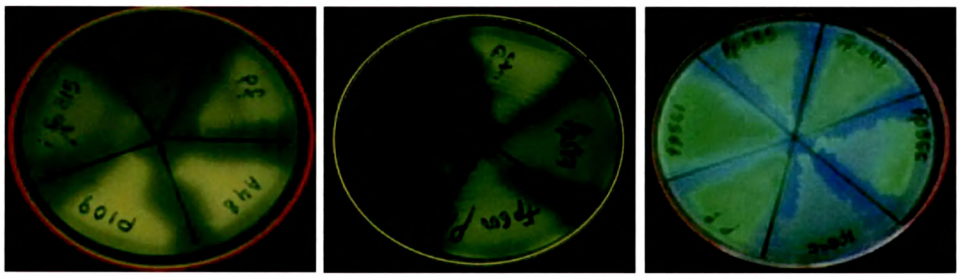


**Fig. 7.1: Fluorescence of 10 wheat rhizosphere isolates on Pseudomonas Agar.** *P. fluorescens* 13525 and *E. coli* DH5α were used as positive and negative controls for fluorescence, respectively. The fluorescence was as monitored after overnight incubation (~16h) at 30°C.

| Antibiotic | A48 | Fp600 | Fp585 | Fp587 | Fp561 | p109 | Fp366 | Fp636 | Fp315 | Fp441 | *Pf* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Erythromycin | + | + | + | + | + | + | + | - | + | + | - |
| Kanamycin | - | - | - | - | - | - | + | + | - | - | - |
| Spectinomycin | + | + | + | + | + | + | + | - | + | + | - |
| Chloramphenicol | + | + | + | + | + | + | - | - | + | + | - |
| Trimethoprim | + | + | + | + | + | + | + | + | + | + | - |
| Tetracycline | - | - | - | - | - | - | - | - | - | + | - |
| Carebenicillin | + | + | + | + | + | + | + | + | + | + | - |
| Gentamycin | - | - | - | - | - | - | - | - | - | - | - |
| Ampicillin | + | + | + | + | + | + | + | + | + | + | + |

**Table 7.2**: **Antibiotic sensitivity profile of wheat rhizospheric isolates of fluorescent pseudomonads**. Antibiotic sensitivity was tested with *P. fluorescens* 13525 was used as negative control as described in Section 2.3.

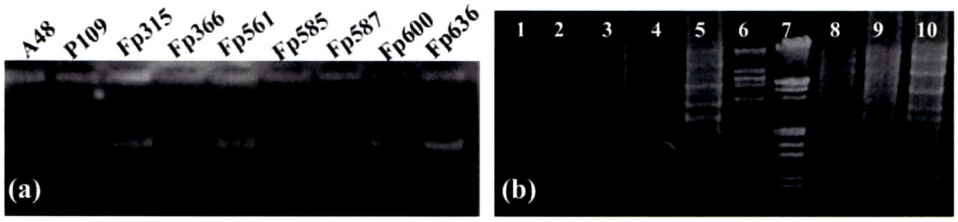
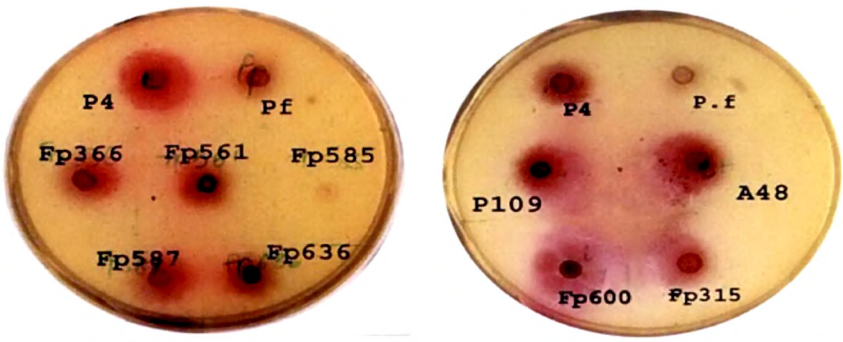



**Fig. 7.2: Native plasmid in wheat rhizospheric isolates. (a)** Native plasmid DNA isolated from the rhizospheric pseudomonads. **(b)** native plasmids digested with BamHI 1-A48; 2-P109; 3-Fp315; 4-Fp366; 5-Fp561, 6-λDNA cut with EcoRI; 7- λDNA cut with EcoRI/HindIII; 8-Fp585; 9-Fp600; 10-Fp636. Plasmid DNA was isolated as in Section 2.4.1.

Although MPS ability of these 10 isolates was tested previously on PVK agar, they solubilized RP to varied extents when subjected to stringently buffered conditions of P-solubilization (**Fig. 7.3**). Based on the P-solubilzation ability on both TRP and PVK media, three strains P109 (mild RP solubilzer), Fp315 (good RP solubilzer) and Fp585 (poor-RP solubilizer) were selected for further genetic modifications.




| Native isolates from wheat rhizosphere | Diameter of zone of acidification (cm) | Native isolates from wheat rhizosphere | Diameter of zone of acidification (cm) |
|---|---|---|---|
| A48 | 0.4 ± 0.1 | **Fp585** | -- |
| **P109** | **0.4** ± 0.2 | Fp587 | 0.5 ± 0.2 |
| **Fp315** | **0.5** ± 0.1 | Fp600 | 0.7 ± 0.2 |
| Fp366 | 0.6 ± 0.2 | Fp636 | 0.4 ± 0.1 |
| Fp561 | 0.3 ± 0.1 | P4 | 0.6 ± 0.1 |

**Fig. 7.3: Organic acid secretion and media acidification by wheat rhizospheric isolates of fluorescent pseudomonads on TRP medium**. The plates here are representative plates. The diameters of the zones of acidification were measured after 5 days of incubation at 30°C. The values are represented as Mean ± S.D. of triplicate observations. *P. fluorescens* (*Pf*) and *Pseudomonas* P4 were used as positive and negative controls respectively. Media composition and other experimental details are as described in Sections 2.2.4 and 2.7

### 7.3.2: Development of *P. fluorescens* 13525, Fp585, Fp315 and P109 transformants harboring pAB3 plasmid expressing *S. elongatus* PCC 6301 *ppc* gene

The *Pseudomonas* transformants/transconjugants were selected on tetracycline and confirmed their citrate utilization on Koser's agar (**Fig. 7.4a**). *P. fluorescens* 13525 as well as the native isolates retained their basic morphology and fluorescence property after genetic manipulations (**Fig. 7.4b**).

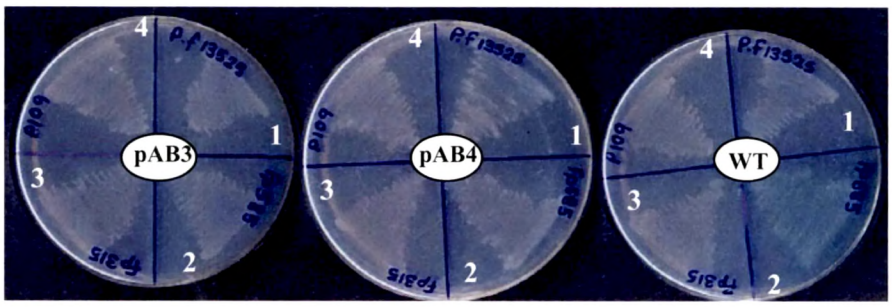



**Fig. 7.4a: Growth of *P. fluorescens* 13525, Fp585, Fp315 and P109 transformant strains of on Koser's Medium.** The growth was monitored after 48h of incubation at 30°C. **1**-*P.fluorescens* 13525, **2**-Fp585, **3**-Fp315 and **4**-P109. The media composition and other experimental details are as described in text (Section 2.6). Tetracycline was added as and when required at the final concentration of 7.5µg/ml.

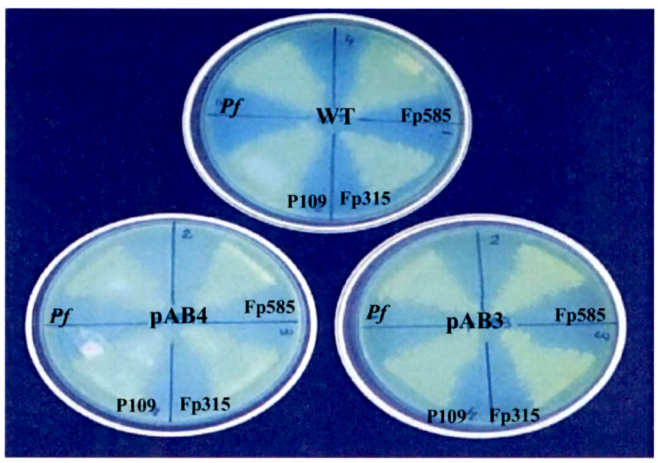



**Fig. 7.4b: Fluorescence of transformant pseudomonads on Pseudomonas Agar.** The fluorescence was monitored after overnight incubation at 30°C. Tetracycline at final concentration of 30µg/ml was used for pAB3 and pAB4 transformants of *P. fluorescens* 13525 (*Pf*) as well as the native isolates.

### 7.3.3: MPS ability of *P. fluorescens* 13525, Fp585, Fp315 and P109 transformants harboring pAB3 plasmid expressing *S. elongatus* PCC 6301 *ppc* gene

P-solubilizing ability of wild type (WT) *P. fluorescens* 13525 and three native isolates on PVK medium varied in the order of Fp315>Fp585>P109=*P. fluorescens* 13525 (**Fig. 7.5a, b**). The pAB3 transformants of *P. fluorescens* 13525, Fp585 and P109 showed enhanced zone of clearance as compared to the control pAB4 (**Fig. 7.5c, d, and e**). On the other hand presence of pAB3 could not improve the P-solubilization ability of Fp315 significantly (**Fig. 7.5f**). P-solubilization by these transformant pseudomonads was also monitored on PVK medium with xylose on which presence of pAB3 enhanced the P-solubilizing ability of *P. fluorescens* 13525, Fp585 and P109 but not of Fp315, as compared to respective controls after an incubation of 72h (**Fig. 7.5g and h**). On PVK-fructose, none of the *ppc* transformants showed zone of clearance (data not shown).

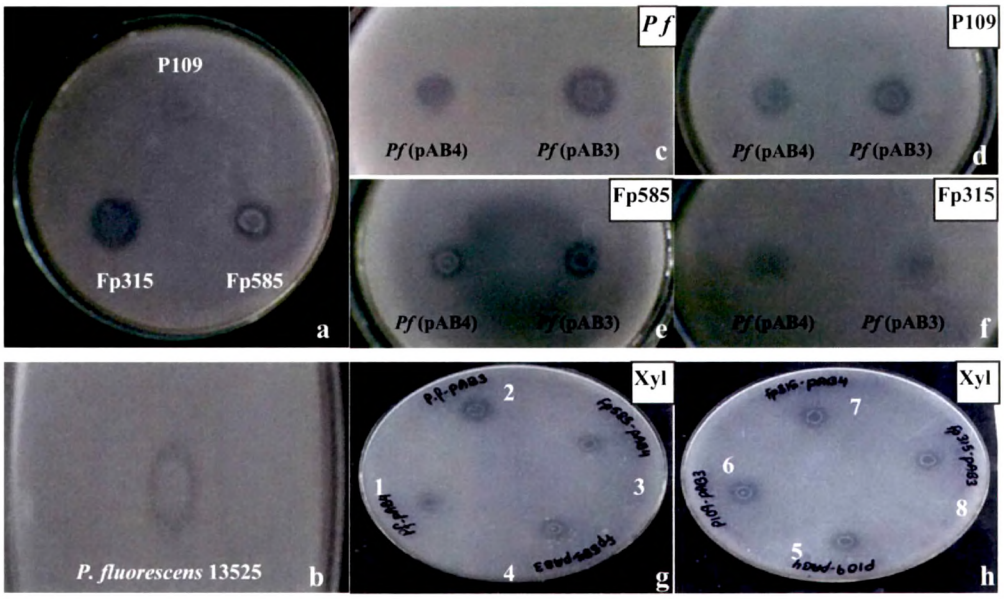



**Fig. 7.5: MPS phenotype of *P. fluorescens* 13525 and native isolates harboring pAB3 plasmid expressing *S. elongatus ppc* gene. a,b-** zone of clearance formed on PVK agar by untransformed 3 native isolates and *P. fluorescens* 13525 respectively. **c, d, e** and **f-** zone of clearance formed by *ppc* transformants of *P. fluorescens* 13525, P109, Fp585 and Fp315 respectively, as compared to their individual controls after incubation of 72-120h.**g, h-** glucose in PVK medium was replaced by same concentration of xylose with other media constituents remaining same. 1-*Pf* (pAB4); 2-*Pf* (pAB3); 3-Fp585 (pAB4); 4-Fp585 (pAB3); 5-P109(pAB4); 6-P109 (pAB3); 7-Fp315 (pAB4); 8- Fp315 (pAB3). Media composition for PVK medium and other experimental details are as described in Section 2.2.5 and 2.7.

### 7.3.4: Growth, RP solubilization and media acidification of *ppc* transformants of *P. fluorescens* 13525 and native fluorescent pseudomonads

Under buffered-RP (TRP) broth conditions, the wild type *P. fluorescens* 13525 and the 3 native isolates showed slower growth (**Fig. 7.6**). pAB4 transformants of *P. fluorescens* 13525, Fp585 and P109 had further reduction in growth rates to varied extents which remained unaltered in their corresponding pAB3 transformants. Growth rate of Fp315 (pAB4) was drastically reduced which could be restored back to that of Fp315 in Fp315 (pAB3).

None of the wild type strains showed media acidification, except Fp315 which dropped the media pH to less than 5 within 48h (**Fig 7.6**). The pAB4 and pAB3 transformants of *P. fluorescens* 13525, Fp585 and P109 failed to acidify the media. On the other hand, control Fp315 (pAB4) acidified the media within 96h and the presence of *ppc* expressing pAB3, time taken for media acidification reduced to 72h, albeit being higher than the wild type. Henceforth, on account of poor cell growth on TRP medium, the effects of further genetic modifications were checked only on PVK medium.

### 7.3.5: Development of *P. fluorescens* 13525 expressing *E. coli cs* gene and its MPS ability on PVK medium

*Pf* (pAB7) and *Pf* (pAB9) expressing *E. coli cs* gene and their respective controls *Pf* (pAB8) and *Pf* (pBBR1MCS-2) were selected on Pseudomonas agar in presence of kanamycin (as described in Section 7.2.3). These transformants retained the property of fluorescence and had normal growth (**Fig. 7.7a, b**). *Pf* (pAB7) and *Pf* (pAB9) showed enhanced MPS phenotype on PVK medium after 120h as compared to its control (**Fig. 7.7c, d, e and f**).

### 7.3.6: Development of *P. fluorescens* 13525 simultaneously expressing *S. elongatus* PCC 6301 *ppc* and *E. coli cs* genes and its MPS ability on PVK medium

*Pf* (pAB37), *Pf* (pAB39) and their respective controls *Pf* (pAB48) and *Pf* (pAB4BBR1MCS-2) when selected on Pseudomonas agar with kanamycin also retained fluorescence and had normal growth (**Fig. 7.8a and b**). However, *Pf* (pAB37) and (pAB39) did not significantly enhance the P-solubilization on PVK medium as compared to the respective controls, *Pf* (pAB48) and *Pf* (pAB4BBR1MCS-2), even after 120h (**Fig. 7.8 c, d, e and f**).

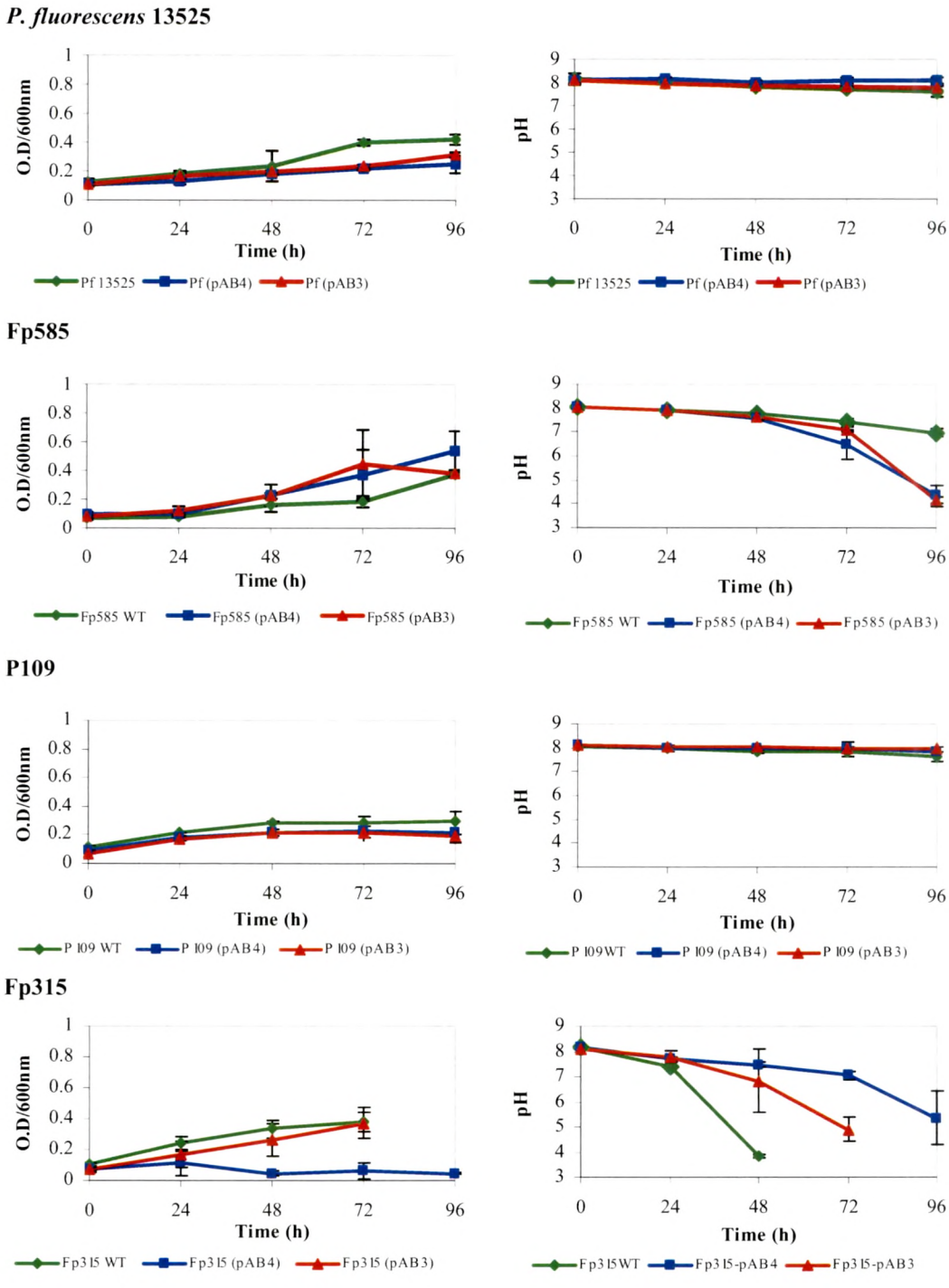



**Fig. 7.6**: **Growth profile and media acidification of wild type and transformants of *P. fluorescens* 13525 and selected native isolates on TRP medium.** The growth and media acidification were monitored on TRP medium with 100mM glucose and RP (Section 2.2.4; 2.9). The values are plotted as Mean ± S.D. of 4 independent observations.

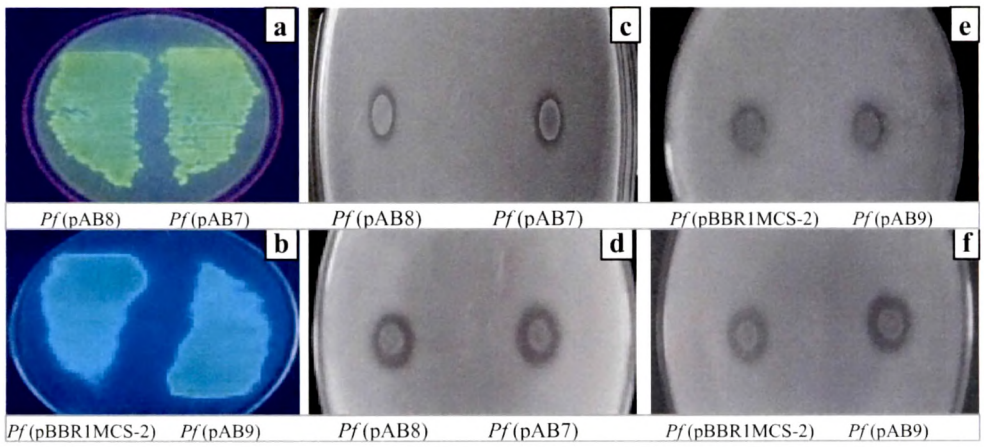



**Fig. 7.7: Fluorescence and MPS ability of *cs* transformants of *P. fluorescens* 13525**

**(a, b)** The fluorescence was monitored after growing *Pf* (pAB7), *Pf* (pAB8), *Pf* (pAB9) and *Pf* (pBBR1MCS-2) on Pseudomonas agar containing kanamycin (50µg/ml) by incubating at 30°C for ~16h (overnight). **(c, d)** and **(e, f)** Zone of clearance formed by *Pf* (pAB7) and *Pf* (pAB8); *Pf* (pAB9) and *Pf* (pBBR1MCS-2) on PVK agar containing 12.5µg/ml kanamycin after incubation for 48h and 120h at 30°C respectively (Section 2.2.5 and 2.7).

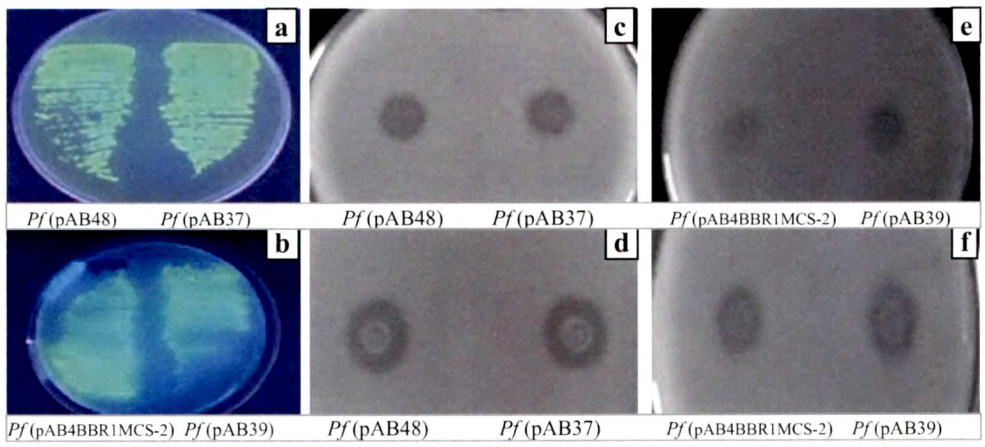



**Fig. 7.8: Fluorescence and MPS ability of *ppc-cs* transformants of *P. fluorescens* 13525. (a, b)** The fluorescence was monitored after growing *Pf* (pAB37), *Pf* (pAB48), *Pf* (pAB39) and *Pf* (pAB4BBR1MCS-2) on Pseudomonas agar containing kanamycin (50µg/ml) and tetracycline (30µg/ml) by incubating at 30°C for ~16h (overnight).**(c, d)** and **(e, f)** Zone of clearance formed by *Pf* (pAB37) and *Pf* (pAB48); *Pf* (pAB39) and *Pf* (pAB4BBR1MCS-2) on PVK agar with kanamycin (12.5µg/ml) and tetracycline (7.5µg/ml) after incubation for 48h and 120h at 30°C respectively (Section 2.2.5; 2.7).

## 7.4: DISCUSSION

Genetic and molecular mechanisms of organic acid secretion by MPS bacteria are confined to involvement of genes responsible for gluconic acid and 2-ketogluconic acid biosynthesis in bacteria like *Enterobacter intermedium*, *Pseudomonas cepacia* and several *Bacillus* spp. The PQQ-GDH and gluconate dehydrogenase (GAD) catalyze the formation of gluconic and 2-ketogluconic acid and are localized in the periplasm. On the other hand, other commonly secreted organic acids including citric, succinic, oxalic, tartaric, lactic, fumaric, glyoxylic, acetic and malic acids are the metabolites of intracellular catabolic pathways. Yet no efforts have been made to genetically manipulate the central metabolic pathways for MPS ability. The present work describes the effect of *S. elongatus* PCC 6301 *ppc* and *E. coli cs* genes on MPS ability of *P. fluorescens* 13525 and native fluorescent pseudomonad isolates. The incorporation of *ppc* expressing pAB3 plasmid in *P. fluorescens* 13525 enhanced its MPS ability on PVK medium with glucose and xylose as carbon sources. Xylose was used as it is one of the predominant monosaccharide found in plant root exudates secreted in the rhizospheric soils where it is a nutrient source for microbial growth (Roy et al., 2002; Kumar et al., 2006). Increased PPC activity benefited the growth of *P. fluorescens* 13525 by increasing the biomass yield on M9 and TrP1 minimal media containing glucose (Chapter 3 and 4).

Although genetically modified strains perform efficiently under laboratory conditions, fail to perform efficiently when used as soil inoculants probably due to competition with other native microorganisms and variation in the nature of the soil and plants (Gyaneshwar et al., 2002; Khan et al., 2006). One of the approaches to overcome this problem is incorporating the *mps* genes in rhizospheric isolates and using them as inoculants which may prove more beneficial, usually because such strains can additionally combat plant pathogenic microbes (Mercado-Blanco and Bakker, 2007). Consequently, 3 rhizospheric isolates Fp585, Fp315 and P109 with varying MPS abilities were chosen to study the effect of *ppc* gene overexpression. Enhanced MPS ability of pAB3 transformants of P109 and Fp585 along with unaltered MPS ability of Fp315 suggested that *ppc* overexpression could enhance the MPS ability of inefficient P-solubilizers but had no effect on efficient P-solubilzers. Although Fp600 and Fp636 had good ACC deaminase activity, their transformant colonies showed very poor growth and were not stably maintained during subculturing.

Enhanced P-solubilization in *Pf* (pAB7) and *Pf* (pAB9) as compared to their respective controls could be attributed to increased gluconic acid secretion as demonstrated for both these strains M9 minimal medium in presence of glucose (Chapter 5). On the other hand, *ppc-cs* co-expression does not appear to enhance the MPS ability which is in accordance with the earlier results where the organic acid secretion remained unaffected in *ppc-cs* double transformants (Chapter 6).

Our results demonstrate that *ppc* and *cs* gene overexpression altered the central glucose catabolic pathways and enhanced MPS ability of *P. fluorescens* 13525. As demonstrated earlier, 2 fold *cs* gene overexpression led to secretion of ~1mM citric acid in *P. fluorescens* 13525. Among MPS bacteria, only few *Bacillus* strains solubilize mineral phosphate via citric acid secretion. But citric acid secretion for P-solubilization has been demonstrated in MPS fungi belonging to *Aspergillus* and *Penicillium* spp. (Khan et al., 2006). In conclusion, this strategy could be employed to incorporate MPS ability into native pseudomonads. Such bacteria would be advantageous as they possess efficient MPS and potent biocontrol abilities, thereby serve the current demand for multipurpose biofertilizers (Vessey et al., 2003; Vassilev et al., 2006).