Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Online International Journal Available at http://www.cibtech.org/jls.htm
2012 Vol. 2 (4) October-December, pp.148-157/Natheer et al.
Research Article

COMPARATIVE PERFORMANCE OF WILD AND *ACD* DEFICIENT MUTANT STRAINS OF *PSEUDOMONAS* AND *PAENIBACILLUS* ON THE MAXIMIZATION OF PLANT GROWTH PROMOTING CHARACTERISTICS IN MAIZE (*ZEA MAYS* L.) CV. CO 1

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ABSTRACT
The comparative performance of the wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on the enhancement of plant growth promoting characteristics and biocontrol against leaf blight disease (*Helminthosporium turcicum*) was studied under gnotobiotic conditions.
The bioinoculation effect of both wild strains of *P. fluorescens* and *P. polymyxa* augmented the maize root elongation and plant growth promoting characteristics *viz*., IAA, siderophore and exopolysaccharide (EPS) production, phosphate solubilization, adhesion to maize root, thermal and desiccation tolerance to a higher level and decreased the incidence of *H. turcicum* in maize plant, when compared to their respective *acd* deficient mutant strains bioinoculation.
Among the two wild strains of *P. fluorescens* and *P. polymyxa*, the wild strain of *P. fluorescens* recorded the highest values for plant growth promoting characteristics when compared to the wild strain of *P. polymyxa*. The results of the present study clearly envisaged the positive effects of *P. fluorescens* and *P. polymyxa* wild strains, exhibiting the ACC-deaminase activity, in augmenting the plant growth promoting characteristics. It may be presumed that the modulation of ethylene level by the rhizobacterial wild strains of *P. fluorescens* and *P. polymyxa* might be the reason for the same.

Key Words: *Maize, ACC-Deaminase, Wild, ACD Deficient Mutant, Plant Growth Promotion*

INTRODUCTION
Rhizosphere bacteria that favourably affect the plant growth and yield of commercially important crops are denominated as "Plant growth promoting rhizobacteria (PGPR)" (Kloepper *et al*., 1980). PGPR are naturally occurring soil bacteria that aggressively colonize plant roots and enhance the plant growth directly by eliciting root metabolic activities by supplying biologically fixed nitrogen (Glick *et al*., 1999; Kloepper *et al*., 1989), hormonal interaction and improvement in root growth, solubilization of nutrients and indirectly by acting against phytopathogens (Timmusk and Wagner, 1999; Jalili *et al*., 2009). PGPR have been reported to be the key elements for plant establishment under nutrient imbalance conditions and their use in agriculture can favour a reduction in the use of synthetic chemical fertilizers and agrochemicals and support eco-friendly crop production (Glick, 1995).
Maize (*Zea mays* L.) is the third major crop of the world after wheat and rice which provides more nutrients for humans and animals than any other cereals and the same is grown in many countries, including India. The positive effects of Maize–PGPR interaction have been reported by many authors (Boddey and Doberiner, 1994; Bashan *et al*., 2004; Gholami *et al*., 2009). PGPR strains may be plant-specific, cultivar specific or non-specific for maize root colonization (Babalola *et al*., 2003) and after colonization, PGPR strains interact with host plant for the improvement of plant growth and induce defense mechanisms against phytopathogens.
1-Aminocyclopropane-1-carboxylate (ACC), an immediate precursor of ethylene, accumulates in the roots of higher plants. Production of ethylene in plants is highly dependent on endogenous levels of ACC (Lürssen *et al*., 1979; McKeon *et al*., 1982). Therefore, in the early stages of plant response to stress,

ACC accumulates concomitantly with a rapid burst in ethylene production (Morgan and Drew, 1997). Ethylene supports the development of adventitious roots, root hair formation and also enhances the seed germination at lower concentration (Pratt and Goeschl, 1969; Esashi, 1991). However, it markedly inhibits the root growth at higher concentration (Jackson, 1991; Belimov *et al*., 2007).

Certain soil bacteria that have ACC-deaminase enzyme are capable of stimulating plant growth by decreasing the level of stress ethylene (Burd *et al*., 1998; Glick *et al*., 1999; Shaharoona *et al*., 2006). ACC-deaminase (*acd*) lowers ethylene levels in plants by converting ACC into α-ketobutyrate and ammonia and the liberated ammonium from ACC is utilized by the bacteria, as a source of nitrogen. Since higher concentrations of ethylene have been reported to inhibit root growth of crop plants (Nukui *et al*., 2000; Arshad and Frakenberger, 2002; Nadeem *et al*., 2010), the bacteria containing ACC-deaminase may completely or partially eliminate the potential inhibitory effects of higher ethylene concentrations in plants (Glick *et al*., 1998). Thus, PGPR strains containing ACC-deaminase activity has the ability to modulate the ethylene level within the host plant by altering the internal ACC concentration and offer promise as a bacterial inoculum for the improvement of plant growth, particularly under stress conditions, such as, flooding, heavy metals, phytopathogens, drought and high salt. Seed inoculation of PGPR strains containing *acd* activity reduces the inhibitory effect of ethylene at spermosphere and leads to the improvement in seed germination (Penrose *et al*., 2001; Mayak *et al*., 2004). Plants treated with ACC-deaminase (*acd*) containing bacteria have longer roots and can better resist the inhibitory effects of stress ethylene on plant growth, while *acd* deficient strains failed to reduce the ethylene synthesis and therefore showed a decreased growth and development of roots under gnotobiotic condition (Lifshitzi *et al*., 1987; Glick *et al*., 1994).

Pseudomonas fluorescens and *Paenibacillus polymyxa* are the two important PGPR genera which are frequently encountered from the rhizosphere of maize. The PGPR characteristics of the same have been described by many authors in different crop plants (Kloepper and Schroth, 1978; Sheng *et al*., 2008; Timmusk *et al*., 2005). The *acd* activity of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* and the resulting plant growth development and yield have been described by Ghosh *et al*. (2003), Saravanakumar and Samiyappan (2007) and Wang *et al*. (2000). However, there were no reports on the comparative performance of *acd* positive wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on the enhancement of plant growth promoting attributes in Maize crop, available.

Hence, the present study has been undertaken with an aim to exploit the comparative performance of *acd* positive (wild) and *acd* deficient mutant strains of *P. fluorescens* and *P. polymyxa* on the enhancement of plant growth promoting attributes and biocontrol of leaf blight disease in Maize.

MATERIALS AND METHODS

Culture Condition

Pseudomonas fluorescens (PF-16) and *Paenibacillus polymyxa* (Pb-16), isolated from the rhizosphere of maize grown at Kadampuliyur, Chidambaram taluk, Cuddalore district, Tamil Nadu state, India were used in the present study. The *Pseudomonas fluorescens* and *Paenibacillus polymyxa* isolates were positive for their ACC-deaminase (*acd*) activity, maintained in King's medium B (King *et al*., 1954) and Nutrient Glucose agar (Englesberg and Ingraham, 1957) slants, respectively, and incubated at 28 ± 2ºC, with monthly transfer. *Helminthosporium turcicum* (AU-1), obtained from the Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar, India, was used as a reference strain for the biocontrol study and the same was maintained in Potato Dextrose agar (PDA) slants and examined periodically for its virulence.

Construction of ACC-deaminase deficient mutant of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* cells (*acd*$^-$ mutant)

The construction of ACC-deaminase deficient mutant (*acd*) of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* isolates was carried out according to Miller (1972) as described below. The *Pseudomonas fluorescens* and *Paenibacillus polymyxa* isolates, namely, PF-16 and Pb-16, which exhibited maximum amount of ACC-deaminase activity under *in vitro* condition, (Data not shown) were used for the spontaneous selection of *acd* deficient mutant and the same microbes were mutagenized with nitrosoguanidine as described by Miller (1972). Following mutagenesis, the diluted cells of the two isolates were individually and immediately plated onto solid Tryptone soybean (TSB) medium and then replica plated onto DF salts minimal medium and glucose medium amended with ammonium sulphate (20 gL^{-1}) and DF salts minimal medium and glucose medium with ACC (3 mM L^{-1}), respectively.

Cells that grow on DFMM or glucose broth (GB) supplemented with ammonium sulphate but not on DFMM or glucose broth supplemented with ACC were selected as *acd* deficient mutants and used for further study. All the mutants that were unable to proliferate on ACC, as a nitrogen source, were shown to yield fluorescent siderophore in the case of *Pseudomonas fluorescens* and white wrinkled colony in case of *Paenibacillus polymyxa* in KB and GB plates plus ammonium sulphate, like the wild type cells, and indicated the absence of ACC-deaminase activity.

Comparative performance of wild (W) and *acd* deficient mutant (M) of *Pseudomonas* and *Paenibacillus* strains on growth promoting characteristics.

Preparation of Inoculum

All the wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* isolates were grown in King´s B broth and nutrient glucose broth, respectively, in a shaking bath at 28 ± 2 ^{o}C for 24 h. Then, the media were centrifuged separately, at 5000 × g for 10 min to harvest the log phase cells of *acd* positive and *acd* deficient mutants of *P. fluorescens* and *P. polymyxa* isolates. Then, the respective pellets were washed three times with 0.1 M phosphate buffer (pH 6.8) individually. Finally, the cells of *P. fluorescens* and *P. polymyxa* were resuspended separately, in the same buffer at a cell concentration of 1×10^{7} CFU mL^{-1} by measuring the OD at 420 nm for *P. fluorescens* and 540 nm for *P.* ***Polymyxa* and used as Inoculum.**

The comparative performance of wild (W) and *acd* deficient mutant of *Pseudomonas fluorescens* and *Paenibacillus polymyxa viz*., PF-16W, PF-16M, Pb-16W and Pb-16M on different PGPR characteristics *viz*., IAA and siderophore production, 'P' solubilization, adhesion to maize roots, EPS production, thermal and dessication tolerance was studied under *in vitro* condition.

IAA Production and Estimation

The IAA production by the wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* was carried out in King's B and nutrient glucose broth respectively together amended with 100 mg/L of DL-Tryptophan and the extraction and estimation of IAA were done according to the Tien *et al*., (1979).

Siderophore production and estimation

The phenolate and hydroxamate type of siderophore production by the wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* were estimated according to Modi *et al*., (1985) and Gibson and Magrath, (1969) respectively.

Phosphate Solubilizing Efficiency

The phosphate solubilizing efficiency of the wild and *acd* deficient mutant strains of *Paenibacillus polymyxa* was carried out in Pikovskaya (PVK) broth according to the procedure of Olsen and Sommers (1982).

Exopolysaccharide (eps) Production

The EPS production of wild and *acd* deficient mutant strains of *Paenibacillus polymyxa* was estimated as per the procedure of Englesberg and Ingraham (1957) as described below. 100 mL of glucose broth was dispensed into 250 mL Erlenmeyer flasks under sterilized condition. One mL culture of *Paenibacillus*

polymyxa isolate (1 x 10^7 CFU/mL) was added to the medium and incubated at 28 ± 2°C for one week. The broth was kept in a rotary shaker at 250 rpm for 15 min every day.

After the incubation period, the cells were harvested by centrifugation at 5000 × g and used for the analysis of alkali stable polysaccharide by anthrone method (Dubois *et al*., 1951) and the supernatant was used for the analysis of water soluble polysaccharide (Sutherland and Wilkinson, 1971).

Effect of wild and *acd* deficient mutant strains of *Pseudomonas* and *Paenibacillus* on maize root elongation

Maize (*Zea mays* L.) seeds cv. Co 1 were surface sterilized by immersion in 95 per cent ethanol for 1 min followed by 20 min in 1 per cent NaCl. After rinsing three times with distilled water, the sterilized seeds were immersed in either sterile water (control) or suspension of wild or *acd* deficient mutant strains of *Pseudomonas fluorescens* or *Paenibacillus polymyxa* separately for 1 h at room temperature. After the bioinoculation, maize seeds were kept in growth pouches (6 seeds/pouch) and kept in a rack. Three replications were maintained for each treatment. The box containing growth pouches were covered with wrapping sheet and kept at 28 ± 2˚C for 4 to 5 days in a light chamber. After the incubation period, the growth pouches were opened and the root length of maize (cv. Co 1) from each treatment was measured and expressed in mm.

Adsorption Assay

After the germination, the three day old sterile seedlings were transferred to slopes of Fahraeus solution (Fahraeus, 1957) solidified with 1.5 per cent agar in test tubes. Sterile Fahraeus solution was added to fill the empty portion of the agar slopes and the tubes were incubated for three more days (24 ºC day/22 ºC night). After the incubation period, the roots were collected from each tube separately, washed first with sterile water and later three times with 0.1 M phosphate buffer (pH 6.8), cut into 5 cm pieces and used for the adsorption study as described by Gafni *et al*. (1986).

Thermal Tolerance

One mL suspension of each PGPR cells, namely, wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa*, was collected from 15 d old King's B broth and glucose broth, respectively. Then, the cells were individually taken in a vial and immersed in water bath adjusted to 50°C temperature. After 20 min exposure, the tubes were removed and cooled rapidly. Then, 100 µl of samples were taken from each tube and plated on nutrient agar plates for determining the viability of the cells.

Desiccation Tolerance

One mL suspension of each PGPR cells, namely, wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* was collected from the 15 d old King's B and glucose broth, respectively. Then, the suspensions were placed individually in sterile 1.5 mL Eppendorf microcapillary tubes and the same were in turn kept open in a sterile petridish. The petridishes with the tubes were then placed in an incubator at 37°C. After 1 week incubation period, the dried cells from the capillary tubes were washed with 100 µl of sterile distilled water with vigorous agitation for the complete removal of PGPR cells and their viability was determined by plating on nutrient agar plates.

Response of wild and *acd* deficient mutant strains of *Pseudomonas* and *Paenibacillus* on leaf blight disease incidence

The experimental studies were performed in a randomized block design with three replications and the following were the treatments, 1) Control, 2) *Pseudomonas fluorescens* wild (PF-16W), 3) *Pseudomonas fluorescens acd* deficient mutant (PF-16M), 4) *Paenibacillus polymyxa* wild (Pb-16W) and 5) *Paenibacillus polymyxa acd* deficient mutant (Pb-16M), respectively.

Preparation of Growth Chamber

The growth chamber used for the study was a desiccator (12 × 10 cm) consisting of two parts. The lower part was filled with a Weaver's medium (Weaver *et al*., 1975) whereas the upper part contained stainless

steel wire mesh (mesh size 3 mm) supports. The lid was placed over the cotton and the chamber was closed before sterilization and then sterilized by autoclaving.

Seeds of maize cv. Co 1 was surface sterilized and germinated as stated elsewhere in the text. Fifty germinated maize seeds with coleoptiles (2 cm high) were transferred aseptically onto the stainless steel wire mesh, incubated for 10 d in growth chamber with temperature ranging from 24ºC at night to 30ºC around noon. By this time, the maize roots yielded many lateral roots, well spread in the Weaver's medium maintained at lower part of the growth chamber.

Challenge Inoculation

Maize plants were challenge inoculated by spraying the spore suspension of *Helminthosporium turcicum* at a spore concentration of 50,000 spores mL^{-1} inoculum level on 10^{th} DAS with an atomizer and control plant was sprayed with sterile Weaver's medium. The spraying was done under proper humid condition. After one week of challenge inoculation, three plants from each treatment were carefully removed and rinsed with sterile distilled water. The blast disease incidence was enumerated with a score chart of 0 to 9 grades devised by IRRI (International Rice Research Institute) (1993).

Statistical analysis

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure described by Gomez and Gomez (1984).

RESULTS AND DISUCSSION

Pseudomonas fluorescens and *Paenibacillus polymyxa* isolates *viz*., PF-16 and Pb-16, respectively, were mutagenized using nitrosoguanidine to produce *acd* deficient mutant strains and then replica plated onto DF salts medium and glucose medium duly supplemented with ACC (3 mM L^{-1}) or ammonium sulphate, respectively as described by Miller (1972). It was observed that the wild strains *viz*., PF-16 and Pb-16 registered a normal growth in the DF salts medium amended with ACC and suggested the ability of the wild strains to utilize ACC, as a sole 'N' source whereas the ACC deaminase (*acd*) deficient strains *viz*., PF-16M and Pb-16M, could not able to metabolize ACC and resulted in poor or no growth (data not shown). Glick *et al*., (1994) constructed an *acd* deficient mutant strain of *Pseudomonas putida* GR-12-2 by chemical mutagenesis (nitrosoguanidine) and reported the inability of the respective mutants to utilize ACC. The results of the present study also clearly in accordance with the earlier findings of Glick *et al*., (1994).

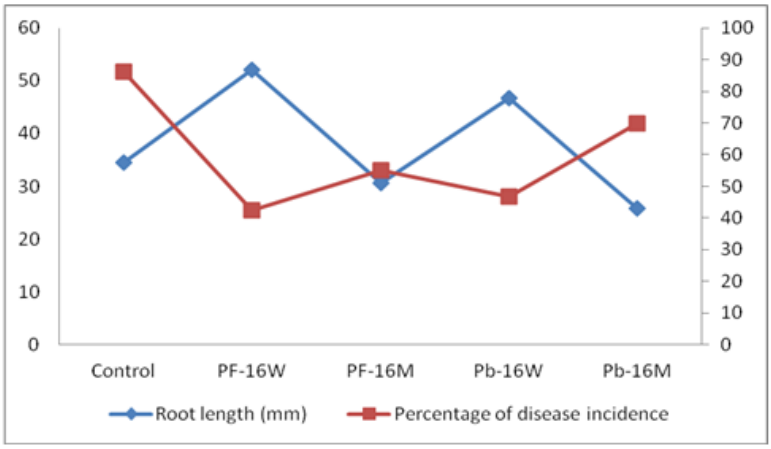

Figure 1: Comparative performance of wild (W) and *acd* deficient mutant (M) strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on maize root elongation and incidence of *Helminthosporium turcicum*

The comparative performance of wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* isolates *viz*., PF-16W, Pb-16W, PF-16M and Pb-16M on the enhancement of

Table 1: Comparative performance of wild (W) and *acd* deficient mutant (M) strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on different plant growth promoting characteristics

Isolate *	IAA production (µg/mL)$^{+}$	Siderophore production (µg/mL)$^{++}$		'P' solubilisation efficiency (µg/mL)**	Adhesion to maize root (10^4/g dry wt of root/h)	EPS production (g/mL)		Thermal tolerance (No. of viable cells/ mL after 50°C treatment for 20 min)	Dessication tolerance (No. of viable cells/ after l week incubation) ^{b,c}
		2,3-DHBA	Salicylic acid			Water soluble polysaccharide	Alkali stable polysaccharide		
PF-16W	5.2±0.46^{a}	4.36± 0.35^{a}	3.82±0.27^{a}	-	367.2±6.25^{a}	-	-	7.73±0.16x10^5^{a}	7.87±0.27x10^5^{a}
PF-16M	3.5±0.21^{c}	3.22±0.26^{c}	2.86±0.18^{c}	-	286.0±5.27^{c}	-	-	6.42±0.11x10^5^{c}	6.22±0.10x10^5^{c}
Pb-16W	4.5± 0.32^{b}	4.09 ±0.20^{b}	2.92±0.15^{b}	186.6±8.6	321.5±4.25^{b}	0.225±0.56	7.161±0.42	6.90±0.18x10^5^{b}	7.01±0.16x10^5^{b}
Pb-16M	2.6±0.12^{d}	2.7±0.19^{d}	1.85±0.03^{d}	152.0±5.1	272.4±4.86^{d}	0.097±0.32	6.561±0.32	5.72±0.07x10^5^{d}	5.95±0.12x10^5^{d}

a - *Values are mean of three replication ± SD*
b - *Values followed by different letters are significantly differed at 5% level according to student "t" test.*
* - *at 1x 10^7 CFU/ml inoculum level*
\+ - *Estimation of Indole acetic acid according to Tien et al. (1979)*
\+ + - *Estimation of Siderophore production according to Modi et al. (1985)*
** - *Estimation of Phosphate solubilization according to Olsen and Sommers (1982)*
2,3 DHBA - *2,3 dihydroxy benzoic acid*
W - *Wild*

different PGPR characteristics *viz*., IAA and siderophore production, adhesion to maize roots and thermal and dessication tolerance was investigated (Table 1). It was observed that the wild strains of *P. fluorescens* and *P. polymyxa viz*., PF-16W and Pb-16W, could augment the above mentioned PGPR characteristics to a higher level when compared to their respective *acd* deficient mutant strains. Between the two wild strains, the wild strain of *P. fluorescens* (PF-16W) recorded the highest values for the above PGPR characteristics when compared to the wild strain of *P. polymyxa* (Pb-16W).

The improved PGPR characteristics of P. fluorescens regarding the IAA and siderophore production, adhesion to maize roots and thermal and dessication tolerance have been reported by many authors (Hayat *et al*., 2010; O'Sullivan and O'Gara, 1992; Puente *et al*., 2009; Wei *et al*., 1996) while the same was reported by Chanway and Holl (1991), Guemouri-Athmani *et al*., (2000) and Holl *et al*., (1988) for P. polymyxa. In the same manner, the *Paenibacillus polymyxa* wild strain exhibited a higher performance for 'P' solubilization and EPS production when compared to their respective *acd* deficient mutant strain. Moreover, the wild strains of *P. fluorescens* and *P. polymyxa viz*., PF-16W and Pb-16W were found to augment the maize root elongation and reduce the leaf blight incidence of maize to a significant level when compared to their respective *acd* deficient mutant strain (Fig. 1). The biocontrol of maize leaf blight disease due to *P. fluorescens* and *P. polymyxa* bioinoculation has already been reported by Belimov *et al*., (2007); Haggag, (2007) and Jalili *et al*., (2009). The mechanism regarding the improved PGPR characteristics by *acd* positive wild strains of *P. fluorescens* and *P. polymyxa* needs further exploitation.

However, there were no earlier reports on the comparative performance of wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on the enhancement of PGPR characteristics in maize, available for discussion. This is the first comprehensive report regarding the beneficial effect of wild and *acd* deficient mutant strains of *Pseudomonas fluorescens* and *Paenibacillus polymyxa* on the enhancement of plant growth promoting characteristics and bio control against *H. turcicum* in maize.

ACKNOWLEDGEMENT

The authors thank to University Grants Commission (UGC) of India for providing funds under the scheme of Maulana Azad National Fellowship (MANF) for the Minority students in support of this research and to Department of Microbiology, Annamalai University, Annamalai Nagar, India for providing necessary facilities to carry out this work.

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