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Isolation and characterization of fluorescent pseudomonads and their effect on plant growth promotion

G. Anitha and B.S. Kumudini*

Department of Biotechnology, Center for Post Graduate Studies, Jain University, Jayanagar, Bangalore – 560 011, India

*Corresponding Author E-mail: kumudini_satyan@yahoo.co.in

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Abstract

Seven isolates of fluorescent pseudomonads were evaluated for their effect on plant growth promoting traits, both under normal and saline conditions using tomato plants. Fifteen rhizosphere samples from crop fields' of rice, chilly, ragi, beans and garden soils from different regions of India were collected and used for further study. They were characterized morphologically and biochemically which led to a conclusion that they may belong to genus *Pseudomonas*. They were also analyzed for their plant growth promoting activities such as production of indole acetic acid, siderophore, hydrogen cyanide and ammonia. It was observed that all the isolates were able to produce these compounds, but to varying extent. But, isolate JUPF37 produced highest followed by JUPF32. Study showed that out of seven isolates of fluorescent pseudomonads, JUPF37 showed highest plant growth promoting traits both under normal and saline conditions.

Key words

Fluorescent pseudomonads, Indole acetic acid, PGPR, Plant growth promotion, Siderophore

Introduction

Rhizosphere supports microbial populations exerting beneficial or detrimental effect on plant growth, which play a vital role in root health maintenance, nutrient uptake and environmental stress tolerance (Ahmed and Khan, 2011). These beneficial microorganisms, including plant growth promoting rhizobacteria (PGPR) form a significant component of plant disease management strategies. PGPR promote plant growth through production of phytohormones, siderophores, antibiotics, enzymes and/or fungicidal compounds (Seleim *et al.,* 2011; Saharan and Nehra, 2011; Sharafzadeh *et al.,* 2012).

Many PGPR have shown the role of rhizosphere as an ecosystem and has gained importance in the functioning of biosphere (Ahmed and Khan, 2011). Various bacterial species *viz*., *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have been reported to enhance plant growth (Wahyudi *et al*., 2011; Kumar *et al*., 2012), germination and seedling vigour in tomato (Rathaur *et al*., 2012), maize (Shahsavani *et al.,* 2009), rice (Mia *et al.,* 2012) and soybean (Kumar *et al*., 2012). Fluorescent pseudomonads, a group of PGPR are the most studied ones. They help in soil health maintenance and are metabolically and functionally most diverse (Lata *et al.,* 2000). Presence of fluorescent pseudomonad inoculant in combination with microbial fertilizer play an effective role in stimulating plant yield and growth (Kloepper *et al.,* 2004; Roesti *et al*., 2006; Rokhzadi *et al.,* 2008). Another widespread characteristic among the rhizosphere bacteria is the ACC deaminase activity, whose regulation is a principal mechanism by which bacteria exert beneficial effects on plants under biotic or abiotic stress (Saleem *et al.,* 2007; Rama *et al*., 2013).

Recognition of PGPR as potential plant growth stimulant has evolved over the past several years to where they are being successfully used under field experiments (Kouchebagh *et al.,* 2012). But, *Pseudomonas* has high level of heterogeneity and biodiversity existing within the species and / or subspecies (Naik *et al*., 2008; Shanmugam *et al.,* 2008). In this study, the effect of new isolates of fluorescent pseudomonads on tomato plant growth has been studied. The study has also been elaborated.

Literature indicates that considerable progress has been made in understanding the molecular, physiological and morphological mechanisms underlying bacteria mediated tolerance to biotic stresses (Van Loon *et al.*, 1998). For environmentally sustainable agricultural systems, bacterial inoculates providing cross protection against different types of biotic and abiotic stresses which would be highly preferable. For it to become an effective tool, in-depth knowledge on the phenomenon of cross-protection is required. This paper focusses on the performance of fluorescent pseudomonad isolates on tomato plant growth promotion under normal and saline conditions.

Materials and Methods

Isolation : Twenty rhizosphere samples were collected from different rice fields, chilly, ragi, beans and garden soils from different regions, stored in sterile containers and isolated as per Alexander (1965). Seven isolates were randomly selected based on their fluorescence under UV which were further characterized (Aneja, 2007).

Characterisation and identification : Phenotypic genus-level characterisation of the isolates was carried out by subjecting the bacterial isolate to cultural (oxygen requirement), morphological (colony morphology), microscopic (Gram staining) and biochemical (utilisation of different carbon sources and enzyme activity) following standard procedures as per Holt *et al*. (1994). All media were obtained from Hi Media.

Plant growth promoting traits : Qualitative (Brick *et al.,* 1991) and quantitative (Patten and Glick, 1996) analyses of indole acetic acid (IAA) were carried out. Absorbance of supernatant mixture was measured at 520 nm and quantified using tryptophan standard. Siderophore production was qualitatively detected (Schwyn and Neilands, 1987) using chrome azurol S (CAS). After 24 hr of incubation, colonies were observed for orange halo formation. Isolates were screened for hydrogen cyanide (Lorck, 2004) synthesis. Nutrient broth was amended with glycine (4.4g.l^{-1}). After incubation, tubes were observed for the development of orange to red color. Bacterial isolates were tested for ammonia synthesis in peptone water as per Joseph *et al*. (2007). After incubation, tubes were observed for the development of brown yellow color. Biofilm formation was studied using overnight culture of isolates in LB broth diluted to OD_{600} = 0.3, using crystal violet as per Iijima *et al*. (2009). Crystal violet attached to the biofilm was dissolved in 5 ml of 95 % ethanol and quantified by measuring absorbance at 590 nm.

Plant growth promotion studies under normal and saline conditions :

Germination and seedling vigour : Studies on both normal and saline conditions (3, 6 and 9 ds m^{-1}) were carried out. Tomato seeds (var. PKM-1; germination - 70%) were considered for the study. Bacterial isolates grown in King's B broth at 37±2 °C for 48 hr, were harvested, centrifuged (6000 rpm) for 15 min and resuspended in phosphate buffer (0.01 M, pH 7.0). Concentration was adjusted to ~1x10^{8} CFU/ml (OD_{600} = 0.3) and used as inoculum (Thompson, 1996). Distilled water treated seeds were considered as control. Germination was studied using top of the paper method (ISTA, 1993). Germination percentage (G %) and vigour index (VI) were calculated (Baki and Anderson, 1973).

Dry weight : Control and treated seedlings were weighed (initial weight) and then dried at 50ºC for two days. They were re-weighed (final weight). Actual dry weight of the biomass was the difference between initial and final weight.

Statistical analysis: Each of the experiments has been carried out in triplicates. ANOVA has been applied for all the parameters.

Results and Discussion

Twelve fluorescent pseudomonad cultures were isolated and seven isolates (JUPF31, JUPF32, JUPF33, JUPF34, JUPF35, JUPF36 and JUPF37) were considered for further study. Colonies on King's B (KB) agar, at λ_{265nm}, showed yellowish-green fluorescence. Fluorescent colonies were selected, maintained on KB slants at 4 °C and used for further studies. Colonies appeared creamy, 1-2 mm diameter and convex. Microscopic studies showed that the isolates were Gram negative rods, which were studied for utilization of sucrose, dextrose, mannitol and lactose. Results showed that the bacterial isolates were positive only for catalase, oxidase, organic acids, citrase, amylase, indole and caseinase. The isolates utilized sucrose, mannitol and lactose to varying extent. Battu and Reddy (2009) also observed gram negative, rod shaped bacterial colonies that produced yellowish green pigment on King's B medium and were positive for gelatinase and oxidase which were identified as *Pseudomonas fluorescens*.

Qualitative analysis showed that all the bacterial isolates produced IAA, ammonia, siderophore and hydrogen cyanide. This is supported by the studies of Gundala *et al.* (2013).

Hydrogen cyanide and ammonia synthesis were observed in all the bacterial isolates. For many pseudomonads, hydrogen cyanide production is the primary mechanism of biocontrol (Kumar *et al*., 2012). Ahmad *et al*. (2006) observed that hydrogen cyanide production was a common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%). Hydrogen cyanide production by rhizospheric bacteria is beneficial from the biocontrol point of view, but detrimental due to its interference with cytochrome P450 system, of certain plants (Kremer and Soussi, 2001). In the present study, seed bacterization did not have a detrimental effect on seed germination or plant growth. Development of brown yellow color was observed after addition of Nessler's reagent indicating ammonia production. Similar observations was earlier made by Yadav *et al.* (2010) in chickpea plant under *in vitro* conditions using PGPR.

As a rhizobacterial trait, the ability of isolates to form biofilm was studied using crystal violet. Results showed that all the isolates had varied biofilm forming ability. It was observed that JUPF31, 32, 33, 34, 35, 36 and JUPF37 showed an optical density of 2.29, 2.91, 2.83, 2.74, 1.97, 1.61, and 2.84 respectively (Fig. 1). This indicated that JUPF32 had highest ability to form biofilm followed by JUPF36, 33 and 34 isolates. Most plant–bacterial associations rely upon the physical interaction between bacteria and plant tissues. Direct observations of bacteria adhered to plant surfaces have revealed formation of biofilms (Ramey *et al.,* 2004). Root-associated pseudomonads have been studied extensively, and many of these promote growth of host plants or are used as biocontrol agents (Lugtenberg *et al*., 2001). Species of *Pseudomonas* form dense biofilms on both abiotic and biotic surfaces, and are primary models in biofilm research (Parsek and Fuqua, 2003). In the present study, results showed that all isolates had biofilm forming ability but in varying quantities.

Qualitative studies on IAA showed pink coloration of the filter paper which indicated its production. Variation in color intensity indicated varied IAA production which was further confirmed by quantitative estimation. Spectrophotometric analysis showed that a maximum of 180 mg ml^{-1} was produced by JUPF37, followed by JUPF32 (145 mg ml^{-1}) and a minimum of 85 mg ml^{-1} by JUPF31 as indicated in Fig. 2.

Among the PGP traits, IAA production by the bacterium has a cascading effect on the plant development due to its ability to influence root growth, which in turn affects the nutrient uptake and ultimately the plant productivity (Mishra *et al.,* 2008). In the present study, all the isolates showed IAA production which varied with the isolates. Similar observations were made by Ramezanpour *et al.* (2010) from the isolates of rice rhizosphere and Kumar *et al.* (2012) from the isolates of french bean rhizosphere. Karnwal (2009) screened 30 strains for their ability to produce IAA. They also observed the significance of bacterial IAA in different microorganism–plant interactions which highlighted the use of IAA as part of their colonization strategy. Similar observations on IAA production were made by Trivedi and Pandey (2008) in *Bacillus megaterium* strain B 388 (MTCC6521) isolated from a temperate Himalayan location.

Selvakumar *et al.* (2009) observed that incubation temperature had an effect on the expression of plant growth promotion traits of *Pseudomonas fragi*, which was able to produce IAA and hydrogen cyanide at all the incubation temperature. It has also been observed that although the bacterium possessed more than one plant growth promotion trait, its ability to produce IAA, which are known to stimulate root growth, could be a major attribute responsible for plant growth promotion. There are many evidences based upon the ability of PGPR to produce phytohormones like auxin and their influence over the morphology, nutrition and plant growth. In most of the observed studies, the growth controller especially IAA, influences most of the root system like primary root growth, side root and piliferous layer formation (Glick, 1995; Karakurt *et al.,* 2011), helps in plant growth promotion.

Iron, despite its abundance in Earth's crust, is largely unavailable for microbial assimilation. Microorganisms, especially rhizospheric bacteria have developed a strategy to scavenge available iron (Fe^{3+}), which involves secretion of high-affinity, low-molecular weight iron-chelating ligands called siderophores (Mercado-Blanco and Bakker, 2007). Beside auxin production, enhanced siderophore production is an important factor contributing towards growth promotion. Fluorescent pseudomonad isolates inoculated on to CAS agar formed orange halo around the colony indicating their ability to produce siderophore. A time course study was carried out on the estimation (λ_{630nm}) of siderophore for 6 days at 24 hr intervals. All the isolates showed increased production of siderophores, but, the quantity of siderophore produced by each of the isolate varied. Of all the isolates, JUPF37 showed highest siderophore production of 98 % units after 4 days which reduced to 74 % units after 7 days (Table 1). This was followed by JUPF32 which

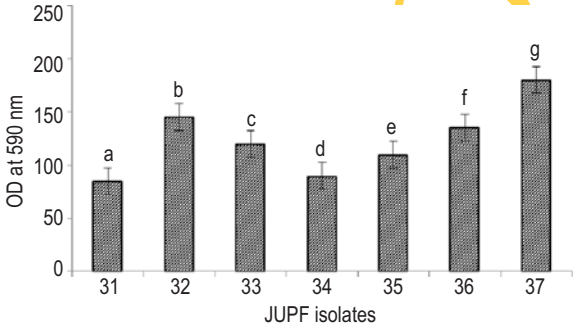

Fig.1 : IAA production by isolates of fluorescent pseudomonads *Bars indicate standard error; **Different letters indicate that the amount of IAA produced are significant from each other

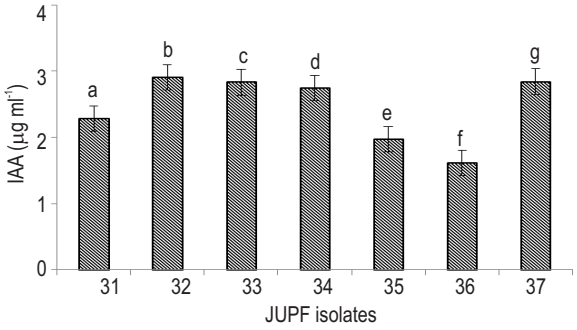

Fig. 2 : Ability of the different isolates of fluorescent pseudomonads to form bilfilm; * Bars indicate standard error; ** Different letters indicate that the amount of biofilm produced are significant from each other

Table 1 : Time course study on siderophore production (% units) by the isolates of fluorescent pseudomonads

JUPF isolates	Days						
	1	2	3	4	5	6	7
31	35±0.01[a]	62±0.10[b]	83±0.05[c]	83±0.01[a]	80±0.05[e]	74±0.05[f]	70±0.03[g]
32	19±0.15[a]	57±0.05[b]	70±0.02[c]	97±0.22[d]	74±0.02[c]	74±0.15[a]	72±0.22[d]
33	29±0.03[a]	50±0.01[b]	71±0.02[c]	84±0.15[d]	79±0.01[b]	74±0.1[f]	70±0.31[g]
34	23±0.12[a]	48±0.01[b]	64±0.01[c]	74±0.2[d]	71±0.05[e]	70±0.1[f]	65±0.42[g]
35	33±0.15[a]	52±0.23[b]	61±0.12[c]	74±0.05[d]	68±0.33[e]	67±0.21[f]	54±0.03[g]
36	24±0.02[a]	47±0.32[b]	73±0.1[c]	74±0.21[d]	74±0.24[e]	65±0.01[f]	65±0.03[g]
37	40±0.10[a]	65±0.22[d]	74±0.10[a]	98±0.3[d]	91±0.13[e]	85±0.25[f]	74±0.17[g]

* Data is represented as Mean ± SE; ** Different letters in the same row indicates that they are significantly different from each other

produced 97 % units of siderophores after 4 days of incubation, not significantly different from JUPF37. JUPF31 showed 83 % units of siderophore production after 3 days of incubation, continued for the 4th day and gradually reduced to 70 % units after 7 days. JUPF33 showed a similar behavior from four days of incubation. Results showed that the siderophore production by all the isolates increased till 4 days of incubation which decreased later. Hence, four-day old cultures were considered for further studies. Ali and Vidhale (2011) observed siderophore production by *Pseudomonas* spp. isolated from hospital environment on CAS agar. Studies by Rakh *et al.* (2011) on biological control of *Sclerotium rolfsii* by *Pseudomonas* cf. *monteilii* 9 reported siderophore production. Wahyudi *et al*. (2011), on screening of *Pseudomonas* sp. isolated from soybean rhizosphere also reported siderophore production. Siderophore plays a very important role in plant growth as it sequesters iron and help in

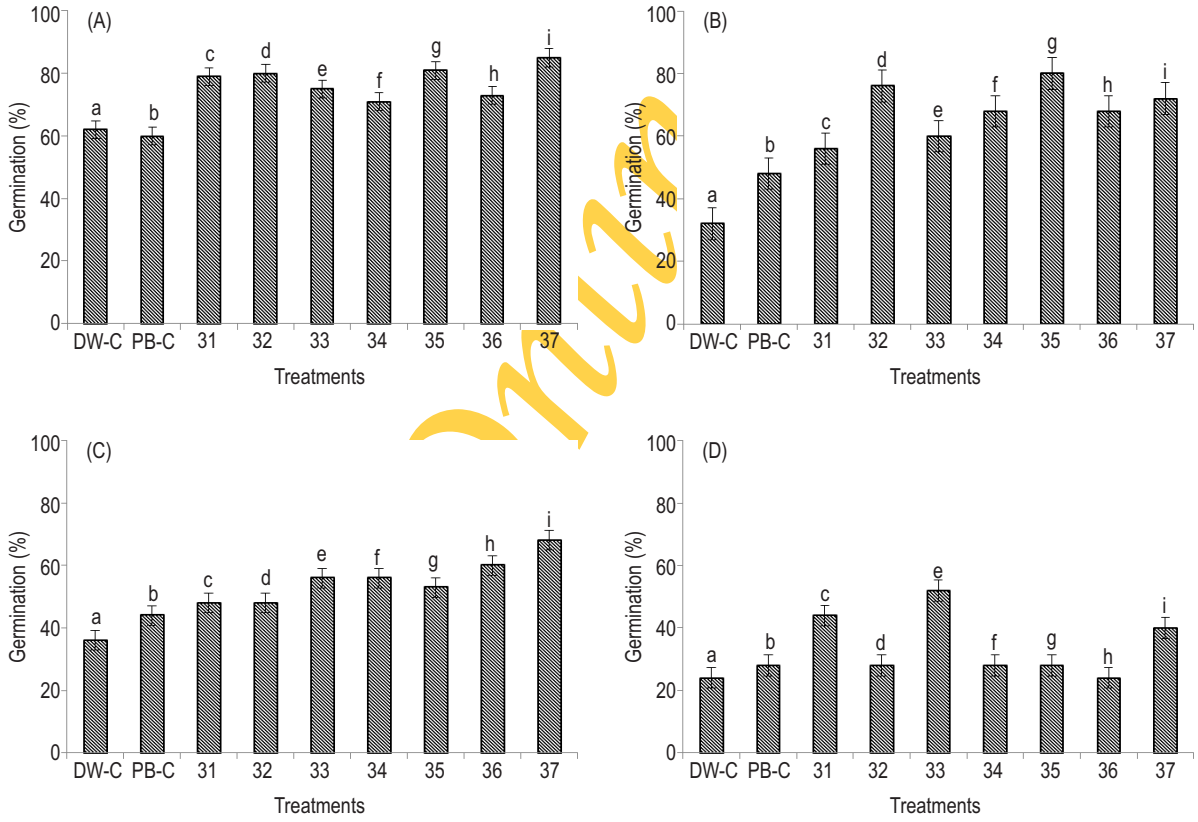

Fig. 3 : Germination (%) under normal (A) and saline conditions after 3 days (B) 6days © 9days (D). *Bar indicate standard error; ** Different letters indicate that the data are significant from each other

plant nutrition (Gokarn *et al.,* 2010). Marques *et al*. (2010) assessed growth promoting abilities of six bacterial isolates using *Zea mays*, which showed that some isolates showed siderophore production. Similar observations were made by Wahyudi *et al*. (2011).

Germination is an indication of growth in plants. As PGPR promotes growth even under stress conditions, studies on plant growth promotion were also carried out under saline conditions. Studies showed increased germination, shoot and root length when compared to distilled water and phosphate buffer controls. Highest germination was observed in seeds treated with JUPF37 (85 %), followed by JUPF35 (81 %) and JUPF32 (80 %) as shown in Fig. 3A. In the present study, three different saline conditions of 3, 6 and 9 ds m^{-1} were considered. At 3 ds m^{-1} salinity, seeds treated with JUPF35 showed highest germination of 80 % followed by JUPF32 (76 %) when compared to distilled water treated seeds as indicated in Fig. 3B. At 6 ds m^{-1} salinity , seeds treated with JUPF37 isolate showed highest germination of 68 %, followed by JUPF36 which showed a germination of 60 %. Least germination was observed in distilled water and phosphate buffer treated control seedlings which showed 36 and 44 % respectively (Fig. 3C). At 9 ds m^{-1} salinity, seeds treated with JUPF35 showed highest germination of 44 % followed by JUPF37 40 % (Fig. 3D).

Increased vigour index was observed in treated when compared to distilled water and phosphate buffer controls. Seedlings treated with JUPF32, showed highest vigour index of 1249, which was followed by JUPF33 and 37 which showed 1176.09 and 1163.40, respectively (Fig. 4A). At 3 ds m^{-1} salinity, JUPF37 showed higher vigour index of 1303.2 followed by JUPF35 which had (944.54) as indicated in Fig. 4B. At 6 ds m^{-1} salinity, JUPF37 showed higher vigour index of 820.70 followed by JUPF32 (661.36) (Fig. 4C). At 9 ds m^{-1}, JUPF33 showed higher vigour index of 483.46 followed by JUPF37 (363.33) as shown in Fig.4D.

Study showed maximum dry weight with JUPF37 (0.47g) treatment followed by JUPF33 with 0.34 g under normal conditions (Fig.5A). At 3 ds m^{-1}, JUPF37 and 34 showed dry weight of 0.31 g followed by JUPF31 and JUPF33 (0.25g) (Fig. 5B). At 6 ds m^{-1}, JUPF37 showed dry weight of 0.25g followed by JUPF32 (0.18g) (Fig. 5C). At 9 ds m^{-1}, JUPF37 showed dry weight of 0.22 g followed by JUPF33 (0.21g) (Fig. 5D), respectively.

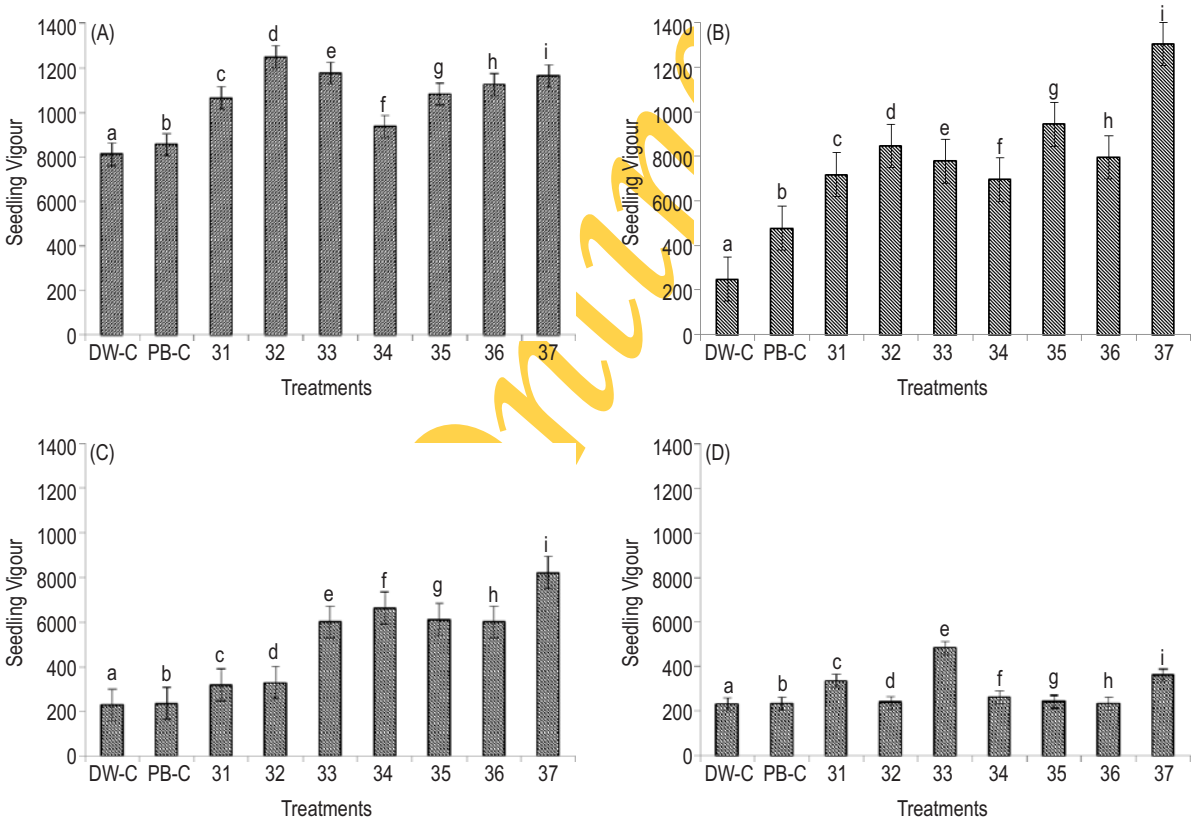

Fig. 4 : Vigour index of fluorescent pseudomonad- treated tomato seedlings under normal (A) and saline conditions after 3days (B), 6days (C) and 9 days (D) . *Bar indicate standard error; ** Different letters indicate that the data are significant from each other

Kumar *et al.* (2007) observed that inoculation with bacteria isolated from the maize rhizosphere of rain-fed Himalayan region resulted in significant enhancement in growth, grain yield and yield attributing characters. Similar observations have also been reported in different plants treated with PGPR, which showed increased growth under different conditions (Saharan and Nehra, 2011; Kumar *et al*., 2012; Mia *et al.,* 2012). Asha *et al.* (2011) observed no negative effect when *P. fluorescens* was applied as biocontrol agent. On the contrary, they exhibited synergism in promoting crop growth and yield of tomato besides controlling the fusarium wilt disease. Three bacterial species, viz. *Bacillus megaterium*, *B. subtilis* and *P. corrugata*, when examined for their growth promoting ability, showed growth promotion of rice (Trivedi *et al.,* 2007).

Plant involves a number of metabolic and physiological changes in response to salt stress and water deficiency which in arid regions is an important limiting factor for cultivating agricultural crops (Yang *et al*., 2009). Increase in soil salinity causes physiological response or disorder in plants (Han and Lee, 2005). In the present study, PGPR strains showed highly root-colonizing capacity, along with higher germination in germinating seeds under high salinity conditions when compared to control. Similar observations are made by Seleim *et al*. (2011). Han and Lee (2005) observed increased N, P and K concentration under salinity stress due to PGPR inoculation. Similarly, Vivas *et al*. (2003) reported that N, P and K concentration in lettuce inoculated by *Bacillus* sp. under stress conditions were increased by about 5, 70 and 50 %, respectively. Salt stress significantly decreases chlorophyll content in plants. It is suggested that PGPR seed treatments can reduce deleterious effects of salt stress by increasing chlorophyll content, photosynthetic activity and mineral uptake.

In the present study, higher vigour index and dry weight, which are indices of plant growth promotion, were observed in treated seeds in comparison with control and treated seeds under saline conditions. The present study indicates the significant role played by rhizobacteria in plant growth promotion as well as increased salt stress tolerance. JUPF37 could be a promising isolate in the development of a new formulation for growth promotion in tomato plants.

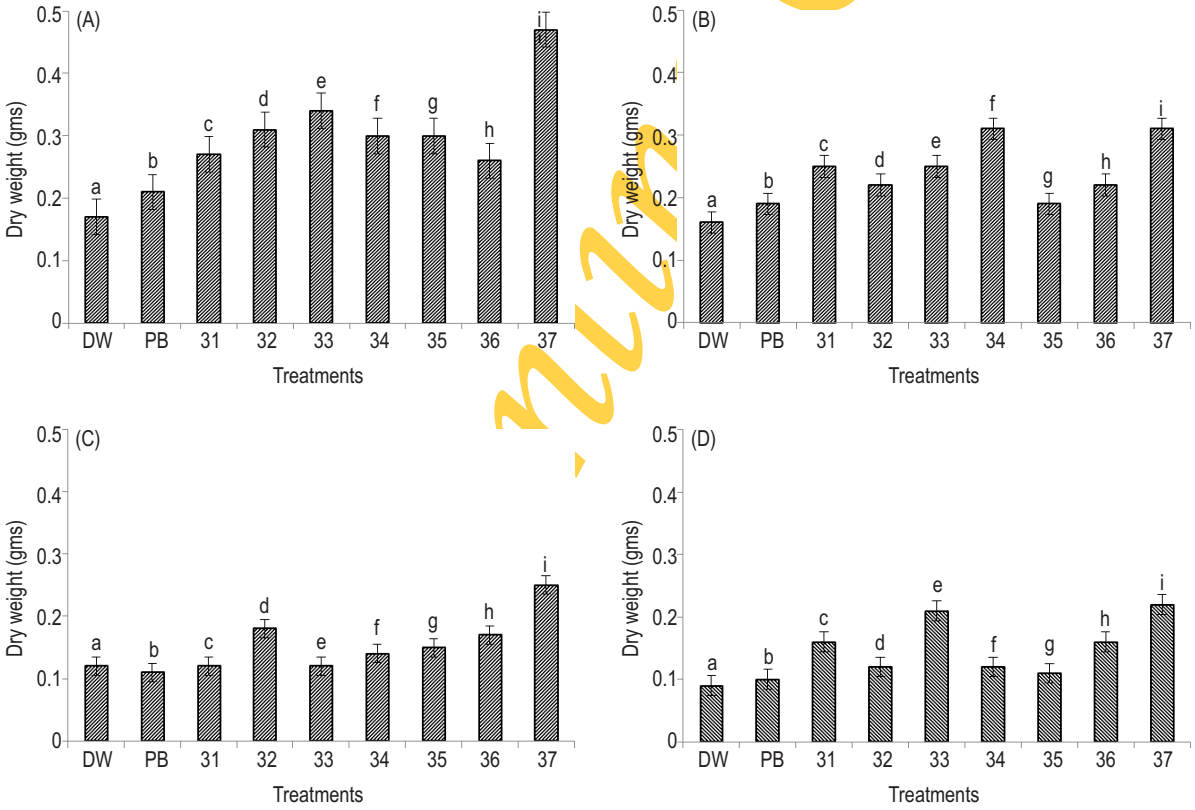

Fig. 5 : Dry weight of fluorescent pseudomonad- treated tomato seedlings under normal (A) and saline conditions 3 days (B), 6 days (C) and 9 days (D); *Bar indicate standard error; **Different letters indicate that the data are significant from each other except in 3ds conditions where the data are not significant from each other

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