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Characterization of fluorescent *Pseudomonas* with crop specific plant growth promoting responses on tomato (*Lycopersicon esculentus*) crop

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Abstract

A total of 5 *Pseudomonas* fluorescence isolates were obtained from tomato plant rhizospheres and were characterized on the basis of biochemical tests and plant growth-promoting activities. In this experiment we were studied to examine its role as a plant growth promoting rhizobia (PGPR) in enhancing the growth and yield of the tomato plant. Subsequently, to investigate the effect of *Fluorescent Pseudomonas* isolates on the growth of okra crop a pot culture experiment were conducted on randomized complete block design with 6 treatments which replicated thrice. Maximum root length (27.35cm) and shoot length (5.935cm) were recorded when seeds were treated with P260 and P66 as compared to control. Significant increase in root length was observed with isolates P260> P66>P141>P229>P200 in respective order.

Keywords: plant, tomato, fluorescent, *Pseudomonas*

Introduction

Introduction of specific microorganisms on plant growth have been reported for numerous crops, including tomato (*Lycopersicon esculentum*) grown under pot. Plant growth promoting rhizobacteria (PGPR) are defined as root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant growth and development. PGPR are known to survive both in rhizosphere and phyllosphere (Krishnamurthy *et al.*, 1998) [7]. Fluorescent pseudomonads are among the most effective rhizosphere bacteria because in addition to disease control, (Dubeikovsky *et al.*, 1993) [4]. Fluorescent *Pseudomonas* as a biocontrol agent offers a promising alternative to manage soil borne plant pathogens (Notz *et al.* 2001) [16].

Such beneficial microorganism referred as PGPR (plant growth promoting bacteria) or PGPF (plant growth promoting fungi) enhance plant growth through numerous mechanisms including the protection of roots against infection by minor and major pathogens (Whipps, 1997, 2001), Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria, which enhance the growth of the plant either directly or indirectly (Kloepper *et al.*, 1980; Glick, 1995) [8]. The direct mechanisms involve nitrogen fixation, phosphorus solubilization, HCN production, production of phytohormones such as auxins, cytokinins and gibberellins, and lowering of ethylene concentration (Kloepper *et al.*, 1989) [9].

Pseudomonas sp. is ubiquitous bacteria in agricultural soils and has many traits that make them well suited as PGPR. Considerable research is underway globally to exploit the potential of one group of bacteria that belong to fluorescent pseudomonas. Recently Pandey *et al.* (2013) [10] reported that *Pseudomonas* strains were plant growth promoting endorhizospheric bacteria inhabiting sunflower (*Helianthus annuus*).

Schippers *et al.* (1987) documented an increase in fresh weight of root and shoot of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains. Kloepper *et al.* (1989) [9] reported 57 per cent yield increase and enhanced plant growth due to seed application of beneficial rhizobacteria in canola.

Among plants growth regulator s indole-3acetic acid (IAA) is the most common natural auxin found in plants and its positive effect on root growth and morphology is believed to increase the access to more nutrient in the soil (Vessey, 2003) [18]. IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and florescence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions (Spaepen and Vanderleyden, 2011) [15].

Material and Methods

The cultures of *Pseudomonas* were inoculated in 100 ml conical flask containing 25ml King's B broth and incubated at 28 ± 2 °C for 48 hrs. For seed soaking inoculums of all the 5 isolates were diluted with 35ml of autoclaved distilled water. 20 ml of the diluted bacterial suspension was dispensed in Tarson tube. Six seeds of tomato were soaked in the each diluted suspension of *Pseudomonas* isolates for 2 hours. Uninoculated seeds served as control. Seeds were planted in plastic pots containing soil mixed with sand and compost in the ratio of 3:1:1 in double replication. In the seedling stage only three plants were kept in each pot. The plants of different crops were uprooted after 25 days after sowing respectively. While uprooting most useful care was taken to avoid root damage. Plants were then washed with tap water, stretched on fixed clean transparent surface followed by measurement of shoot length and root length.

Determination of indole acetic acid by fluorescent *Pseudomonas* spp.

For the production of indolic compounds, an active culture of *Pseudomonas* spp. was inoculated to 20 ml DF salts minimal media (Dworkin and Foster, 1958) [3] in 100ml conical flasks and incubated for 3 days at 28 ± 2 °C. The medium was supplemented with L-Tryptophan at a concentration of 1.02 g/L from a 5mM filtersterilized stock prepared in warm distilled water. After incubation for 72 hours, the grown bacterial cells were removed from the culture medium by centrifugation at 5,000 rpm for 5 min and the pH of the medium of all isolates was recorded. Then 1ml of aliquot from the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Webber, 1951) [5] with blank as uninoculated DF salts minimal media. It was allowed to stand at RT for 20 min, before the absorbance at 535 nm was measured in colourimeter. The standard curve was prepared by adding 1M IAA in the increasing concentration: 0.0µl, 5µl, 10µl, 20µl, 30µl, 40µl, 50µl, 60µl, 70µl, 80µl, 90µl, 100µl in DF salts minimal media with decreasing concentration: 1ml, 995µl, 990µl, 980µl, 970µl, 960µl, 950µl, 940µl, 930µl, 920µl, 910µl, 900µl respectively.

Screening isolates of *Pseudomonas* spp. for Phosphate solubilization

Qualitative screening

Qualitative screening of phosphate solubilizing fluorescent *Pseudomonas* was performed on Pikovskaya's agar medium (HiMedia) containing tricalcium phosphate as a phosphate source and Bromo Cresol Purple (0.1 g/l) as a pH indicator for acidification (Vazquez *et al.*, 2000). After incubation of fresh cultures of fluorescent *Pseudomonas* at 28 ± 2 °C for 48 h, phosphate solubilizing isolate turned the media colour from purple to yellow in the zones of acidification.

Polyhydroxybutyrate production

Qualitative screening

Pseudomonas isolates were screened for PHB accumulation qualitatively by following the viable colony method using Sudan Black B dye (Juan *et al.*, 1998) [6]. Sterilized Nutrient agar (HiMedia) supplemented with 1 % glucose was spot inoculated with the isolates and incubated at 30°C for 24 h. Ethanolic solution (0.02 %) of Sudan Black H₂SO₄ they were washed with ethanol (96 %) to remove the excess stain from the colony. The dark blue coloured colony was taken as positive for PHB production.

Quantitative spectrophotometric assay for siderophore production (liquid assay)

The chrome azurolsulfonate (CAS) assay (Schwyn and Neilands, 1987) [13] was used to evaluate the siderophore production. For siderophore quantification, actively growing cultures of *Pseudomonas* was inoculated to 20 ml King's B broth (Hi Media) media in 100ml flasks and incubated for 3 days at 28 ± 2 °C. The bacterial cells were removed by centrifugation at 3000 rpm for 5 min. 0.5 ml of the culture supernatant was then mixed with 0.5 ml CAS solution and 10µl shuttling solution (sulfosalicylic acid). After 20 mins of incubation, the colour obtained was determined using the spectrophotometer at 630 nm. Only King's B broth was used as blank while reference solution was prepared by adding CAS dye and shuttle solution to King's B and absorbance was recorded. Values of siderophore released in King's B was expressed in per cent siderophore units and calculated using the formula:

Where,

$$\frac{(Ar-As)}{Ar} \times 100$$

Ar = OD of reference solution,

As = OD of samples

Result and Discussion

In the present investigation, five isolates of *Pseudomonas* were screened by six different siderophore assay *viz.*, CAS assay, CAS agar medium, Hydroxyquinoline test, Tetrazolium test, FeCl₃ tests and Arnow's assay. CAS assay is the universal assay for detection of siderophores. The principle of this assay is based on a colour change of CAS from blue to orange resulting from siderophoral removal of Fe from the chrome azurol dye (Guan *et al.*, 2001). All the isolates showed positive response for siderophore production on CAS agar plate *i.e.* orange color halo was observed in all the isolates. For selection of fluorescent *Pseudomonas* isolates with high ability to produce siderophore, isolates were inoculated on KMB supplemented with a strong chelator 8-Hydroxyquinoline. All 5, isolates were able to grow on HQ supplemented medium. All isolates produced deep red colour on addition of tetrazolium salt and NaOH which used to test presence of hydroxamate type of siderophore, indicating that the isolates have capacity to reduce tetrazolium salt by hydrolysis of hydroxamate group in presence of strong alkali. FeCl₃ test gave positive response with isolate P66 P141, P200, and P260. Arnow's assay was performed to detect catechol type of siderophores. All the 5 isolates gave negative response for Arnow's test. Carboxylate type of siderophore was determined by spectrophotometric test and the percentage of siderophore unit ranged from 75% to 88.365%. Among the 5 isolates, isolate P229 produced highest (88.365%) siderophore units. Minimum siderophore unit was observed for isolate P260 (75%). In the order of decreasing siderophore producing ability the following isolates were also identified as candidate's P229>P141>P66>P200>P260.

Quantification of Indole acetic acid (IAA) production by *Pseudomonas* spp.

Efficacy of different isolates of *Pseudomonas* used in the present investigation to produce IAA from L-tryptophan as precursors varied, the isolates P229, P141 and P66 were identified as the high producer of indole acetic acid thus

indicating their ability to synthesized IAA from L-tryptophan as precursors *in vitro*. The maximum production of IAA was observed for the isolate P229 (17.81 µg/ml) whereas P200 (10.9 µg/ml) was the lowest producer. Microbial IAA could be involved in the growth stimulation observed in our greenhouse assay. Production of plant growth regulators by the microorganisms is another important mechanism often associated with growth stimulation (Vessay, 2003).

Screening of phosphate solubilizing *Pseudomonas spp.* and its quantification

1. Quantitative estimation of soluble phosphate concentrations in Pikovskaya's broth was expressed as µg/ml and it varied significantly from 313.500 to 382.00µg/ml.
The lowest value was observed for isolate P141 and highest for isolate P229.
2. Isolate P229 can be considered as promising inducer of phosphate mobilization. The amount of inorganic phosphate solubilized was 382.00µg/ml. Phosphate solubilization by isolates P66, P200 and P260 were significantly highest among all the other isolates that they solubilized 351.00µg/ml, 338.500 µg/ml and 314.00 µg/ml.
3. All the rhizospheric isolates of fluorescent *Pseudomonas spp.* showed variable phosphate solubilizing potential with P229> P66>P200>P260>P141 being the best phosphate solubilizers.

4. These candidate isolates can be used as microbial inoculants to improve soil fertility by releasing bound phosphorus thereby increasing the crop yield potential. Stimulation of different crops by plant growth promoting *Pseudomonas* isolates with potential phosphate solubilization ability may help in exploiting larger reserves of phosphorus present in most agricultural soils. Several *Pseudomonas* species have been reported among the most efficient phosphate-solubilizing bacteria and as important bio-inoculants due to their multiple biofertilizing activities of improving soil nutrient status, secretion of plant growth regulators and suppression of soil-borne pathogens (Rodriguez and Fraga 1999; Gulati *et al.* 2008; Vyaset *al.* 2009; Agrawal *et al.* 2014) ^[11, 19].

Plant growth promoting response of *Pseudomonas* on tomato (*Lycopersicon esculentus*)

Efficacy of different isolates of *Pseudomonas* for tomato plants varied to induce root and shoot length ranging from 13.73 to 27.35 cm and 3.28 to 5.935 cm respectively. Maximum root length (27.35cm) and shoot length (5.935cm) were recorded when seeds were treated with P260 and P66 respectively. Seed treated with isolate P260 has 13.61cm (49.76%) more root length and Seed treated with isolate P66 has 2.65cm (44.65%) more shoot length as compared to control. Significant increase in root length was observed with isolates P260> P66>P141>P229>P200 in respective order. Significant increase in shoot length was observed with isolates P66> P260>P141>P200>P229

Table 1: Production of various siderophores by *Pseudomonas* isolates

Isolate	% Siderophore units	Arnow's test (development of pink color)	FeCl ₃ test (instant Development of deep red color)	Tetrazolium test (instant development of deep red color)	HQ test
P66	83.05 ^c ±0.449	Negative	Positive	Positive	***
P141	85.15 ^b ±0.350	Negative	Positive	Positive	*
P200	80.93 ^d ±0.930	Negative	Positive	Positive	*
P229	88.365 ^a ±0.095	Negative	Positive	Positive	*
P260	75 ^e ±0.300	Negative	Positive	Positive	**
Max	88.365 ^a ±0.095				
Min	75 ^e ±0.300				
CD(0.01)	2.891				
CD(0.05)	1.84				
Fcal	97.409				

Table 2: Quantification of Indole acetic acid (IAA) production by fluorescent *Pseudomonas* isolates.

Isolate	Identification	IAA Production (µg/ml)	pH of the minimal medium 7DAI
P66	Pa	16.72	7.24
P141	Pp	17.63	7.12
P200	Fp	10.9	6.84
P229	Fp	17.81	7.71
P260	Fp	11.09	7.11
Max		17.81	
Min		10.9	

Pa: *P. aeruginosa*; Pp: *P. putida*; Fp: *Fluorescent Pseudomonas*

Table 3: Inorganic phosphate solubilization of fluorescent *Pseudomonas* isolates

Isolate	Phosphate solubilized in Pikovskaya's broth (µg/ml)	pH of the Pikovskaya's broth 7DAI	Phosphate solubilization in Pikovskaya's agar medium (with Bromo cresol purple)	Phosphate solubilization in Pikovskaya's agar medium (without Bromo cresol purple)
P66	351.00	3.61	+++	+++
P141	313.500	3.72	++	++
P200	338.500	4.12	+	+
P229	382.00	3.48	+++	+++
P260	314.00	3.67	++	+
Control	81			
Mix	382.00			

Min	314.00			
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+++ : luxuriant/high phosphate solubilization; ++ : medium phosphate solubilization; + : low phosphatesolubilization; - : no phosphate solubilization.

Isolates	Average value					
	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Shoot fresh weight(g)	Root dry weight(g)	shoot dry weight (g)
P66	22.300 ^b ±1.100	5.935 ^{bc} ±0.165	0.046 ^{ab} ±0.003	0.185 ^{ab} ±0.055	0.018 ^d ±0.001	0.045 ^b ±0.015
P141	21.00 ^{bc} ±1.170	4.883 ^{bc} ±0.185	0.055 ^a ±0.001	0.225 ^a ±0.005	0.038 ^a ±0.01	0.035 ^b ±0.008
P200	17.835 ^a ±1.665	4.516 ^{bc} ±0.115	0.035 ^b ±0.007	0.135 ^b ±0.005	0.025 ^c ±0.002	0.030 ^b ±0.000
P229	19.920 ^{bc} ±1.150	4.216 ^c ±0.285	0.040 ^{ab} ±0.001	0.115 ^{bc} ±0.030	0.026 ^b ±0.001	0.035 ^b ±0.005
P260	27.350 ^a ±1.220	5.233 ^{ab} ±0.500	0.051 ^a ±0.003	0.225 ^a ±0.015	0.035 ^a ±0.004	0.125 ^a ±0.017
C	13.735 ^b ±0.335	3.283 ^d ±0.150	0.016 ^c ±0.001	0.035 ^c ±0.005	0.016 ^e ±0.001	0.020 ^b ±0.000
Max.	27.350 ^a ±1.220	5.935 ^{bc} ±0.165	0.051 ^a ±0.003	0.225 ^a ±0.015	0.035 ^a ±0.004	0.125 ^a ±0.017
Min.	13.735 ^d ±0.335	3.283 ^d ±0.150	0.016 ^c ±0.001	0.035 ^c ±0.005	0.016 ^e ±0.001	0.020 ^b ±0.000
CD. 0.01	6.153	1.398	0.013	0.124	0.018	0.059
CD.0.05	4.068	0.92	0.014	0.081	0.001	0.039
F.cal	41.392	11.533	18.807	0.019	14.283	15.374

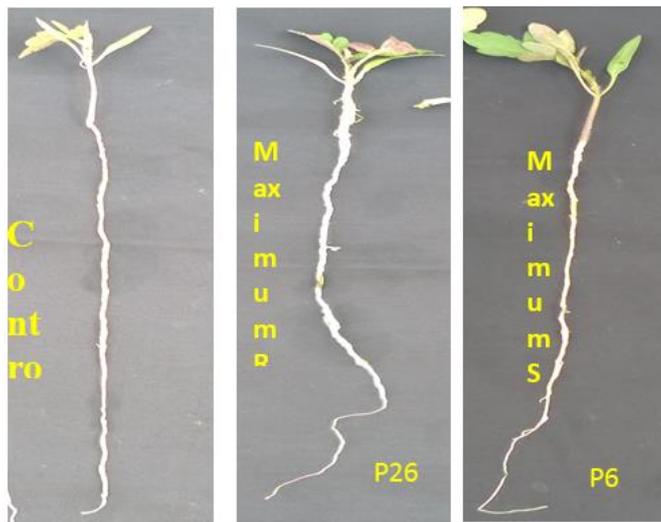


Plate 1: PGP activity of fluorescent *Pseudomonas* isolates in tomato crop

Conclusion

The cultures of *Pseudomonas* were inoculated in 100 ml conical flask containing 25ml King's B broth and incubated at 28±2 °C for 48 hrs. For seed soaking inoculums of all the 5 isolates were diluted with 35ml of autoclaved distilled water. 20 ml of the diluted bacterial suspension was dispensed in Tarson tube. Six seeds of okra were soaked in the each diluted suspension of *Pseudomonas* isolates for 2 hours. Uninoculated seeds served as control, it was recorded that maximum root length shoot length were recorded when seeds were treated with P260, and P66 respectively.

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