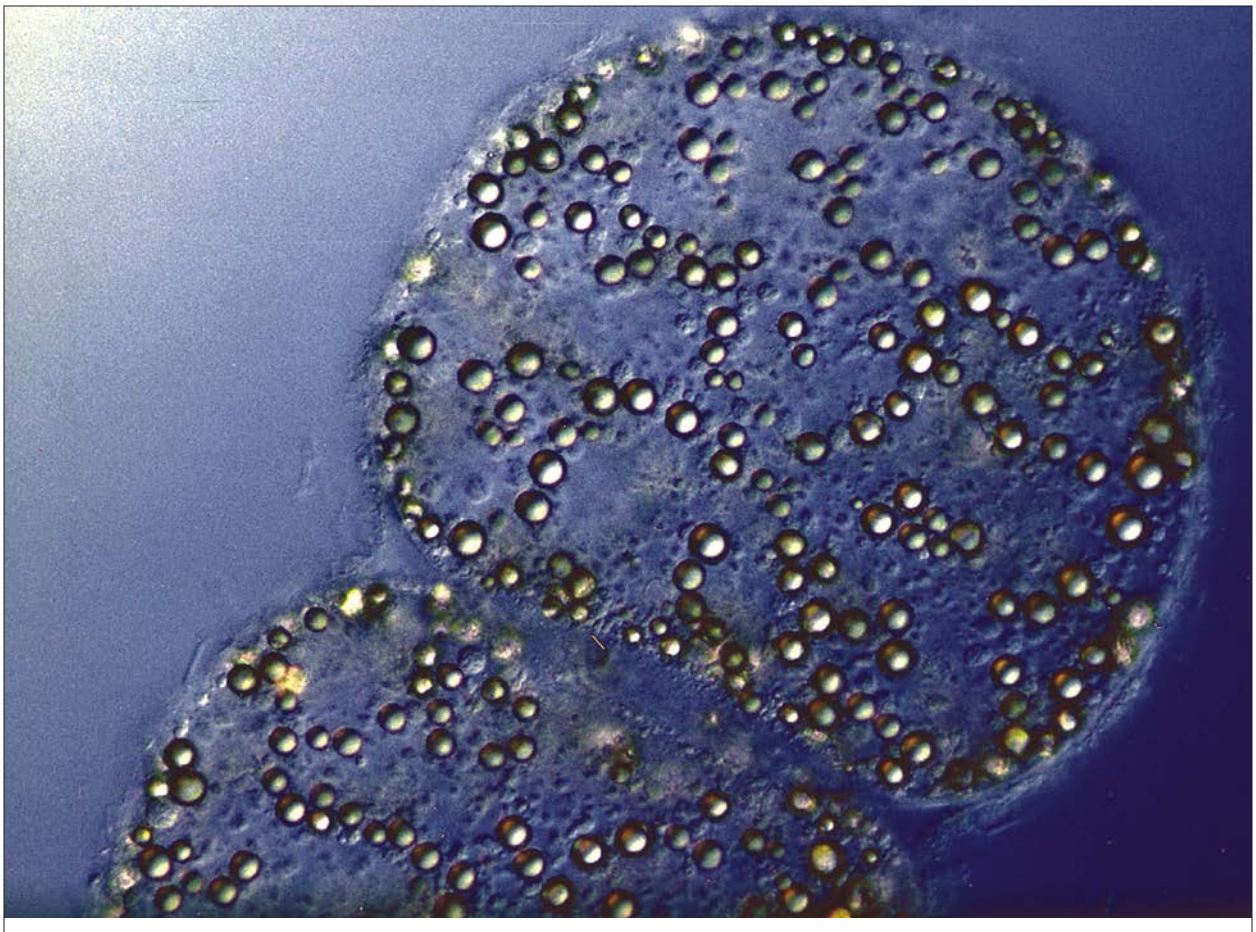

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Differential Response of Inoculation with Indole Acetic Acid Producing *Pseudomonas* sp. In Green Gram (*Vigna radiata* L.) and Black Gram (*Vigna mungo* L.)

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ABSTRACT

Rhizosphere bacteria promote plant growth by improving the availability of nutrients, suppressing the growth of plant pathogens or by production of hormones such as auxins. Influence of indole acetic acid (IAA) producing bacterial mutants was studied on nodulation and plant growth promotion in green gram (*Vigna radiata* L.) and black gram (*Vigna mungo* L.) in the present studies. IAA producing *Pseudomonas* strain MPS90 was mutagenized with transposon Tn5 to obtain mutants with variation in IAA production ability. Low amount of IAA production was observed in 35.14% mutants whereas, 3.43% mutants produced higher amount of IAA in comparison to parent strain. Seed inoculation of three mutants i.e., MPS90-14, MPS90-106 and MPS90-150 caused slight stimulation of root growth of green gram seedlings at both 5 and 10 days of observation whereas four mutants i.e., MPS90-39, MPS90-133, MPS90-145 and MPS90-157 caused stimulation of shoot growth at 5 days. In black gram, majority of the *Pseudomonas* mutants enhanced the root growth of seedlings at 5 days of observation whereas at 10 days, only four mutants i.e., MPS90-39, MPS90-157, MPS90-102 and MPS90-106 caused stimulation of root growth. The shoot growth of black gram seedlings at 5 days observation was retarded with most of the mutants except the mutants MPS90-14, MPS90-51, MPS90-133 and MPS90-280. Coinoculation studies of *Pseudomonas* mutants with *Bradyrhizobium* sp. strain S24 caused increase in shoot dry weight that varied from 110 to 137 per cent in green gram and from 105 to 198 per cent in black gram in comparison to *Bradyrhizobium*-inoculated plants at 60 days of growth. The stimulation effect varied from 280-390% in green gram and 179-357% in black gram in comparison to uninoculated control treatment. Mutants MPS90-133, MPS90-145 and MPS90-51 showed more nodule formation by *Bradyrhizobium* strain S24 in green gram whereas mutants MPS90-102, MPS90-39 and MPS90-280 caused more stimulation for nodule formation in black gram at 60 days of plant growth. Thus, mutants altered in IAA production ability showed differential responses on nodulation and plant shoot weight in the two hosts.

Key words: Indole acetic acid, *Pseudomonas* sp., nodulation, *Bradyrhizobium* strain, plant growth, green gram, black gram

INTRODUCTION

Indole-3-acetic acid (IAA) is an important naturally occurring auxin with broad physiological effects on plants (Davies, 2010). Many plant growth-promoting rhizobacteria (PGPR), including

Azospirillum, Azotobacter, Bacillus, Burkholderia, Pseudomonas and *Rhizobium* produce IAA or related auxins (Dubeikovsky *et al*., 1993; Taghavi *et al*., 2009). Auxins have been implicated in signaling between microorganism and plant (Spaepen *et al*., 2007) leading to stimulation of cell division, initiation of lateral and adventitious roots (Malamy and Benfry, 1997), increase rate of seedling emergence (De Freitas and Germida, 1990) and results into elongation of stems and roots (Yang *et al*., 1993). The stimulation of growth of roots results in enhanced uptake of nutrients by the associated plants (Lifshitz *et al*., 1987). Therefore, promotion of plant growth after inoculation with rhizobacteria has been attributed to IAA production in *Azospirillum brasilense* (Okon and Vanderleyden, 1997), *Rhizobium* species (Hirsch and Fang, 1994) and in *Pseudomonas* (Patten and Glick, 1996; Malik and Sindhu, 2011). Similarly, coinoculation of legumes with *Rhizobium* and IAA-producers such as *Azospirillum brasilense*, *Bacillus* and *Pseudomonas* have been found to increase the number of nodules, nodule fresh weight and nitrogenase activity in comparison to *Rhizobium/Bradyrhizobium* inoculated plants (Yahalom *et al*., 1990; Malik and Sindhu, 2011).

Low concentration of IAA was found to promote plant growth, whereas high concentrations inhibited root growth, thus indicating that effect of IAA depends on the concentration (Arshad and Frankenberger, 1991; Keyeo *et al*., 2011). Similarly, inoculation of canola seeds with *P. putida* GR 12-2 which produced low level of IAA, resulted in two or four-fold increase in length of seedling roots and treatment of plant roots with an IAA over producing mutant significantly inhibited the growth of canola root (Xie *et al*., 1996). In contrast, mutant strains of *A. brasilense* that synthesized very low levels of auxins did not promote the formation of lateral roots of wheat seedlings (Barbieri and Galli, 1993) whereas inoculation with IAA overproducing mutant of *Pseudomonas putida* was found to cause extensive development of adventitious roots on mung bean cuttings (Mayak *et al*., 1999). On the other hand, some deleterious rhizobacteria including *Enterobacter taylorae, Klebsiella planticola, Alcaligenes faecalis, Xanthomonas maltophila, Pseudomonas* sp. and *Flavobacterium* sp. exerted inhibitory effect on root and shoot growth as well as on the rate of seedling emergence through IAA secretion (Sarwar and Kremmer, 1995; Suzuki *et al*., 2003; Keyeo *et al*., 2011). Mutants of *Bacillus megaterium* with altered IAA production levels (overproducers and underproducers) showed a negative effect on the symbiotic parameters in *Phaseolus vulgaris* (Srinivasan *et al*., 1996).

Cultivar-specific growth promotion of spring wheat (*Triticum aestivum* L.) has been observed by coexistent *Bacillus* species (Chanway *et al*., 1988). A mutant strain of *Pseudomonas fluorescens* BSP53a that overproduced auxin showed a stimulatory effect on the root development of black currant softwood cuttings and had an inhibitory effect with cherry tree plants (Dubeikovsky *et al*., 1993). Moreover, inoculation with an *Azospirillum brasilense* Sp245 mutant strain (strongly reduced in auxin biosynthesis) or addition of increasing concentrations of exogenous auxin to the plant growth medium, showed a differential response among the common bean (*Phaseolus vulgaris* L.) genotypes (Remans *et al*., 2008). Thus, effect of IAA on the growth of seedlings/plants varies with a range of effective IAA concentrations depending upon the plant species used or to the sensitivity of the plant organ to auxin. Generally, roots were found more sensitive to stimulation by lower concentrations of exogenous auxin than the shoots (Evans *et al*., 1994).

Green gram (*Vigna radiata* L.) and black gram (*Vigna mungo* L.) are the most widely cultivated important pulse crops in India, grown during the summer season in arid and semi-arid zones. India is the largest producer of black gram in the world. Despite the potential of growth-mediating auxins and other allelochemicals in agriculture, it is one of the poorly understood areas

of plant-microbe interactions. Further work is needed to characterize IAA-producing bacteria from the rhizosphere soil. In this study, mutants were derived by Tn5 mutagenesis of *Pseudomonas* strain MPS90 having variation in IAA production ability. Inoculation of different mutants showed stimulation/retardation effect on the growth of root and shoot of green gram and black gram seedlings. However, coinoculation of IAA-producing *Pseudomonas* strain/mutants with *Bradyrhizobium* sp. strain S24 in these two legume crops increased the number of nodules and plant dry weights in comparison to *Bradyrhizobium*-inoculated plants.

MATERIALS AND METHODS

This work was carried out in the Department of Microbiology, CCS Haryana Agricultural University, Hisar during the month of April 2004 to July 2007.

Bacterial cultures and seeds: *Pseudomonas* sp. strain MPS90 was isolated from the rhizosphere of green gram. This strain produced IAA and inhibited the growth of *Fusarium oxysporum, Rhizoctonia solani* and *Pythium aphanidermatum* on medium plates by production of siderophores (Sahu and Sindhu, 2011). *Bradyrhizobium* sp. strain S24 was obtained from the Department of Microbiology, CCS Haryana Agricultural University, Hisar. *Pseudomonas* strain was maintained on Luria-Bertani (LB) medium and *Bradyrhizobium* strain was maintained on yeast-extract mannitol agar medium (YEMA; Vincent, 1970) by periodic transfers. *E. coli* strain S17-1 containing suicidal plasmid pSUP2021 (having Tn5 transposon) was obtained from Department of Microbiology and maintained on LB agar medium containing kanamycin (50 μg mL^{-1}). Seeds of green gram (*Vigna radiata* L. Wilczek) variety Asha and black gram (*Vigna mungo*) cultivar T9 were obtained from Pulses Section, Department of Plant Breeding, CCS Haryana Agricultural University, Hisar.

Determination of IAA production: IAA production by *Pseudomonas* strain and different mutants was determined using Salkowski's reagent (Gordon and Weber, 1951; Mayer, 1958). Bacterial culture/mutants from LB medium slopes were transferred into tubes containing 5 mL LB broth supplemented with L-tryptophan (100 μg mL^{-1}) and were incubated at 28±1°C for 5 days. The broth was centrifuged for 5 min at 10,000 rpm and equal volume of Salkowski's reagent was added in the supernatant. The contents were mixed and allowed to stand at room temperature for 30 min to develop colour. The optical density was recorded at 500 nm. Uninoculated broth served as control. Standard curve was prepared with 5-100 μg mL^{-1} of IAA (Sigma Chemicals) for quantification.

Determination of intrinsic antibiotic resistance pattern of *Pseudomonas* strain: Intrinsic antibiotic resistance pattern of *Pseudomonas* strain MPS90 was determined on LB plates containing different concentrations of antibiotics (Dadarwal *et al*., 1987). Filter sterilized stock solutions of antibiotics were added in LB molten agar kept at 60°C in water bath. The medium was gently shaken after adding the desired concentration of antibiotic and plated. After solidification of the medium, the *Pseudomonas* strain was spot inoculated on to antibiotic incorporated plates and incubated at 28±1°C for 2-3 days. Control plate without antibiotic was also kept for comparison of growth. On the basis of appearance of growth on antibiotic plates, the antibiotic resistance pattern of *Pseudomonas* strain was determined.

Derivation of mutants from IAA producing *Pseudomonas* strain by Tn5 mutagenesis: The *E. coli* strain S17-1 (donor) and *Pseudomonas* sp. strain MPS90 (recipient) were mixed and grown overnight on LB plates at 28±1°C (Simon *et al.*, 1989). The growth after conjugation was inoculated into LB broth and incubated for 24 h. Serial dilutions of this growth were prepared up to 10^{-6} and plated on LB medium plates containing kanamycin (Kan^r) (50 μg mL^{-1}) and streptomycin (Sm^r) (50 μg mL^{-1}). Plates were incubated for 3 days at 28±1°C. Kan^r and Sm^r mutant colonies were selected and transferred to LB slants for further studies.

Effect of *Pseudomonas* strain/mutants on seedling growth: Healthy seeds of green gram var. Asha and black gram cultivar T9 were surface sterilized with acidic alcohol (H_2SO_4: ethanol, 7:3, v/v) for 3 min followed by thorough washing with repeated changes of sterilized distilled water (Sindhu *et al.*, 1999). The surface sterilized seeds were inoculated with broth culture of different *Pseudomonas* strain/mutants and allowed to be adsorbed for 30 min. Inoculated seeds were germinated on water agar plates (10 g agar L^{-1} distilled water) at 28±1°C in a BOD incubator. Uninoculated seeds treated with LB broth alone were sown as control. The root and shoot lengths were measured at 5 and 10 days after sowing.

Coinoculation of *Pseudomonas* strain/mutants with *Bradyrhizobium* sp. strain S24 under chillum jar conditions: For preparing chillum jar assemblies, thoroughly washed and dried coarse river sand was used to fill the upper assembly while the lower assembly was filled with quarter-strength of Sloger's nitrogen-free mineral salt solution (Sloger, 1969). The whole assembly was autoclaved at 15 lbs. pressure for 3 h. Surface-sterilized seeds of green gram and black gram were inoculated with broth culture of *Bradyrhizobium* sp. strain S24 alone or as coinoculant with *Pseudomonas* strain/mutants by mixing the broth of the two in a ratio of 1:1 (v/v). Two milliliter of the mixed inoculum was inoculated on 15 seeds and left for 30 min for adsorption. In case of *Bradyrhizobium* strain S24 alone, 1 mL of broth and 1 mL water was added to have relatively the same level of inoculum.

Seeds were sown in sterilized chillum jar assemblies. Uninoculated seeds were sown as control, keeping 3 replications for each treatment. In each chillum jar, 3 seedlings were kept. The jars were kept in a net house under day light conditions. Quarter strength Sloger's nitrogen-free mineral salt solution was used for watering as and when required. The plants were uprooted at 30, 45 and 60 days after sowing and observations were taken for nodule number, nodule fresh weight and plant dry weights.

Nodule fresh weight and plant dry weight: The nodules were detached from the roots, washed with water and blotted in folds of filter paper. The nodules were counted and nodule fresh weight was recorded. Shoot portion was dried in oven at 90°C for 24 h and weighed.

RESULTS

Successful crop production is highly dependent on the availability of micro- and macro-nutrients in the soil. Microorganisms in their natural habitats such as soil or rhizosphere are crucial to the functioning of the world's ecosystems and they are major contributors to the biogeochemical cycles (Vessey, 2003; Woyessa and Assefa, 2011). Pseudomonads and other PGPR enhance plant growth by different mechanisms including atmospheric nitrogen fixation (Sindhu *et al.*, 2010), increased mobilization of insoluble nutrients such as bound phosphorus and iron (Kloepper *et al.*, 1980;

Table 1: Characteristics of *Pseudomonas* strain MPS90 used for Tn5 mutagenesis

Pseudomonas strain	Rhizosphere source	IAA production (μg mL^{-1}) 5 days	Growth inhibition of *Fusarium oxysporum*	Antibiotic resistance (50 μg mL^{-1})			
				Km, Nal, Tc, Rif	Cm, Sm, Tm	Amp	Spc
MPS90	Green gram	16.2	+	-	+	+	+

Antibiotics used: Km, Kanamycin; Nal, Nalidixic acid; Tc, Tetracycline; Rif, Rifampicin; Cm, Chloramphenicol; Sm, Streptomycin; Tm, Trimethoprim; Amp, Ampicillin and Spc, Spectinomycin

Sindhu *et al*., 2009), suppression of phytopathogenic organisms (Sindhu *et al*., 2011) and/or by production of phytohormones including auxins, gibberllins and cytokinins (Dubeikovsky *et al*., 1993; Weyens *et al*., 2009).

Screening of *Pseudomonas* mutants for IAA production, inhibition of fungal growth and antibiotic resistance: *Pseudomonas* strain MPS90 showed 16.2 μg mL^{-1} of IAA production at 5 days of growth (Table 1). Strain MPS90 also inhibited the growth of *Fusarium oxysporum, Rhizoctonia solani* and *Pythium aphnidermatum* in spot test on King's B medium plates and showed large zones of fungal growth inhibition (Sahu and Sindhu, 2011). Spot inoculation of *Pseudomonas* strain MPS90 was done onto LB plates supplemented with different antibiotics at a concentration of 50 μg mL^{-1} and the strain MPS90 showed resistance to five antibiotics namely chloramphenicol, streptomycin, trimethoprim, amipicillin and spectinomycin (Table 1).

Derivation of Tn5-derived mutants in *Pseudomonas* strain for altered IAA production level: *E. coli* strain S17-1 containing the suicidal plasmid pSUP2021 with transposon Tn5 (donor) was used for transfer of Tn5 to *Pseudomonas* strain MPS90 (recipient) by conjugation using patch cross method. From the Tn5 mutagenesis, a total of 350 Kan^r and Sm^r mutants were selected and screened for IAA production in LB broth supplemented with tryptophan (100 μg mL^{-1}). About 35.14% mutants showed low production of IAA and only 3.43% mutants were found with high IAA production ability (Table 2). Rest of mutants i.e., 61.43% showed IAA production equal to the parent strain MPS90.

Production of IAA by different *Pseudomonas* mutants: Quantitative production of indole-acetic acid was carried out using Salkowski's reagent. Mutants derived from *Pseudomonas* strain MPS90 i.e., MPS90-39, MPS90-51, MPS90-133, MPS90-150 and MPS90-157 produced less amount of IAA in comparison to parent strain at 5 days of growth (Table 3). Mutants MPS90-102, MPS90-106 produced IAA equal to parent strain. Mutant MPS90-14 produced less amount of IAA at 2 days of growth but it was at par with parent strain at 5 days of growth. Two mutants MPS90-145 and MPS90-280 produced higher amount of IAA in comparison to parent strain.

Effect of seed inoculation with *Pseudomonas* mutants on root and shoot elongation in green gram and black gram: Ten mutants selected on the basis of variation in IAA production level, were used as seed inoculants to study its effect on root and shoot elongation on plain water agar plates using seeds of green gram cv. Asha. Only three mutants i.e., MPS90-14, MPS90-106 and MPS90-150 caused shght stimulation of root growth of green gram seedlings at both 5 and 10 days of observation (Table 4). All other mutants showed stunting effect on root growth of green gram seedlings. Four mutants i.e., MPS90-39, MPS90-133, MPS90-145 and MPS90-157 caused stimulation of shoot growth at 5 days whereas all the mutants except MPS90-39 and MPS90-157

Table 2: Screening of Tn5-derived mutants from *Pseudomonas* strain MPS90 for alteration in IAA production level

Pseudomonas sp. strain	Total mutants selected	Parental type	IAA Low producer mutants	IAA over producer mutants
MPS90	350	215 (61.43%)	123 (35.14%)	12 (3.43%)

The values in parenthesis show the per cent frequency of mutants altered in IAA production level

Table 3: IAA production by selected mutants obtained from *Pseudomonas* strain MPS90

	IAA production (μg mL^{-1})	
Strain/mutants	2 days	5 days
MPS 90	14.0	16.2
MPS90-14	9.6	15.4
MPS90-39	9.5	9.7
MPS90-51	9.3	12.7
MPS90-102	10.5	13.9
MPS90-106	12.0	14.4
MPS90-133	10.4	12.4
MPS90-145	17.1	24.6
MPS90-150	11.4	12.0
MPS90-157	9.4	10.2
MPS90-280	25.3	34.1

Control contains LB broth and the Salkowski reagent. IAA production was calculated on the basis of equal 1.0 optical density of bacterial growth suspension

Table 4: Inoculation effect of *Pseudomonas* sp. strain MPS90 or its mutants on seedling growth of green grain

	Root length (cm)		Shoot length (cm)	
Treatment	5 days	10 days	5 days	10 days
Control	10.3±0.48	11.2±0.57	16.0±0.42	25.1±0.45
MPS90	8.9±0.32	10.0±0.60	15.4±0.23	23.8±1.10
MPS90-14	12.1±0.59	15.9±0.53	14.7±0.47	20.9±0.86
MPS90-39	10.2±0.54	11.3±0.51	18.0±0.36	25.3±0.56
MPS90-51	10.6±0.36	11.2±0.38	16.3±0.47	24.5±0.65
MPS90-102	9.8±0.22	10.2±1.28	16.3±0.23	24.1±1.40
MPS90-106	11.6±0.45	11.7±0.75	16.3±0.39	23.3±0.43
MPS90-133	10.7±0.45	11.3±0.40	17.3±0.32	24.6±0.98
MPS90-145	10.9±0.91	11.4±0.46	17.4±0.21	24.2±1.19
MPS90-150	11.5±0.39	12.0±0.43	16.9±0.48	24.6±0.57
MPS90-157	10.9±0.73	11.2±0.39	17.5±0.16	25.4±0.74
MPS90-280	8.7±0.35	11.2±0.39	15.9±0.48	24.0±1.26

Data are as Mean±SE and average values of 8 replications

showed stunting effect on shoot growth at 10 days of observation in comparison to uninoculated control treatment.

In black gram, majority of the *Pseudomonas* mutants enhanced the root growth of seedlings at 5 days of observation except the mutants MPS90-106 and MPS90-280. At 10 days of seedling growth, only four mutants i.e. MPS90-39, MPS90-157, MPS90-102 and MPS90-106 caused slight stimulation of root growth (Table 5). Whereas, most of the mutants showed retardation effect on the shoot growth of black gram seedlings except the mutants MPS90-14, MPS90-51, MPS90-133 and MPS90-280 at 5 days. Only the mutant MPS90-39 showed shoot growth stimulation at 10 days

Table 5: Inoculation effect of *Pseudomonas* sp. strain MPS90 or its mutants on seedling growth of black gram

	Root length (cm)		Shoot length (cm)	
Treatment	5 days	10 days	5 days	10 days
Control	9.8±0.85	16.1±0.96	13.7±0.45	22.4±0.50
MPS90	9.4±0.89	15.6±1.15	13.6±0.61	21.1±0.70
MPS90-14	12.1±0.61	13.9±0.90	14.7±0.29	21.0±0.74
MPS90-39	11.1±0.43	17.4±1.21	13.2±0.29	25.9±0.76
MPS90-51	12.4±0.51	15.9±0.46	14.0±0.32	22.3±0.63
MPS90-102	10.5±0.27	18.6±1.22	12.6±0.32	21.4±0.49
MPS90-106	9.7±0.46	19.5±1.44	10.7±0.46	22.7±1.07
MPS90-133	12.1±0.69	13.2±0.58	14.6±0.23	18.2±0.88
MPS90-145	11.6±0.65	12.1±1.08	12.6±0.83	21.4±0.68
MPS90-150	11.5±0.32	14.7±0.27	13.2±0.45	19.8±0.43
MPS90-157	11.6±0.36	16.4±0.65	13.7±0.33	21.8±0.48
MPS90-280	8.2±0.19	13.1±1.18	14.0±0.42	20.9±0.29

Data are as Mean±SE and average of 8 replications

shoot growth of black gram seedlings except the mutants MPS90-14, MPS90-51, MPS90-133 and MPS90-280 at 5 days. Only the mutant MPS90-39 showed shoot growth stimulation at 10 days of observation. Maximum retardation effect on shoot growth was observed by inoculation of mutants MPS90-133, MPS90-150 and MPS90-280 at 10 days of seedling growth in comparison to uninoculated control treatment. Thus, inoculation of different *Pseudomonas* mutants showed host-dependent variation in growth responses.

Effect of coinoculation of *Pseudomonas* mutants with *Bradyrhizobium* strain S24 in green gram and black gram under sterile conditions: Seed inoculation of green gram with *Pseudomonas* mutants and *Bradyrhizobium* strain S24 enhanced the shoot dry weights in green gram at 30 days of plant growth except the mutant MPS90-157 (Table 6). The shoot dry weight ratio of coinoculated plants over control or *Bradyrhizobium* inoculated plants varied from 2.89 to 3.58 times and 1.10 to 1.37 times, respectively at 60 days of plant growth. Significant gains in shoot dry weights were observed with mutants MPS90-14, MPS90-106, MPS90-133, MPS90-145 and MPS90-280. The parent strain MPS90 and all the mutants also increased nodule number and nodule biomass at 60 days of growth indicating stimulation of nodulation by *Bradyrhizobium* on coinoculation with *Pseudomonas* mutants.

IAA low producer and over producer mutants were also coinoculated with *Bradyrhizobium* strain S24 in black gram. Five *Pseudomonas* mutants MPS90-39, MPS90-51 (low IAA producer), MPS90-14 and MPS90-102 (IAA produced equal to parent strain) and MPS90-280 (IAA overproducer) showed significant gains in shoot dry weights at both 30 and 45 days of plant growth (Table 7). Four *Pseudomonas* mutants namely MPS90-14, MPS90-102, MPS90-157 and MPS90-280 caused more nodule formation by *Bradyrhizobium* strain S24 in comparison to stimulation by coinoculation of parent *Pseudomonas* strain at 30 and 45 days of growth. The shoot dry weight gains varied from 1.05 to 1.99 times those of *Bradyrhizobium*-inoculated treatments. However, inoculation with two mutants MPS90-106 and MPS90-133 adversely affected the symbiotic effectiveness at 30 and 45 days as compared to *Bradyrhizobium*-inoculated treatment. At 60 days of plant growth, increase in shoot dry weight varied from 1.79 to 3.57 times those of uninoculated

Table 6: Effect of coinoculation of green gram with *Pseudomonas* strain MPS90 or its mutants and *Bradyrhizobium* sp. strain S24 on symbiotic parameters at 30, 45 and 60 days of plant growth

	Nodule no. (plant^{-1})			Nodule fresh weight (mg plant^{-1})			Shoot dry weight (mg plant^{-1})		
Treatments	30 days	45 days	60 days	30 days	45 days	60 days	30 days	45 days	60 days
Control	1	1	4	3	5	22	193	310	448
MPS90	0	1	3	0	4	16	220	342	485
Bradyrhizobium sp. strain S24	26	30	35	108	206	278	377	683	1170
S24+MPS90	27	41	45	114	283	302	447	715	1312
S24+MPS90-14	28	36	45	148	271	352	448	864	1592
S24+MPS90-39	32	38	47	102	214	296	385	586	1295
S24+MPS90-51	28	46	52	136	325	408	544	694	1406
S24+MPS90-102	34	42	46	182	260	338	536	667	1438
S24+MPS90-106	24	39	42	160	256	324	518	782	1470
S24+MPS90-133	29	44	58	127	262	417	379	725	1452
S24+MPS90-145	27	44	52	167	302	396	447	658	1496
S24+MPS90-150	28	39	46	172	248	336	619	712	1430
S24+MPS90-157	23	31	36	107	179	284	350	437	1054
S24+MPS90-280	26	39	50	153	274	405	632	845	1604

Data are given as average values of 3 plants

Table 7: Effect of coinoculation of black gram with *Pseudomonas* strain MPS90 or its mutants and *Bradyrhizobium* sp. strain S24 on symbiotic parameters at 30, 45 and 60 days of plant growth

	Nodule no. (plant^{-1})			Nodule fresh weight (mg plant^{-1})			Shoot dry weight (mg plant^{-1})		
Treatments	30 days	45 days	60 days	30 days	45 days	60 days	30 days	45 days	60 days
Control	2	3	5	6	10	18	187	302	410
MPS90	-	2	3	-	8	16	174	310	428
Bradyrhizobium sp. strain S24	28	32	37	148	224	256	295	466	816
S24+MPS90	29	34	49	142	192	267	337	475	921
S24+MPS90-14	35	42	43	224	268	295	442	558	903
S24+MPS90-39	26	38	74	168	240	516	415	720	1078
S24+MPS90-51	28	33	70	190	262	375	401	615	1159
S24+MPS90-102	48	57	76	238	360	517	532	817	1625
S24+MPS90-106	29	34	35	172	215	264	285	436	721
S24+MPS90-133	24	30	32	115	170	205	310	412	784
S24+MPS90-145	30	36	38	184	235	292	312	514	932
S24+MPS90-150	27	32	50	146	218	286	335	623	1133
S24+MPS90-157	34	45	50	204	250	300	395	565	858
S24+MPS90-280	45	60	74	226	328	498	408	718	1346

Data are given as average values of 3 plants

controls. Five mutants MPS90-39, MPS90-51, MPS90-102, MPS90-150 and MPS90-280 caused significant gains in shoot dry weight ratios i.e., 1.32, 1.42, 1.99, 1.37 and 1.15 times those of *Bradyrhizobium* inoculated plants, respectively. Maximum increase in nodulation behaviour i.e., nodule number and nodule biomass was observed in black gram plants inoculated with mutants MPS90-39, MPS90-51, MPS90-102 and MPS90-280 at 60 days of plant growth.

DISCUSSION

The root colonizing microorganisms in and around the growing roots, interact with each other and with the plant resulting into either symbiotic, associative, neutralistic or detrimental effects depending upon the microorganisms involved, abiotic and biotic soil environment and the plant defense system (Benizri *et al*., 2001; Somers *et al*., 2004). These microbial populations in the rhizosphere release specific allelochemicals in soil, cause mineralization of nutrients, produce growth hormones and antagonize the plant pathogens (Ahmad *et al*., 2008; Martinez-Viveros *et al*., 2010; Sindhu *et al*., 2011). Present study was aimed at deriving low as well as over IAA producing mutants from the *Pseudomonas* strain MPS90 and to study their effect on nodulation and plant growth in green gram and black gram on coinoculation with *Bradyrhizobium* strain.

Different bacterial strains have been found to produce IAA in varying amounts (Prikryl *et al*., 1985; Keyeo *et al*., 2011). *Pseudomonas* strain MPS90 showed 16.2 μg mL^{-1} of IAA production at 5 days of growth (Table 1). Barea *et al*. (1976) reported that among 50 bacterial isolates obtained from the rhizosphere of various plants, 86, 58 and 90% isolates produced auxins, gibberellins and kinetin-like substances, respectively. The production of phytohormones has also been reported in other PGPR strains including *Azotobacter chroococcum* (Muller *et al*., 1989), *Azospirillum* spp. (Remans *et al*., 2008), *Rhizobium* species (Hirsch and Fang, 1994), *Pseudomonas fluorescens* (Dubeikovsky *et al*., 1993) and *P. putida* (Taghavi *et al*., 2009). Barazani and Friedman (1999) reported that high levels of IAA (76.6 μm) were excreted by four deleterious rhizobacteria (*Micrococcus luteus, Streptoverticillium* sp., *Pseudomonas putida* and *Gluconobacter* sp.) and lower amounts of IAA (16.4 μm) were secreted by plant growth promoting rhizobacterial isolates including *Agrobacterium* sp., *Alcaligenes piechaudii* and *Comamonas acidovorans*.

A total of 350 kanamycin-resistant mutants with altered IAA production level under cultural conditions (overproducers and low producers) were selected. About 35.14 per cent mutants showed low production of IAA and only 3.43 per cent mutants were found with high IAA production ability (Table 2). Mutants i.e., MPS90-39, MPS90-51, MPS90-133, MPS90-150 and MPS90-157 produced low amount of IAA in comparison to parent strain (Table 3). Comai and Kosuge (1983) also reported that insertion of IS51 (1.3 kb DNA element) on the plasmid borne *iaa*M locus in *P. syringae* pv. *savastanoi* resulted in the loss of indole acetic acid production and attenuation of virulence. Mazzola and White (1994) generated IAA-deficient mutant by Tn5 transposition into the *iaa*M gene of *P. syringae* pv. *syringae* Y30. The IAA^- mutant retained the ability to colonize the bean phylloplane and induced disease symptoms on beans which were similar to those produced by the parental strain. In this study, two mutants MPS90-145 and MPS90-280 produced higher amount of IAA in comparison to parent strain. The elevated IAA levels in these mutants may be a consequence of transposon insertion into a region of bacterial genome that elevated the expression of IAA biosynthesis genes either directly or by overproduction of tryptophan.

Production of IAA has been found to affect plant growth in diverse ways, varying from pathogenesis and growth inhibition to plant growth stimulation (Somers *et al*., 2004; Spaepen *et al*., 2007; Fassler *et al*., 2010). Only three mutants i.e., MPS90-14, MPS90-106 and MPS90-150 caused slight stimulation of root growth of green gram seedlings at both 5 and 10 days of observation on plain water agar plates (Table 4) and all other mutants showed stunting effect on root growth of green gram seedlings. Four mutants i.e., MPS90-39, MPS90-133, MPS90-145 and MPS90-157 caused stimulation of shoot growth at 5 days whereas other mutants showed stunting effect on shoot growth at 10 days of observation in comparison to uninoculated control treatment.

Moreover, the inoculation of the *Pseudomonas* mutants in black gram showed host-dependent variation in growth responses. Majority of the *Pseudomonas* mutants enhanced the root growth of black gram seedlings at 5 days of observation except the mutant MPS90-106 and MPS90-280, whereas at 10 days of seedling growth, only four mutants i.e. MPS90-39, MPS90-157, MPS90-102 and MPS90-106 caused slight stimulation of root growth (Table 5). Most of mutants showed stunting effect on the shoot growth of black gram seedlings except the mutants MPS90-14 and MPS90-133 at 5 days of seedling growth. Maximum retardation effect on shoot growth was observed by inoculation of mutants MPS90-133, MPS90-150 and MPS90-280 at 10 days of seedling growth in comparison to uninoculated control treatment.

Similar concentration dependent effect of IAA on stimulation or retardation of root/shoot growth has been reported in earlier studies (Arshad and Frankenberger, 1991). Loper and Schroth (1986) observed a significant linear relationship between IAA accumulation of the rhizobacterial strains and decreased root elongation of sugar beet seedlings. The initial stunting effect on seedlings could be due to contact of bacterial cell with legume seeds or due to synthesis or secretion of excessive amount of IAA or by production of some inhibitory agent/toxin by the bacterium when grown in synthetic medium or in root exudates of legumes (Gealy *et al*., 1996; Bolton and Elliott, 1989). It is also possible that phytoalexins produced by seedlings as a host defense response after inoculation (infection) of rhizobacteria could be inhibitory for seedling growth initially. The production of toxic metabolites by other non-fluorescent *Pseudomonas* strains with an inhibitory effect on wheat root growth has also been reported (Fredrickson *et al*., 1987). The inhibitory effect of some deleterious rhizobacteria (DRB) was also related to their high amount of IAA excretion in *Enterobacter taylorae* (Sarwar and Kremer, 1995).

Inoculation of legumes or cereal plants with PGPR strains has been found to show a wide range of effects that varied among strains of PGPR. Some PGPR strains stimulated plant growth by affecting some plant physiological events such as photosynthesis, nodulation and nitrogen fixation whereas a few PGPR strains inhibited these processes under certain environmental conditions (Zhang *et al*., 1996). Seed inoculation of green gram with *Bradyrhizobium* sp. strain S24 alone or on coinoculation with different mutants of *Pseudomonas* strain MPS90 increased the nodule number and shoot dry weights in comparison to uninoculated controls (Table 6). Mutant dependent variations in shoot dry weights were observed in various treatments at different stages of plant growth. At 30 days of plant growth, coinoculation of *Pseudomonas* mutants with *Bradyrhizobium* strain S24 enhanced the shoot dry weights in green gram except the mutant MPS90-157 (Table 6). The shoot dry weight ratio of coinoculated plants over control or *Bradyrhizobium* inoculated plants varied from 2.89 to 3.58 times and 1.10 to 1.37 times, respectively at 60 days of plant growth. Mutants MPS90-14, MPS90-106, MPS90-133, MPS90-145 and MPS90-280 showed significant gains in shoot dry weights. The parent *Pseudomonas* strain MPS90 and the mutants also increased nodule number and nodule biomass.

Coinoculation of *Pseudomonas* mutants with *Bradyrhizobium* strain S24 in black gram showed differential effect on nodulation and plant growth. Two IAA low producer mutants MPS90-39, MPS90-51 and mutants MPS90-14 as well as MPS90-102 (IAA producer equal to parent strain) and one IAA over producer mutant MPS 90-280 enhanced shoot dry weights at both 30 and 45 days of plant growth (Table 7). The shoot dry weight gains varied from 1.05 to 1.99 times those of *Bradyrhizobium*-inoculated treatments. However, the coinoculation of different mutants showed gains in shoot dry weight varying from 179 to 357% to those of uninoculated controls at 60 days of plant growth. Maximum increase in nodulation behaviour i.e., nodule number and nodule

biomass was observed in black gram plants inoculated with mutants MPS90-39, MPS90-51 (low IAA producer), MPS90-102 (IAA equal to parent strain) and MPS90-280 (IAA over producer) at 60 days of plant growth. Hafeez *et al.* (2004) also reported that inoculation with IAA producing (*Brady*)*Rhizobium* strains increased root dry weight, root length and root area of cotton by 248, 332 and 283%, respectively. The increased root growth due to IAA production by these strains enhanced shoot dry weight and N uptake through efficient nutrient uptake. Beneficial effects of coinoculation of PGPR with *Rhizobium* on symbiotic parameters have also been reported in other legumes like chickpea (Sindhu *et al.*, 2002), clover (Derylo and Skorupska, 1993), green gram (Sindhu *et al.*, 1999), pea (Berggren *et al.*, 2001) and soybean (Dashti *et al.*, 1998).

In this study, coinoculation of *Bradyrhizobium* with majority of *Pseudomonas* mutants enhanced the nodule formation on both green gram and black gram hosts. Similar three-fold increase in the number of root nodules was observed after inoculation of soybeans with spontaneous mutants of *Rhizobium japonicum* that overproduced 30-fold more IAA than the wild-type strain (Kaneshiro and Kwolek, 1985). On the other hand, mutants of *Bradyrhizobium elkanii* deficient in IAA production induced fewer nodules on soybean roots than did the parental strain and the normal number of nodules was reestablished following application of exogenous IAA (Fukuhara *et al.*, 1994). Coinoculation with IAA-producing *Pseudomonas* strains with *Mesorhizobium* sp. *Cicer* strain Ca181 also resulted in increased nodule number and nodule fresh weight in chick pea (Malik and Sindhu, 2011). In contrast, similar experiments using mutants of *Bacillus megaterium* with altered IAA production levels (overproducers and underproducers) had a negative effect on these parameters (Srinivasan *et al.*, 1996). It was suggested that inoculation with these free-living bacteria may increase the number of infection sites on roots for attachment and nodulation by *Rhizobium*. In addition, rhizobacteria have been found to enhance the production of flavonoid-like compounds or phytoalexins in roots of several crop plants (Goel *et al.*, 2001) which induce the transcription of rhizobial nodulation (*nod*) genes (Peter and Verma, 1990) and may result into enhanced nodulation.

Pseudomonas strain/mutants that showed initial stunting effect on root and shoot growth of the seedlings, did not show adverse effect on nodulation and plant growth when these bacteria were used as coinoculants with *Bradyrhizobium* under sterilized chillum jar conditions. For example, coinoculation of two over-producer mutants i.e., MPS90-145 and MPS90-280 caused maximum gains in shoot dry weight ratio of green gram at 60 days of growth. In black gram also, IAA over-producer mutant MPS90-280 as well as MPS90-102 (IAA equal to parent strain) caused significant gains in shoot dry weights at 60 days of growth. Thus, the concentration of IAA may exert differential response on higher plants in relation to nodulation and plant dry weight. Therefore, inoculation tests under field conditions are essential for evaluating the impact of allelochemicals secreted by soil-borne microorganisms. Moreover, the interactions between plants and rhizobacteria usually encourage the establishment of specific beneficial rhizospheres and such associations between different crop species can also be cultivar-specific (Merharg and Killham, 1995). Results of this study also suggested that screening of rhizosphere bacteria for production of hormones, inhibition of growth of phytopathogenic fungi and for growth promotion under gnotobiotic conditions, could be a better approach for selection of effective PGPR strains (Sahu and Sindhu, 2011). In future, a better understanding of the molecular biology of plant-PGPR interactions will enable scientists to design strategies in selecting, modifying and using PGPR for plant growth promotion and their use as biofertilizers for specific crops (Vessey, 2003).

CONCLUSION

Beneficial microorganisms in the soil maintain the fertility status and improve physical characteristics of the soil which is essential for improving biomass production, remediation of pollutants and for enhancing crop productivity (Weyens *et al*., 2009; Sindhu *et al*., 2010). In the present study, mutants with variation in IAA production ability were obtained from *Pseudomonas* strain MPS90 using Tn5 mutagenesis. Only 3.43% mutants produced higher levels of IAA and 35.14% mutants produced low amount of IAA in comparison to parent strain. Seed inoculation of mutants in green gram and black gram showed stimulation/retardation effect on growth of the seedlings. Coinoculation studies of *Pseudomonas* mutants with *Bradyrhizobium* sp. strain S24 increased shoot dry weight that varied from 110 to 137 per cent in green gram and from 105 to 198 per cent in black gram in comparison to *Bradyrhizobium*-inoculated plants at 60 days of growth. Maximum nodule formation was observed after coinoculation of mutants MPS90-133, MPS90-145 and MPS90-51 with *Bradyrhizobium* strain S24 in green gram whereas mutants MPS90-102, MPS90-39 and MPS90-280 caused more stimulation for nodule formation in black gram at 60 days of plant growth. Thus, differential behaviour on enhancement of nodulation and plant shoot weight was observed in the two hosts by coinoculation of *Bradyrhizobium* with different mutants altered in IAA production ability.

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