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Disease Control and Plant Growth Promotion of Green Gram by Siderophore Producing *Pseudomonas* sp.

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ABSTRACT

Siderophore production by rhizosphere bacteria contributes to fungal growth inhibition resulting into suppression of plant diseases. Therefore, siderophore-producing *Pseudomonas* isolates were studied for root rot disease control and plant growth stimulation of green gram. Fifty-eight *Pseudomonas* strains were obtained from the rhizosphere soil of chickpea and green gram using dilution plating technique. These strains were tested for siderophore production on MM9 medium and succinate medium plates containing chromo-azurol S by spot test method. Siderophore-producing strains formed the halo zone around the colony growth and diameter of halo zone varied with different *Pseudomonas* strains. Siderophore production was found more on MM9 medium as compared to succinate medium. Bacterial colony growth (G) and halo zone (H) size ratios varied from 1.44 to 10.24 in different *Pseudomonas* strains on MM9 medium. Siderophore-producing *Pseudomonas* strains inhibited the growth of phytopathogenic fungi i.e., *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium aphanidermatum*. Large zones of fungal growth inhibition were observed on PDA medium in comparison to nutrient agar medium plates. Seed inoculation of *Pseudomonas* cultures in green gram (*Vigna radiata* L.) caused reduction in root rot disease (*R. solani* induced) symptoms under pot house conditions and disease control varied from 33.4 to 100% with different *Pseudomonas* cultures. Coinoculation of different *Pseudomonas* strains with *Bradyrhizobium* strain SMR15 in green gram also enhanced the nodule number, nodule fresh weight and plant dry weight at 30 and 60 days of plant growth. Coinoculation of *Pseudomonas* strain CP56 with *Bradyrhizobium* strain and *R. solani* showed maximum 275.8% increase in plant dry weight at 60 days in comparison to control uninoculated plants and completely suppressed the root rot disease under pot house conditions. Thus, inoculation with siderophore-producing *Pseudomonas* sp. could be utilized for disease control and plant growth promotion of legumes.

Key words: Siderophore, *Pseudomonas* sp., phytopathogenic fungi, *Bradyrhizobium* strain, disease suppression, nodulation, green gram

INTRODUCTION

Iron is fourth most abundant element on the earth's crust and is essential for the growth of all living organisms (Neiland, 1981). However, its availability to plants and microorganisms is hindered due to its easy chemical oxidation and formation of insoluble ferric salts (Neilands, 1982). Most of the microorganisms produce extracellular iron transport agents called siderophores, to

solubilize and sequester iron (Leong, 1986; Buyer and Sikora, 1990). These siderophores play prominent role in the physiology of symbiotic diazotrophs, soil microflora and in biological control of soil borne plant pathogens (Crowley and Kraemer, 2007; Lemanceau *et al*., 2007).

The plant rhizosphere is colonized by saprophytic, pathogenic and plant growth promoting strains of bacteria (Miller *et al*., 1989). The interactions of rhizospheric microorganisms with each other and the environment surrounding the plant root, leads to either beneficial or detrimental effects on plant growth (Weyens *et al*., 2009; Sindhu *et al*., 2010; Woyessa and Assefa, 2011). Many species of *Azotobacter*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Pseudomonas*, *Rhizobium* and *Serratia* have been found to improve plant growth and are termed as plant growth-promoting rhizobacteria (PGPR) (Kloepper *et al*., 1980). Some PGPR are also involved in control of plant diseases by suppression of phytopathogenic organisms (Weller, 2007) by various mechanisms viz. antibiosis, production of hydrolytic enzymes and hydrocyanic acid, stimulation of phytoalexins or flavonoid-like compounds in roots and by induction of systemic resistance. PGPR have also been reported to produce siderophores which chelate metal cations and inhibit the spore germination of pathogenic fungi leading to disease suppression (Raaijmakers *et al*., 1995; Sindhu *et al*., 2009). Thus, PGPR have emerged an environment friendly alternative to the hazardous pesticides.

The pathogenic fungi i.e., *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium aphanidermatum* have been found to cause wilt, root rot and damping off disease, respectively in various crops leading to significant losses in crop yield (Siddiqui *et al*., 2001; Validov *et al*., 2007; Scherwinski *et al*., 2008). Recently, seed bacterization with specific PGPR strains inhibited the growth of soil borne root infecting fungi, suppressed the disease and enhanced the growth of various plants under green house and field conditions (Ehteshamul-Haque and Ghaffar, 1993; Kumar, 1996; Ahmad *et al*., 2008). The fluorescent siderophores were found to control the growth of pathogenic fungi i.e., *Pythium ultimum* (Gill and Warren, 1988), *Gaeumannomyces graminis* (Brisbane and Rovira, 1988) and inhibited the germination of *Fusarium oxysporum* chlamydospores (Simeon *et al*., 1987). The inhibition was totally or partially reversed if the medium or field soil was amended with iron (III) in the form of Fe-EDTA. Siderophore negative mutants were not inhibitory to these fungi indicating that siderophores contribute to the suppression of certain fungal diseases (Buysens *et al*., 1996).

Cucumber and spinach seedlings supported more growth of the siderophore producing *Pseudomonas* strains than nonproducers and also promoted rootlet elongation on cucumber (De Bellis and Ercolani, 2001). Moreover, the coinoculation of *Pseudomonas* sp. with *Bradyrhizobium*/*Mesorhizobium* sp. have been found to cause a significant increase in nodule number, nodule weight and plant dry weight of green gram and chickpea when grown under sterilized chillum jar conditions (Sindhu *et al*., 1999, 2002). Present study was carried out to find out the prevalence of siderophores in *Pseudomonas* strains and to understand the role of siderophores in disease control and plant growth promotion of green gram.

MATERIALS AND METHODS

This study was carried out in the Department of Microbiology, CCS Haryana Agricultural University, Hisar on the crop green gram during the month of July, 2008 to June 2010.

Microbial cultures: Fifty-eight *Pseudomonas* isolates were obtained from the rhizosphere soil of chickpea and green gram by plating of serial dilutions on King's B (KB) medium plates.

Pseudomonas colonies were selected based on typical morphological and pigment production characteristics. The different *Pseudomonas* isolates were grown on KB medium plates for 2 days at 28+2°C. One percent solution of tetramethyl-p-phenyl-diamine dihydrochloride was added to cover surface of plates. The oxidase positive *Pseudomonas* isolates gave purple colour on these plates.

Twenty-two reference strains of *Pseudomonas* and one *Bradyrhizobium* sp. strain SMR15 were obtained from the Department of Microbiology, CCS Haryana Agricultural University, Hisar. The *Pseudomonas* strains were maintained by periodic transfer on Luria Bertani agar slants (Sambrook *et al*., 1989) and *Bradyrhizobium* strain was maintained on yeast extract mannitol agar (YEMA) medium slants (Somasegaran and Hoben, 1994). The bacterial cultures were stored at 4°C in refrigerator for further use. Three fungal cultures i.e., *Fusarium oxysporum, Pythium aphanidermatum* and *Rhizoctonia solani* were obtained from the Department of Plant Pathology, CCS Haryana Agricultural University, Hisar and maintained on potato dextrose agar (PDA) medium slants. Seeds of green gram (*Vigna radiata*) var. Asha were obtained from RDS Seeds Farm, CCS Haryana Agricultural University, Hisar.

Screening of *Pseudomonas* sp. for siderophore production: The Universal chemical assay utilizing chromo-azurol S (CAS/iron (III) and hexadecyltrimethyl ammonium bromide) agar plates was used for qualitative detection of siderophores produced by different *Pseudomonas* strains as described by Schwyn and Neilands (1987b). Siderophore production was also determined on iron deficient succinate medium used by Mayer and Abdallah (1978). Different *Pseudomonas* cultures were grown on fresh King's B medium (King *et al*., 1954) slants. Growth suspension (10 μL) of different *Pseudomonas* strains was spotted on the CAS incorporated plates and incubated at 28±2°C for 3-4 days. Orange halo zone around the colonies on blue agar plates indicated the presence of siderophores (Schwyn and Neilands, 1987a). The diameter of orange halo zone formed and diameter of the *Pseudomonas* colony was measured as described by Van Rossum *et al*. (1994).

Growth inhibition of pathogenic fungi: Three fungi i.e., *Fusarium oxysporum, Pythium aphanidermatum* and *Rhizoctonia solani* were transferred to fresh PDA medium plates and incubated for four days at 28±2°C to get complete spore formation. The fungal spore suspension (3.0 mL) was added into sterilized nutrient agar (NA) and PDA medium, mixed uniformly and plated. Growth suspension (10 μL) of different *Pseudomonas* strains was spotted on fungal spore-incorporated plates as described by Sindhu *et al*. (1999). The plates were incubated in the BOD incubator at 28±2°C for 4 days. The growth inhibition zones of different fungi were recorded.

Seed bacterization of green gram with *Pseudomonas* strains: Three siderophore-producing *Pseudomonas* strains, i.e., MP20, MPS54 and CP56 and one siderophore-lacking strain CPS67 were tested for disease control of green gram under pot house conditions. The earthern pots of 10 kg capacity were filled with sandy loam soil and river sand mixed in 70:30 ratio. *Pseudomonas* strains were grown on LB medium for 2 days and a *Bradyrhizobium* sp. strain SMR15 was grown on YEMA medium slopes for 7 days. The growth suspension of each *Pseudomonas* and *Bradyrhizobium* cultures was made in 5 mL of sterilized water.

Surface sterilized seeds of green gram (*Vigna radiata*) cv. Asha were inoculated with broth culture of *Bradyrhizobium* strain SMR15 alone or with different *Pseudomonas* strains. Coinoculation of different *Pseudomonas* strains was done by mixing with growth suspension of *Bradyrhizobium* in 1:1 ratio (v/v). The viable count in the broth was kept 10^8-10^9 cells mL^{-1} and

10 g seeds were inoculated with 1 mL of bacterial growth suspension (Sindhu *et al.*, 1999). There were nineteen treatments in this experiment. Each treatment had three replications. Growth of 4 days-old *R. solani* was harvested from PDA plates and fungal growth suspension was prepared in sterilized saline water. Fungal growth suspension (100 mL) was mixed in the 10 kg soil: sand mixture in earthern pots with treatments T_2, T_6, T_7, T_{10}, T_{11}, T_{14}, T_{15}, T_{18} and T_{19}. The growth suspension of fungus was also inoculated on the roots of green gram plants in the *R. solani* treatments only.

The plants were grown in the pot house under day light conditions during the month of May-June 2010. Sloger's nutrient solution was added in the pots as and when required (Sloger, 1969). The plants were uprooted at 30 and 60 days of plant growth and observations were taken for nodule number, nodule fresh weight, plant dry weight and disease index. After washing with tap water, nodules were detached from the roots and dried in the folds of filter paper. The nodules were counted and weighed. Shoot portions of the plants were dried in oven at 90°C for 24 h and weighed.

Disease index and reduction in disease: On the basis of symptoms observed, percent disease index, percent final stand and percent disease control were calculated by following formulae:

$$\%\ \text{Disease incidence}(\text{DI}) = \frac{\text{Total No. of disease plants}}{\text{Total No. of plants}} \times 100$$

$$\text{Final stand} = 100 - \%\ \text{Disease incidence}$$

$$\%\ \text{Disease control} = \frac{100 - \text{DI in treatment}}{\text{Disease incidence in control}} \times 100$$

Disease control and disease incidence were recorded after 30 and 60 days of sowing. It was calculated on the average of six plants grown per pot.

RESULTS

Certain bacteria such as pseudomonas and bacilli exert beneficial effects on plants either by enhancing crop nutrition, by reducing damages caused by pathogens or pests and by their ability to degrade xenobiotic compounds. These rhizosphere bacteria have emerged as important biological components of agriculture soils with the ability to stimulate plant growth and for protection of plants from pathogens. In this study, siderophore-producing *Pseudomonas* species were isolated from the rhizosphere soil and were evaluated for plant growth promotion and disease control in green gram.

Screening of *Pseudomonas* isolates for siderophore production: All the *Pseudomonas* strains were screened for siderophore production using universal chromo-azurol assay. The presence of iron chelator siderophore was indicated by decolourization of blue coloured ferric CAS complex in an orange halo zone around the colonies on CAS agar plates. Out of 80 *Pseudomonas* strains screened for siderophore production, 37 strains were found to produce siderophore on MM9 medium (Table 1). The diameter of halo zone varied with different *Pseudomonas* strains. The *Pseudomonas* strains MPS52, MPS54, MPS90, CPS59, PS31, PS39, CP20, CP28, CP56, MP14, MP20, MP33 and MP38 showed large halo zone size measuring more than 10 mm on MM9 medium plates. Siderophore production was also determined on iron deficient succinate medium. Only two *Pseudomonas* strains MPS52 and CP20 showed large halo zone formation (above 10 mm). Rest of

Table 1: Screening of selected *Pseudomonas* strains for siderophore production using universal chromazurol assay method

	Halo zone size (mm, diameter)	
Pseudomonas strains	MM9 medium	Succinate medium
MRS23	4	4
MPS52	15	12
MPS54	16	8
MRS55	2	3
MPS90	16	9
CPS59	14	4
CPS67	-	2
PS31	22	8
PS39	16	3
CP17	6	4
CP20	17	10
CP28	14	3
CP56	16	6
MP14	14	4
MP20	10	2
MP33	20	6
MP38	14	4

Different *Pseudomonas* strains were tested for siderophore production by spotting 10 μL growth suspension on MM9 or iron deficient succinate medium plates (pH 6.8). Observations were recorded after 3 days of growth at 28±2°C

the strains showed variation in diameter of halo zone formed. It was found that siderophore production was more on MM9 medium as compared to succinate medium. Only 46.25% of *Pseudomonas* strains showed siderophore production on the two types of media tested in these studies.

The relative siderophore production ability of different *Pseudomonas* strains was determined by comparing the bacterial colony diameter to halo zone size on MM9 medium plates. It was measured by dividing the halo zone area $(0.5H)^2$ to colony growth area $(0.5G)^2$ Out of 37 *Pseudomonas* strains, 20 strains showed more than 3.0 ratio of halo zone to colony growth (Table 2). *Pseudomonas* strain MPS54 showed maximum halo zone to growth ratio (10.24) and minimum halo zone to growth ratio (less than 2.0) was obtained in *Pseudomonas* strains, namely MRS23, MRS55, MPS90, CPS59, CPS67, CP17, CP28 and MP14.

Interactions of siderophore-producing *Pseudomonas* strains with phytopathogenic fungi: Selected siderophore-producing *Pseudomonas* strains were screened for antagonistic activity against three fungi namely, *Fusarium oxysporum, Rhizoctonia solani* and *Pythium aphanidermatum* on PDA and NA medium plates. The growth inhibition zones in different fungi varied with different *Pseudomonas* strains tested (Table 3). The *Pseudomonas* strains MRS23, MPS54, MRS55, CP17 and CP56 showed maximum antagonistic activity against *F. oxysporum* on PDA and NA medium plates and inhibition zone diameter was more than 4 mm. Two strains MPS52 and CP20 did not inhibit *F. oxysporum* growth but these strains were found to inhibit other two fungi *R. solani* or *P. aphanidermatum*. On the other hand, *Pseudomonas* strains MRS23, MPS54, MRS55, CP17 and CP56 showed large growth inhibition zones (more than 4 mm) against *R. solani* on PDA and NA medium plates. When different *Pseudomonas* strains were tested for antagonistic activity against *P. aphanidermatum*, MPS52, MPS54, PS31, CP20 and MP38 showed

Table 2: Relative siderophore production by different strains of *Pseudomonas* sp. on MM9 medium plates

Pseudomonas strains	Colony growth (G)	Halo zone (H)	Halo zone to growth ratio
MRS23	4	5	1.56
MPS52	8	15	3.51
MPS54	5	16	10.24
MRS55	5	7	1.96
MPS90	12	16	1.77
CPS59	10	14	1.96
CPS67	8	-	-
PS31	11	22	4.00
PS39	9	16	3.16
CP17	5	6	1.44
CP20	8	17	4.51
CP28	9	14	2.41
CP56	11	20	3.30
MP14	10	14	1.96
MP20	5	10	4.00
MP33	8	20	6.25
MP38	8	16	4.00

G: Growth expressed as colony diameter (mm), H: Siderophore production expressed as halo zone diameter (mm), H/G: Halo zone area $(0.5\ H)^2$ divided by growth area $(0.5\ G)^2$

Table 3: Antagonistic activity of *Pseudomonas* strains on growth inhibition of phytopathogenic fungi on PDA and NA medium plates

	Inhibition zone of fungal growth (mm, diameter)					
	Fusarium oxysporum		*Rhizoctonia solani*		*Pythium aphanidermatum*	
Pseudomonas strains	PDA	NA	PDA	NA	PDA	NA
MRS23	4	8	6	8	-	2
MPS52	-	-	-	-	12	5
MPS54	8	6	6	8	16	3
MRS55	6	8	6	6	8	-
MPS90	2	4	-	2	5	10
CPS59	2	4	2	4	-	-
CPS67	-	-	-	-	-	-
PS31	4	4	4	2	12	6
PS39	2	-	2	-	6	5
CP17	6	8	6	6	7	9
CP20	-	-	-	-	10	12
CP28	4	4	4	4	5	2
CP56	8	6	8	4	-	6
MP14	4	2	-	2	2	8
MP20	4	4	3	2	-	6
MP33	2	4	2	2	4	4
MP38	4	2	-	4	10	-

Antagonistic activity of the selected siderophore-producing strains was determined on potato dextrose agar (PDA) and nutrient agar (NA) medium by spot test method

maximum antagonistic activity against this fungus on PDA medium plates (Table 3). Two strains MPS90 and CP20 showed large growth inhibition zone (more than 10 mm) against *P. aphanidermatum* on NA medium plates.

Pseudomonas strain CPS67 did not inhibit the growth of any of the fungi. It was observed that *Pseudomonas* strains that showed large inhibition zones against *P. aphanidermatum* were comparatively less inhibitory to *F. oxysporum* and *R. solani* on the PDA as well as on NA medium plates. In majority of strains, the diameter of inhibition zone was larger on NA medium than to PDA medium plates. Usually, the *Pseudomonas* strains which showed more siderophore production (large H:G ratio) (Table 3) showed more growth inhibition of the phytopathogenic fungi. For example, *Pseudomonas* strains having large H:G ratio such as MPS54, PS31, PS39, CP56 and MP33 showed sufficient growth inhibition of different phytopathogenic fungi.

Effect of *Pseudomonas*, *Bradyrhizobium* and *R. solani* inoculation on symbiotic parameters and disease control: Green gram plants were inoculated either singly with *Bradyrhizobium* sp. strain SMR15 or siderophore-producing *Pseudomonas* strains MP20, MPS54, CP56 or siderophore-lacking strain CPS67 and with root rot causing fungi *Rhizoctonia solani*. Coinoculation of *Bradyrhizobium* strain, *Pseudomonas* strains or *R. solani* was also done and inoculated plants were harvested after 30 and 60 days of plant growth under pot house conditions. At 30 days of plant growth, the coinoculation of *Bradyrhizobium* strain with *Pseudomonas* strains MP20, MPS54 or CP56 enhanced the nodule number, nodule weight and plant dry weights as compared to single inoculated or control plants (Table 4). The inoculation with *R. solani* caused root rot disease in 83.7% of inoculated plants. The coinoculation of *Pseudomonas* strains with *R. solani* lowered the disease incidence and 16.3% disease reduction was observed with *Pseudomonas* strains MP20 and CPS67 whereas 66.7% disease reduction was observed with MPS54. Maximum plant

Table 4: Coinoculation of selected *Pseudomonas* strains with *Bradyrhizobium* strain for nodulation and control of root rot disease of green gram at 30 days of plant growth

Treatments	Nodule No. (No. pL^{-1})	Nodule fresh wt. (mg pL^{-1})	Plant dry wt. (mg pL^{-1})	Disease incidence (%)	Disease control (% reduction)
Control (Soil alone)	4.3	09.1	187.1	16.7	-
Control + *R. solani*	13.5	22.0	251.3	83.7	-
Control + *Bradyrhizobium* sp. SMR15	17.2	35.4	281.0	-	-
Pseudomonas strain MP20	16.2	28.1	262.5	-	-
MP20 + *B.* sp. strain SMR15	19.3	38.0	330.0	-	-
MP20 + *R. solani*	14.5	27.0	258.3	83.7	16.3
MP20 + *R. solani* + *B.* sp. strain SMR15	14.2	25.4	250.5	66.6	33.4
Pseudomonas strain MPS54	14.8	31.0	335.0	-	-
MPS54+ *B.* sp. strain SMR15	18.2	48.0	356.0	-	-
MPS54 + *R. solani*	15.2	34.2	265.5	33.3	66.7
MPS54 + *R. solani* + *B.* sp. strain SMR15	13.8	29.2	231.1	33.3	66.7
Pseudomonas strain CP56	17.3	52.0	379.0	-	-
CP56 + *B.* sp. strain SMR15	20.7	53.0	567.0	-	-
CP56 + *R. solani*	16.3	26.1	275.0	66.6	33.4
CP56 + *R. solani* + *B.* sp. strain SMR15	22.5	77.1	474.6	33.3	66.7
Pseudomonas strain CPS67	16.3	48.0	447.1	-	-
CPS67 + *B.* sp. strain SMR15	16.7	42.4	314.0	-	-
CPS67 + *R. solani*	18.5	62.2	308.0	83.7	16.3
CPS67 + *R. solani* + *B.* sp. strain SMR15	15.7	46.5	330.0	66.6	33.4

The figures are average values of 6 plants. Disease incidence is the % of plants infected and disease control is the % reduction of diseased plants after inoculation with bacteria. The values of nodule number, nodule fresh weight and plant dry weight are calculated on per plant basis

Table 5: Coinoculation of selected *Pseudomonas* strains with *Bradyrhizobium* strain for nodulation and control of root rot disease of green gram at 60 days of plant growth

Treatments	Nodule No. (No. pL^{-1})	Nodule fresh wt. (mg pL^{-1})	Plant dry wt. (mg pL^{-1})	Disease incidence (%)	Disease control (% reduction)
Control (Soil alone)	12.5	87.0	651.0	16.7	-
Control + *R. solani*	26.3	135.4	604.2	66.6	-
Control + *Bradyrhizobium* sp. SMR15	30.5	162.0	1235.6	-	-
Pseudomonas strain MP20	25.7	142.2	805.0	-	-
MP20 + *B.* sp. strain SMR15	32.2	176.4	1012.3	-	-
MP20 + *R. solani*	19.3	123.2	737.0	66.6	33.4
MP20 + *R. solani* + *B.* sp. strain SMR15	18.8	122.0	1025.0	33.3	66.7
Pseudomonas strain MPS54	26.5	149.0	1364.5	-	-
MPS54 + *B.* sp. strain SMR15	29.2	194.4	1352.3	-	-
MPS54 + *R. solani*	18.7	109.8	810.0	66.6	33.4
MPS54 + *R. solani* + *B.* sp. strain SMR15	27.3	175.0	881.5	16.7	83.3
Pseudomonas strain CP56	27.2	182.5	1283.5	-	-
CP56 + *B.* sp. strain SMR15	32.8	216.0	1678.3	-	-
CP56 + *R. solani*	18.2	104.8	752.6	66.6	33.4
CP56 + *R. solani* + *B.* sp. strain SMR15	28.5	192.4	1795.3	-	-
Pseudomonas strain CPS67	27.8	213.0	1342.0	-	-
CPS67 + *B.* sp. strain SMR15	36.2	245.4	1067.5	-	-
CPS67 + *R. solani*	20.7	144.5	1115.2	66.6	33.4
CPS67 + *R. solani* + *B.* sp. strain SMR15	22.5	132.3	1172.6	33.3	66.7

The figures are average values of 6 plants. Disease incidence is the % of plants infected and disease control is the % reduction of diseased plants after inoculation with bacteria. The values of nodule number, nodule fresh weight and plant dry weight are calculated on per plant basis

biomass enhancement (3.03 times) was found on coinoculation of *Pseudomonas* strain CP56 with *Bradyrhizobium* strains SMR15 as compared to uninoculated control plants and 2.01 times increase was observed in comparison to *Bradyrhizobium* inoculated plants.

At 60 days of plant growth, single inoculation with *Bradyrhizobium* strain SMR15 or with either *Pseudomonas* strains MPS54, CP56 or CPS67 or coinoculation treatments of *Pseudomonas* with *Bradyrhizobium* resulted in significant increase in nodule number, nodule fresh weight and plant dry weight as compared to control uninoculated plants (Table 5). The coinoculation of *Pseudomonas* strain CP56, *Bradyrhizobium* strain and *R. solani* showed maximum 275.8% increase in plant dry weight in comparison to control uninoculated plants. Similarly, the coinoculation of *Pseudomonas* strain CPS67 with *Bradyrhizobium* strain and *R. solani* caused 180.1% increase in plant dry weight as compared to uninoculated control plants. Maximum increase in nodule number and nodule fresh weight was observed on coinoculation of *Bradyrhizobium* strain with *Pseudomonas* strains CPS67 or CP56. Single inoculation of different *Pseudomonas* cultures with *R. solani* caused only 33.4% disease control whereas coinoculation of *Bradyrhizobium* and *Pseudomonas* strains MP20 or CPS67 caused 66.7% disease control. There were no disease symptoms on green gram plants when coinoculated with *Bradyrhizobium* strain and *Pseudomonas* strain CP56, indicating that this bacterial strain completely controlled the root rot disease under pot house conditions.

DISCUSSION

The plant rhizosphere is an important ecological environment in the soil for plant-microbe interactions (Weyens *et al*., 2009; Tahat *et al*., 2010). The early root colonizing microorganisms, in

and around the growing roots, may interact with each other and with the plant in a way that may result in symbiotic, associative, neutralistic or detrimental effects (Vikram and Hamzehzarghani, 2008; Saber *et al*., 2009; Osman *et al*., 2011), depending upon the type of microorganisms involved, nutrient status in soil, abiotic and biotic soil environment and the plant defense system (Benizri *et al*., 2001). Fluorescent pseudomonads in the plant rhizosphere have been found to improve the plant growth through multitudinous factors viz. production of plant growth promoting substances (Ahmad *et al*., 2008; Sindhu *et al*., 2010), early colonization of root surfaces (Beniziri *et al*., 2001), secretions of vitamins (Derylo and Skorupsca, 1993) and through suppresion of plant diseases by production of antibiotics, siderophores, hydrolytic enzymes and HCN (Stockwell and Stack, 2007; Sindhu *et al*., 2009; Ahemad and Khan, 2011).

Both fluorescent and nonfluorescent *Pseudomonas* isolates were obtained in this study from the chickpea and green gram rhizosphere soil samples. Fifty-eight of the selected 65 Gram negative bacterial isolates showed oxidative metabolism, were Kovac's oxidase positive and belonged to *Pseudomonas*. Similarly, Gupta *et al*. (1998) isolated 121 rhizobacteria from the rhizotic zones of green gram using 7 selective and 4 non-selective media. Gram negative bacteria accounted for 65% and the dominant genera were *Pseudomonas*, *Bacillus*, *Enterobacter*, *Proteus* and *Klebsiella*. Similarly, *Pseudomonas* was reported as the most predominant genera (42%) followed by *Bacillus* (28%) and *Enterobacter* (21%) from a total of 105 bacteria isolated from rhizosphere and rhizoplane of groundnut (Baig *et al*., 2002).

Pseudomonas species have been found to produce different types of siderophores such as pyoverdines, pseudobactins, colourless nocardamine, pyochelin, salicyclic acid and cepabactin (Meyer and Abdallah, 1980; Verma and Chincholkar, 2007). In this study, out of 80 *Pseudomonas* strains screened for siderophore production, only 46.25% strains produced siderophore on MM9 medium and succinate medium (Table 1). Ksiezniak and Kobus (1993) tested 1653 microbial species including bacteria, actinomycetes and fungi for siderophore production and highest siderophore producing bacteria belonged to genera *Pseudomonas* and *Erwinia*. Similarly, siderophore production was observed in 74.2% of *Rhizobium*/*Bradyrhizobium* strains infecting pigeon pea, out of 31 strains tested (Duhan *et al*., 1998).

The diameter of halo zone varied with different *Pseudomonas* strains. Thirteen *Pseudomonas* strains MPS52, MPS54, MPS90, CPS59, PS31, PS39, CP20, CP28, CP56, MP14, MP20, MP33 and MP38 showed large halo zone (more than 10 mm) on MM9 medium. On iron-deficient succinate medium, only two *Pseudomonas* strains MPS52 and CP20 showed large halo zone formation (above 10 mm) (Table 1). Thus, it was found that siderophore production was more on MM9 medium as compared to succinate medium. On the contrary, maximum siderophore production (216.23 μg mL^{-1}) was reported in deferrated standard succinate medium (SSM) by *Pseudomonas* strain PRS9 after 72 h of incubation (Sharma and Johri, 2003a). Succinate was found better carbon source than citrate (SCM) media for siderophore production; however, defferation of media resulted in increased siderophore production in all the strains. Djibaoui and Bensoltane (2005) also reported that the ability of *Pseudomonas* to grow and produce siderophores is dependent on the iron content and the type of carbon sources in the medium. Highest siderophores concentration was obtained in succinate medium with *Pseudomonas fluorescens*. Alexander and Zuberer (1991) found that several *Pseudomonas* isolates obtained from grass species did not show siderophore on CAS agar but produced siderophore in liquid culture. Sharma and Johri (2003b) also reported low levels of siderophore production by *Pseudomonas* strains in complex media like King's B medium, trypticase soya medium and nutrient agar medium.

Growth inhibition zones of the three fungi namely, *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium aphanidermatum* varied with different *Pseudomonas* strains tested on PDA and NA medium plates. The *Pseudomonas* strains MPS54, MRS55, CP17 and CP56 showed maximum antagonistic activity against *F. oxysporum* and inhibition zone diameter was more than 6 mm (Table 3). *Pseudomonas* strains MRS23, MPS54, MRS55, CP17 and CP56 showed large growth inhibition zones (more than 4 mm) against *R. solani* on PDA and NA medium plates. Strains MPS52, MPS54, PS31, CP20 and MP38 showed maximum antagonistic activity against *P. aphanidermatum* on PDA medium plates. *Pseudomonas* strains that showed large inhibition zones against *P. aphanidermatum* were comparatively less inhibitory to *F. oxysporum* and *R. solani*. The diameter of inhibition zone was larger on NA medium than to PDA medium plates in majority of the strains. Similar effects of medium composition on inhibition of fungal growth were observed by Goel *et al.* (2000). They reported that *Pseudomonas* strain MRS16 inhibited growth of different pathogenic fungi (*Aspergillus* sp., *Fusarium oxysporum*, *Pythium aphanidermatum* and *Rhizoctonia solani*) *in vitro*. Larger inhibition zones were obtained on nutrient agar and King's B media compared to potato dextrose agar and pigment production media.

Becker and Cook (1988) observed zone of inhibition against *Pythium ultimum* var. *sporangiferrum* by pseudomonads isolated from roots of wheat. Siderophore-negative mutants of strain B324 were not inhibitory to *Pythium* indicating that fungal growth inhibition was due to production of siderophores by this strain. Inhibition of germination of chlamydospores of *Fusarium oxysporum* in soil was also correlated with production of siderophores by fluorescent pseudomanads (Simeon *et al.*, 1987). The inhibition was totally or partially reversed by adding Fe (III) to the system. Yeole *et al.* (2001) found that twelve fluorescent pseudomonad isolates obtained from chilli, cotton, groundnut and soybean inhibited the growth of 12 test soil borne plant pathogenic fungi in Fe-deficient King's B medium. The inhibition ranged from 3.3 to 15% and all *Pseudomonas* isolates produced siderophores under iron-deficient conditions. The inhibition was curtailed by 24-60% in the presence of iron (50 μm) when siderophore production was abolished. Screening of 563 bacteria obtained from the roots of pea, lentil and chickpea showed that 76% isolates produced siderophores, 5% isolates showed ACC deaminase activity and 7% isolates were capable of indole production (Hynes *et al.*, 2008). Twenty-six isolates (5%) suppressed the growth of *Pythium* species strain p88-p3, 7% suppressed the growth of *Fusarium avenaceum* and 9% suppressed the growth of *R. solani* CKP7. Ahmad *et al.* (2008) reported that siderophore production and antifungal activity was exhibited by 10 to 12.77% of *Azotobacter* and *Pseudomonas* isolates. *Pseudomonas* Ps5 and *Bacillus* B1 isolates showed broad-spectrum antifungal activity on Muller-Hinton medium against *Aspergillus*, *Fusarium* and *Rhizoctonia bataticola*.

Different microbial antagonists have been found to control the root rot disease caused by *Rhizoctonia solani* in different crops (Abeysinghe, 2009; Dar *et al.*, 2011; Osman *et al.*, 2011). In this study, inoculation of green gram with *R. solani* caused root rot disease in 83.7% of inoculated plants (Table 4) and the coinoculation of *Pseudomonas* strains with *R. solani* lowered the disease incidence. Disease reduction varied from 16.3% to 66.7% in coinoculated plants. At 60 days of plant growth, inoculation of different *Pseudomonas* cultures with *R. solani* caused only 33.4% disease control whereas coinoculation of *Bradyrhizobium* and *Pseudomonas* strains caused 66.7% (with MP20 and CPS67 strains) to 83.3% (with MPS54 strain) disease control. On coinoculation of *Bradyrhizobium* strain with *Pseudomonas* strain CP56, no disease symptoms were observed on green gram plants indicating that this bacterial strain completely controlled the root rot disease under pot house conditions. Similar effects of siderophore-producing bacteria on disease suppression

were reported on wilt disease caused by *F. oxysporum* (Duijff *et al.*, 1994) and *Pythium*-induced post emergence damping off disease in tomato (Buysens *et al.*, 1996). The observed antagonism and protection of plants was attributed to the siderophore-mediated iron competition. Kloepper *et al.* (1980) showed that inoculation with *Pseudomonas* strain B10 as a cell suspension and pure pseudobactin at 10 μm concentrations caused significant increases in plant growth compared with water treated controls. Boruah and Kumar (2002) reported that seed bacterization with *Pseudomonas* strains that displayed *in vitro* antibiosis against many pathogenic fungi, improved germination, shoot height, root length, fresh and dry mass, enhanced yield and chlorophyll content of leaves in the five test crop plants under field conditions. They reported that the plant growth promotion was due to siderophore production where disease suppression was due to the antibiotic substance. Similarly, Sharma and Johri (2003a) found that strains GRP3A and PRS9 which showed antagonistic activity against *Colletotrichum dematium*, *Rhizoctonia solani* and *Sclerotium rolfsii* showed significant increase in germination percentage and plant growth of maize seeds.

During these studies, inoculation with *Bradyrhizobium* strain SMR15 or with *Pseudomonas* strains MPS54, CP56 or CPS67 or coinoculation of *Pseudomonas* with *Bradyrhizobium* resulted in significant increase in nodule number, nodule fresh weight and plant dry weight as compared to control uninoculated plants at 30 and 60 days of plant growth (Table 4, 5). The coinoculation of fungi with *Pseudomonas* strain CP56 and *Bradyrhizobium* strain showed maximum enhancement in nodule number (1.93 times) and plant dry weight (4.4 times) in comparison to uninoculated control plants. The coinoculation of *Pseudomonas* strain CP56 with *Bradyrhizobium* strain and *R. solani* showed maximum 275.8% increase in plant dry weight whereas coinoculation of *Pseudomonas* strain CPS67 caused 180.1% increase in plant dry weight in comparison to control uninoculated plants. Similar enhancement of nodulation and nitrogen fixation after coinoculation of *Bradyrhizobium* and *Pseudomonas* were also observed in other legumes. For example, Bolton *et al.* (1990) studied coinoculation effect of *R. leguminosarum* and a toxin-producing *Pseudomonas* sp. in pea roots under Leonard jar conditions and found that *Pseudomonas* sp. increased the number of nodules formed by *R. leguminosarum* on pea roots. Mahmoud and Abd-Allah (2001) reported that siderophore producing *Penicillium chrysogenum* and *P. aeruginosa* significantly enhanced nodulation and nitrogen fixation of mungbean compared with plants infected with *Bradyrhizobium* strain alone. The enhancement of nodulation after coinoculation of *Bradyrhizobium* and *Pseudomonas* could be attributed to increased bradyrhizobial population and colonization of legume roots (Chebotar *et al.*, 2001), elongation and stimulation of rootlets (De Bellis and Ercolani, 2001) and enhancement of flavonoids in roots after application of *Pseudomonas* strains which act as inducer of nodulation genes (Peter and Verma, 1990).

CONCLUSION

Siderophore-producing *Pseudomonas* strains isolated from the rhizosphere soil of chickpea and green gram were found to inhibit the growth of wilt-causing fungi (*Fusarium oxysporum*), root rot-causing fungi (*Rhizoctonia solani*) and damping-off disease-causing fungi (*Pythium aphanidermatum*) on medium plates. Seed inoculation of antagonistic *Pseudomonas* cultures caused 33.4 to 100 per cent reduction of root rot disease in green gram under pot house conditions. Coinoculation of *Pseudomonas* strains with *Bradyrhizobium* strain SMR15 in green gram enhanced the nodule number, nodule fresh weight and plant dry weight as compared to *Bradyrhizobium*-inoculated or uninoculated control plants. The results suggested that microorganisms from the soil could replace the use of pesticides and nitrogenous fertilizers to reduce the pollution of soil and water.

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