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Effect of antagonistic Rhizobacteria coinoculated with *Mesorhizobium ciceris* on control of fusarium wilt in chickpea (*Cicer arietinum* L.)

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The antagonistic activity against *Fusarium oxysporum* f. sp. *ciceris* was determined for 40 chickpea rhizobacteria. Twenty eight isolates showed antagonistic activity against test fungus ranging from 18.2 to 41.8%. Characterization of the antagonistic attributes showed that all the antagonistic isolates produced diffusible and volatile antifungal metabolites in terms of growth inhibition, maximum being with the isolates 39P (77.8%) and 15B (64.2%), respectively. Nineteen of the isolates showed catechol and hydroxamate type siderophore production. All the isolates produced ammonia and twelve showed HCN production. On the basis of their antagonistic and PGP functionality traits, five isolates (2B, 7B, 28P, 34P and 38P) were selected for glass house studies on two chickpea varieties (JG-62 and GPF-2). Isolates 28P, 34P and 38P were found to be most promising for wilt control and plant growth promotion. Isolate 38P reduced the wilt incidence to 44.6% which was at par with fungicide treatment (55.5%) and had a significant edge over negative control (85%) in the chickpea variety JG-62. Similar trend of wilt incidence was observed in GPF-2 variety. Green house experiments on two varieties of chickpea JG-62 and GPF-2 showed that seed treatment with plant growth promoting rhizobacteria (PGPR) + *Mesorhizobia* had a synergistic effect in terms of disease control and growth promotion as compared to use of single bioinoculants, thus positively influencing plant microbe interaction.

Key words: Antagonism, chickpea, *Fusarium*, plant growth promoting rhizobacteria (PGPR), *Rhizobium*.

INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is a major constraint to chickpea (*Cicer arietinum* L.) cultivation throughout the world and especially in Indian subcontinent where chickpea is a commonly grown pulse crop. Presently, it occupies an area of 8 million hectare and its production is 7.1 million tons (Nikamet al., 2011). Despite the high total production, yields of chickpea are low due to attack of different diseases including fusarial wilts which can cause up to 100% yield losses annually (Pandeet al., 2010).

Plant growth promoting rhizobacteria (PGPR) have been proved as biocontrol agents of soil borne plant pathogens, offer an attractive alternative to chemical fertilizers, pesticides and supplements. Thus, the use of PGPR is steadily increasing in agriculture (Ashrafuzzaman et al., 2009). Plant growth promoting rhizobacteria are an heterogeneous group of bacteria that can be found in the rhizosphere at root surfaces and in association with roots which can improve the extent or quality of plant growth directly or indirectly (Joseph et al., 2007; Datta et al., 2011). The direct mechanism involves the N_2 fixation (Wani et al., 2007), solubilization of insoluble phosphorus (Khan et al., 2009), sequestering of iron by production of siderophore (Rajkumar et al., 2006), production of phytohormones such as, auxins, cytokinins and gibberellins (Godhino et al., 2010) and their transport to the developing plants or facilitating the uptake of nutrients from the recipient environment. The indirect mechanisms involve

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production of siderophores that chelate iron, making it unavailable to phytopathogens, antagonism by synthesis of volatile and diffusible antifungal metabolites such as phenazine and hydrogen cyanide, the ability to successfully compete with pathogens for nutrients and niches on the root to induce systemic resistance (Nelson, 2004; Saharan and Nehra, 2011).

Rhizobacteria are reported to play an important role in biocontrol via production of volatile antifungal compounds such as ammonia, aldehydes, alcohols, ketones and sulfides (El-Katatany et al., 2003). The production of diffusible antifungal metabolites with biocontrol activity is also reported. Phenazine is a diffusible potent green-pigmented antimicrobial metabolite implicated in antagonism (Tjeerdvan et al., 2004). A large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter* (Ahmad et al., 2008), *Bacillus* (Cakmakci et al., 2007; Ahmad et al., 2008), *Beijerinckia* (Thuler et al., 2003), *Burkhoderia* (Govindarajan et al., 2006), *Klebsiella* (Govindarajan et al., 2007), *Serratia* (Gyaneshweret al., 2001) have been reported to enhance plant growth, reduce pathogenic growth and disease development in various crops.

The objective of the present study was to isolate rhizobacteria from the chickpea rhizosphere with potent antagonistic activity against *F. oxysporum* f. sp. *ciceris* and to determine the ability of the selected bacterial antagonists alone and in combination with *Mesorhizobium ciceris to* suppress fusarium wilt under green house conditions.

MATERIALS AND METHODS

Isolation of rhizobacteria

The rhizobacteria were isolated from twenty different soil samples collected from different chickpea growing fields in Punjab, Uttar Pradesh and Palampur. All the microbial strains were isolated on their respective media; *Bacillus* and *Serratia* spp. on Nutrient agar, *Pseudomonas* spp. on King's B agar.

Characterization of rhizobacteria isolates

Selected bacterial isolates were characterized on the basis of Gram's reaction, catalase production, nitrate reduction, starch hydrolysis and methyl red test as per the standard methods (Cappuccino and Sherman, 1992).

Screening for antagonistic rhizobacteria against *F. oxysporum* sp.*ciceris*

Antagonistic activity

Antagonistic activity of the 48 bacterial isolates against *F. oxysporum*f. sp. *ciceris* (Department of Plant Breeding and Genetics PAU, Ludhiana, India) was evaluated based on dual culture technique (Lemessa and Zeller, 2007) and replicated thrice. Radial growth of the test fungus was measured and percentage growth inhibition was calculated using the formula:

Inhibition (%) = (R – r)/R×100

Where, r is the radius of the fungal colony opposite the bacterial colony and R, is the maximum radius of the fungal colony in the absence of the bacterial colony.

Inhibition of fungal mycelium proliferation

One milliliter of 24 h old bacterial culture and a 5 mm disc of test fungus were inoculated in 50 ml of potato dextrose media in 250 ml conical flasks at 25°C on a rotary shaker (three replication per isolate). Broth inoculated only with fungus served as control. The differences in dry weights between the fungus and the bacterium or the control cultures were recorded by passing 48 h grown dual cultures through preweighed filter paper. The filter papers were dried for 24 h at 70°C and weighed. The percent reduction in weight of the test fungus was calculated using the formula:

Reduction in weight (%) = (w1-w2)/ w1×100

Where, w1 represents the weight of the test fungus in control flasks and w2 with the bacterial antagonists (Trivedi and Pandey, 2006).

Elucidation of antagonistic traits

Volatile antifungal compounds

The production of volatile antifungal compounds by the isolates was assayed by a sealed plate method by Fiddman and Rossal (1993). On a lawn of test bacteria a second Petri dish containing PDA inoculated with a 6-mm plug of the test fungus was placed over the bacterial culture, each culture was replicated thrice. The two plates were sealed together with parafilm and further incubated at 25°C. Radial growth of the test fungus was measured over 24 h intervals for a period of 5 days.

Diffusible antifungal metabolites

Production of diffusible antifungal metabolites was assayed by the method of Montealegre et al. (2003). PDA plates covered with a cellophane membrane were overlaid with nutrient agar and inoculated with 100 µl of bacterial suspension. After incubation for 72 h at 28°C, the membrane along with the grown bacterial growth was removed and an 10 mm disc of a pure culture of *F. oxysporum* was placed in the centre of the plate and incubated at 28°C. The growth of the fungal culture was measured up to a week and compared with growth in the control.

Microbial siderophore production

Qualitative detection of siderophore (plate assay)

Siderophore production by bacterial isolates was detected by the universal method of Schwyn and Neilands (1987) using chrome azurol S (CAS). Cultures positive for siderophore produced an orange halo around the colony where siderophores had chelated iron that had been bound to the dye.

Detection of catechol and hydroxamate type siderophores

Catechol-type siderophores was detected and estimated in culture supernatant by Arnow (1937). Hydroxamate-type siderophores was detected and estimated in culture supernatant by Csaky (1948) assay.

Production of HCN

All the isolates were screened for the production of hydrogen

cyanide as per the method described by Bakker and Schippers (1987). Petri plates containing 10% trypticase soya agar supplemented with 4.4 g of glycine per litre were inoculated with the bacteria and inverted with a lid containing filter paper, impregnated with 0.5% picric acid and 2% sodium carbonate, over each Petri plate. The plates were incubated at 28°C for 3 to 5 days. A change in color from yellow to orange-brown on the filter paper indicated cyanide production.

Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water (Cappuccino and Sherman, 1992).

Evaluation of bioantagonistic potential under glass house condition

A bagculture experiment was conducted to study the influence of the 5 selected antagonists (on the basis of relative antagonistic traits) as seed treatment on seedling emergence, wilt control and growth of chickpea. The polyethylene bags (15 x 10 cm) were filled with sterilized soil inoculated with 1 g fresh weight of fungus at 100 g soil^{-1}. Two chickpea (desi) genotypes, JG-62 (susceptible to *Fusarium* wilt) and GPF-2 (wilt tolerant) (Department of Plant Breeding and Genetics PAU, Ludhiana, India) were selected. The seeds of both varieties of chickpea were surface sterilized with 0.1% mercuric chloride and rinsed thoroughly with sterilized water then dipped in the 5 PGPR cultures (10^6 ml $^{-1}$ broth) individually and in combination with *Mesohizobium* for half an hour before sowing of seeds. After germination and seedling emergence the bags were watered regularly to maintain optimum moisture and other routine care was taken during the experimentation. Each treatment was replicated 5 times with 10 seeds/ bag, seeds treated with fungicide captan@2 g/kg seeds were sown similarly and parameters for disease incidence in terms of wilting and yellowing of leaves and percentage wilt incident was calculated. The plant growth in terms of percent germination, plant height was recorded up to 60 days after sowing. The statistical analysis was done using CPCS1 software developed by Department of Mathematics, Statistics and Physics, PAU, Ludhiana.

RESULTS AND DISCUSSION

Isolation and biochemical characterization of rhizobacterial isolates

A total of 40 rhizobacterial isolates were isolated from chickpea rhizospheric soil from 20 different locations. Out of these 40 isolates, 22 were selected from Kings B medium and 20 of these showed yellowish green pigment whereas two of them showed blue green pigmentation, characteristic of *Pseudomonas* sp., 16 isolates selected from NA medium showed typical colony morphology which was predominantly off-white to creamish in colour, typical of genus *Bacillus* whereas two of the isolates produced pink color pigmentation with entire margin representing genus *Serratia.* The predominance of *Pseudomonas* and *Bacillus* sp. in legume rhizosphere has been reported by many workers. Parmar and Dadarwal (2001) reported that the fluorescent pseudomonads and spore forming *Bacillus* group predominantly colonise the rhizosphere of healthy chickpea plants. Yadav et al. (2010) reported that population of *Pseudomonas, Bacillus, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia and Serratia* dominated the chickpea rhizosphere. Joseph et al. (2007) in their studies for characterization of PGPR associated with chickpea reported that out of 150 isolates, 40 belonged to genus *Bacillus*, 35 to *Pseudomonas*, 40 to *Azotobacter* and 35 to *Rhizobium*. All the isolates were assessed for cultural, morphological and biochemical characteristics as per Bergey's manual of systematic bacteriology. Sixteen (40%) of the isolates were found to be Gram-positive, rod-shaped bacteria and were found to show profuse growth on NA media at 28°C. Most of the *Bacillus* isolates were positive for indole, Voges-Proskauer, catalase, nitrate reductase and citrate but were negative for methyl red test. Most of them were positive for starch hydrolysis. Twenty two (55%) of the isolates isolated from chickpea rhizosphere showed rapid growth on King's B media at 28°C in 48 h with production of fluorescent yellow to green pigment but two out of these cultures produced dark blue coloured pigment. All were Gram negative rods, indole, methyl red, Voges-Proskauer negative, citrate and catalase positive. They were also able to hydrolyze starch and reduce nitrates. Two of the isolates were Gram negative rod and showed a characteristic production of red coloured pigment on NA media. These pigment producing isolates were positive for Voges-Proskauer, citrate and catalase and nitrate reduction test and Negative for methyl red test (Table 3). On the basis of these tests, the isolates were tentatively placed into three genera: *Bacillus* (1B-16B), *Serratia* (17-S and 18S) and *Pseudomonas* (19P-40P).

In vitro screening for antagonistic potential

Out of the 40 isolates tested against *F. oxysporum* f. sp. *ciceris* in dual culture under *in vitro* conditions, fifteen isolates of *Pseudomonas* spp., eleven of *Bacillus* spp. and both the *Serratia* isolates showed antagonistic potential against *F. oxysporum*. However variation in inhibition potential was observed although inoculum load was same for all isolates. Out of positive antagonists 2B, 38P, 12B, 7B and 4B isolates showed maximum percent inhibition of test fungus (Figure 1).

As compared to the control, zone of inhibition was clearly visible at 5th day after incubation. The percent growth inhibition was found to range between 18.2-41.8% (Table 1). Kumar et al. (2010) reported that *Sinorhizobium fredii* KCC5 and *P. fluorescens* LPK2 inhibited the growth of plant pathogenic fungus *Fusarium udum* in dual culture and increase in fungal inhibition corresponded to incubation period. Strains KCC5 and LPK2 inhibited *F. udum* by 56 and 83% after 6 days of incubation. Similar findings recorded by Kaur et al. (2007), reported that 14 out of 96 *Pseudomonas* isolates from chickpea rhizosphere were highly antagonistic to *F. oxysporum* sp. Growth inhibition of *F. oxysporum* may be due to fungistatic effect or might be attributed to the secretion of antibiotics by the fungi or

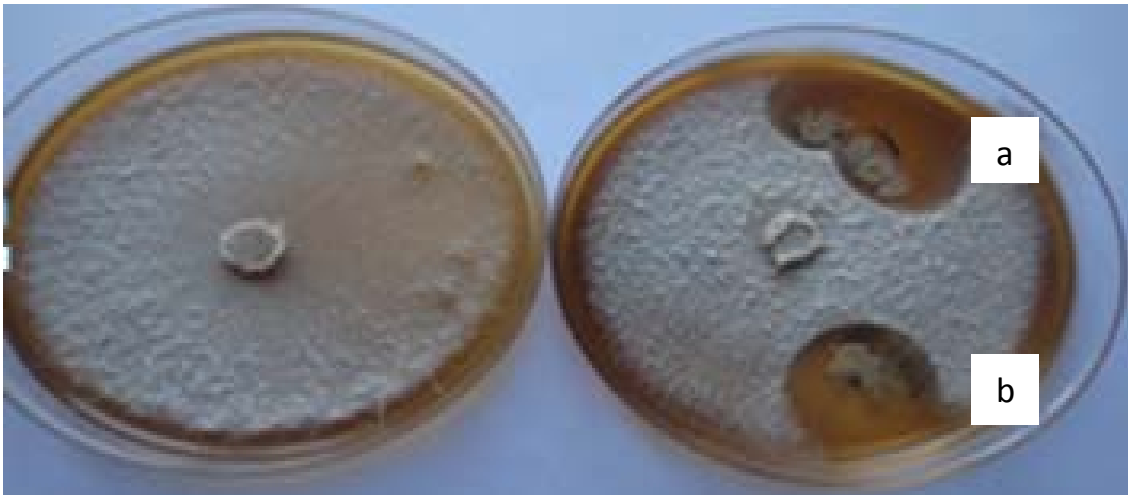


Figure 1. Growth inhibition of *F. oxysporum* by rhizobacterial isolates.

Table 1. Growth inhibition of *F. oxysporum* by rhizobacterial isolates.

Isolate	Growth Inhibition (%)	Isolate	Growth Inhibition (%)
Control	-	20P	18.2
2B	41.8	21P	23.6
4B	32.7	22P	18.2
5B	27.2	25P	23.6
6B	25.4	26P	21.8
7B	32.7	27P	27.3
8B	27.3	28P	30.9
12B	32.7	29P	27.3
13B	25.4	32P	23.6
14B	23.6	34P	32.7
15B	27.3	35P	23.6
16B	21.8	36P	20.0
17S	20.0	37P	23.6
18S	23.6	38P	38.2
-	-	39P	29.1

other inhibitory substances produced by the antagonists.

Quantitative evaluation of antagonism

The maximum percent biomass inhibition on dry weight basis was recorded after 5 days of incubation, 2B showed maximum inhibition (93.9%) followed by isolates 34P (84.4%), 28P (79.8%) and 20P (79.8%) (Table 2). Hassanein et al. (2009) reported 75% reduction in dry weight of *F. oxysporum* by *Pseudomonas aeruginosa.* Trivedi and Pandey (2006) also recorded 41% reduction in biomass of *F. oxysporum* by *Pseudomonas corrugata* in liquid media. All the isolates showed different ability to inhibit mycelial growth of the fungus and a notable reduction in mycelial biomass was observed as compared to the control. *In vitro* broth-based dual cultures offer a better method for evaluation of antagonistic efficiency of the biocontrol agents as the liquid medium provides a better environment to allow the antagonistic activities from all possible interacting sites.

Elucidation of antagonistic traits

All the 28 antagonistic rhizobacterial isolates were found to produce toxic volatiles. A reduction in the radial growth of the test fungus *F. oxysporum* sp. *ciceris* was observed after 120 h of incubation due to production of volatile antifungal compounds (Figure 2). However growth inhibition

Table 2. Inhibition in mycelial proliferation of *F. oxysporum* by rhizobacterial isolates.

Isolate	Reduction in dry weight (%)	Isolate	Reduction in dry weight (%)
Control	-	20P	74.8
2B	93.9	21P	64.8
4B	66.0	22P	69.9
5B	61.1	25P	15.4
6B	40.2	26P	66.0
7B	73.7	27P	69.2
8B	33.4	28P	79.8
12B	70.6	29P	72.4
13B	15.2	32P	29.6
14B	74.1	34P	84.4
15B	14.9	35P	83.7
16B	24.0	36P	30.6
17S	61.3	37P	26.6
18S	67.4	38P	74.1
-	-	39P	67.7

varied between 14.2-64.2% which was in accordance with findings of the dual culture studies where some isolates showed varying antagonistic potential. Maximum inhibition was observed in 15B isolate (64.2%) after 5 days of incubation (Table 3). Arafoui et al. (2006) reported that 8 of the 21 rhizobacterial isolates from chickpea rhziosphere significantly inhibited the growth of *F. oxysporum* sp. *ciceris* by producing volatiles. The production of volatile antifungal compounds by *Pseudomonas cepacia* and fluorescent pseudomonads has also been reported by other workers (Tripathi and Johri, 2002; Fernando et al., 2006).

Production of diffusible antifungal compounds

Diffusible antimetabolites are the non-volatile antibiotics produced by bacteria in low concentrations such as phenazine-1-carboxyclic acid, 2-hydroxyphenazines, phenazine-1-carboxamide (PCN), etc. and such compounds are implicated in disease suppression in plants (Kim et al., 2011). All the isolates produced toxic diffusibles. A notable reduction in the radial growth of *F. oxysporum* sp. *ciceris* was observed after 120 h of incubation due to diffusible antifungal metabolites (Figure 3). Growth inhibition varied between 22.2 - 77.8%, maximum being observed with 39P (77.8%) after 5 days of inciubation (Table 4). Thus, these isolates produced both volatiles as well as diffusible antifungal metabolites which are strongly implicated in antifungal activity. Tjeerdvan et al. (2004) in their studies reported that *P. fluorescens* produces a broad-spectrum antibiotic phenazine-carboxylic acid (PCA), which is active against a variety of fungal root pathogens.

Production of siderophore

Nineteen of the antagonistic rhizobacteria showed a distinct orange halo on CAS plates indicating siderophore production. Out of these eleven belonged to *Pseudomonas* spp., seven to *Bacillus* spp. and one belonged to *Serratia* spp. Siderophore production was found to start after 24 h of incubation, reaching a maximum after 72 h, when organism had entered into stationary phase. Maximum siderophore production was shown by 38P followed by 34P and 28P. Gupta and Goel (2002) reported similar results on CAS agar where diameter of halo ranged from 0.72-2.6 cm after 24-72 h of incubation. In a similar study conducted by Akhtar and Siddiqui (2009), siderophore production by *Pseudomonas* sp. isolated from chickpea rhizosphere was reported and the halos formed on CAS medium ranged from 1.6-1.7 cm. The results of these workers are in corroboration with the present study.

Out of the 19 isolates producing orange halo on CAS plates, 14 were found to produce catechol-type siderophore which ranged from 21.2-93.2 $\mu g.ml^{-1}$ (Table 5). Highest amount of catechol type siderophore was produced by 34P (93.2 $\mu g.ml^{-1}$) followed by 38P (92.4 $\mu g.ml^{-1}$), 7B (89.2 $\mu g.ml^{-1}$) and 28P (82.1$\mu g.ml^{-1}$). However, isolate 34P and 2B also produced higher amount of hydroxamate type siderophore (65.2µg.ml and 63.5$\mu g.ml^{-1}$), respectively whereas overall hydroxamate type siderophores were produced by only 12 isolates. Maximum production was recorded in isolate 26P (100.2$\mu g.ml^{-1}$) followed by 38P (70.7µg.ml-1), 34P (65.2 µg.ml-1) and 29P (63.5µg.ml-1) (Table 5). Only seven of the isolates produced both types of siderophores. In a study conducted by Siddiqui et al. (2007), it was found that *Pseudomonas putida* caused greater root colonization and greater siderophore production than the other species of PGPR. Sayyed et al. (2005) found that *P. putida* and *P. fluorescens* produced 83 and 87% units of siderophores in succinate media.

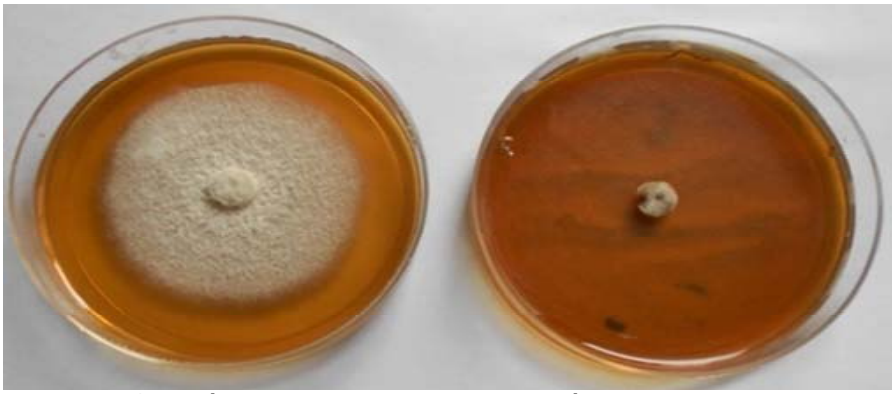


Figure 2. Antagonistic effect of volatile antifungal compounds on growth of *F. oxysporum*.

Table 3. Effect of volatile antifungal metabolites on growth of *F. oxysporum*.

Treatment	Growth inhibition (%)	Treatment	Growth inhibition (%)
Control	-	20P	42.8
2B	30.0	21P	20.0
4B	28.6	22P	31.4
5B	35.7	25P	28.5
6B	40.0	26P	38.5
7B	44.3	27P	27.1
8B	14.2	28P	42.8
12B	21.4	29P	35.7
13B	28.5	32P	34.2
14B	40.0	34P	50.0
15B	64.2	35P	28.5
16B	38.6	36P	30.0
17S	21.4	37P	25.7
18S	30.0	38P	35.7
-	-	39P	30.0

Figure 3. Antagonistic effect of diffusible antifungal compounds on growth of *F. oxysporum*.

Table 4. Effect of diffusible antifungal metabolites on growth of *Fusarium oxysporum*.

Isolate	Growth inhibition (%)	Isolate	Growth inhibition (%)
Control	-	20P	33.3
2B	22.2	21P	48.8
4B	37.8	22P	44.4
5B	37.8	25P	22.2
6B	31.1	26P	33.3
7B	24.4	27P	15.5
8B	37.8	28P	66.6
12B	31.1	29P	71.1
13B	28.9	32P	44.4
14B	37.8	34P	44.4
15B	42.2	35P	31.1
16B	33.3	36P	42.2
17S	42.2	37P	48.9
18S	48.9	38P	53.3
-	-	39P	77.8

Table 5. Production of catechol and hydroxamate-type siderophore by rhizobacterial isolates.

Isolate	Catechol type siderophore* (µg.ml^{-1})	Hydroxamate type siderophore* (µg.ml^{-1})
2B	73.6	28.1
4B	65.4	-
5B	21.2	-
7B	89.2	33.4
8B	32.7	40.6
12B	-	48.8
14B	-	51.9
18S	25.4	43.2
21P	49.4	-
22P	68.2	-
26P	-	100.2
28P	82.1	-
29P	-	63.5
34P	93.2	65.2
36P	59.5	-
37P	53.2	-
38P	92.4	70.7
39P	56.2	50.4
40P	-	46.5

Production of HCN

Out of 28 antagonistic bacterial isolates only twelve of the isolates were found positive for HCN production, of which seven belonged to *Pseudomonas* spp. and five belonged to *Bacillus* spp. Isolates 3B, 9B, 12B, 20P, 27P, 29P, 34P, 38P and 39P were found to be strong producer of HCN, causing colour change from yellow to reddish brown whereas 4B, 6B and 25P were moderate producers indicated by orange brown colour. Siddiqui and Shakeel (2009) reported HCN production by twenty-one *Pseudomonas* isolates from pigeonpea rhizosphere, out of which three were potent producers of HCN, whereas eleven were moderate producers. Saraf et al. (2008) iso-lated 10 strains of *Pseudomonas* from chickpea rhizo-sphere, out of which three produced HCN, the strongest producer being *Pseudomonas* M1P3.

Production of NH_3

All the antagonistic isolates were found to be ammonia

Table 6. Effect of bioantagonist on germination, plant growth and disease control under glass house conditions in chickpea variety (JG-62 and GPF-2).

Treatment	Seedling emergence (%)		Shoot length (cm)		Incidence of wilt (%)	
	JG-62	GPF-2	JG-62	GPF-2	JG-62	GPF-2
Negative control	66.6	69.0	85.0	14.5	85.0	74.4
Captan (fungicide)	75.0	68.3	55.5	16.4	55.5	33.7
2B	88.3	88.3	60.3	18.0	60.3	39.6
7B	88.3	83.3	56.6	18.4	56.6	40.0
28P	83.3	81.7	68.0	18.3	68.0	42.8
34P	81.6	90.0	47.7	19.0	47.7	40.7
38P	93.3	91.7	44.6	19.1	44.6	38.1
2B+*Rhizobium*	86.6	93.3	36.5	21.5	36.5	26.7
7B+*Rhizobium*	78.3	85.0	59.5	18.6	59.5	33.3
28P+*Rhizobium*	85.0	86.7	56.8	20.7	56.8	38.4
34P+*Rhizobium*	83.3	95.0	40.0	20.5	40.0	29.8
38P+*Rhizobium*	81.6	93.3	36.7	19.6	36.7	30.3
CD at 5%	1.32	1.10	1.68	1.26	1.68	1.56

producers. Out of these, 11 were *Bacillus* isolates, 15 were *Pseudomonas* isolates and both the *Serratia* isolates. A marked variation in the ability to produce ammonia was observed amongst the isolates as indicated by the intensity of the color developed. Isolates 17S, 18S, 21P, 32P, 34P, 37P, 38P and 39P were strong producers of ammonia. Ammonia production was detected in 95% of the isolates from the rhizosphere of rice influencing plant growth promotion (Samuel and Muthukkaruppan, 2011). Joseph et al. (2007) reported ammonia production by 95% of *Bacillus* isolates and 94.2% of *Pseudomonas.* Mishra et al. (2010) also reported that *B. subtilis MA-2* and *P. fluorescens* MA-4 were found to be efficient in ammonia production and significantly increased biomass of medicinal plant Geranium.

Evaluation of wilt control and plant growth promoting potential under glass house condition

Effect of rhizobacteria on seedling germination

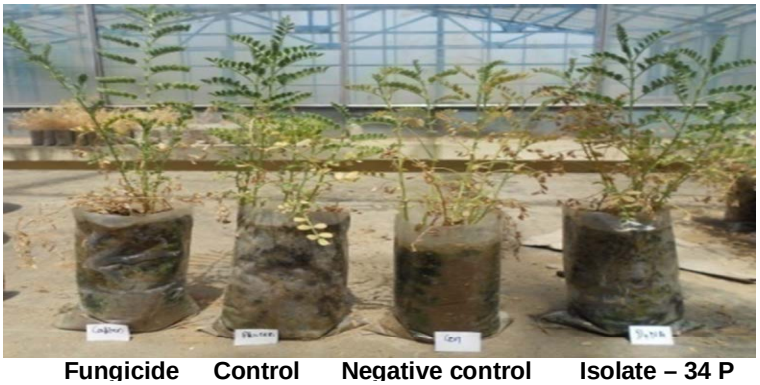
The effects of seed treatment with PGPR and PGPR + *Mesorhizobia* on two varieties of chickpea, JG-62 and GPF-2 revealed a synergistic interaction between the inoculants as evidenced by seedling emergence. An enhancement in percent germination was observed in treatments comprising of PGPR + *Mesorrhizobia* as compared to PGPR alone that also has shown some degree of enhancement in percent germination. Most of the rhizobacterial isolates enhanced seedling emergence, as compared to negative control as well as fungicide (captan) treatment. Maximum seedling emergence was recorded in 38P (93.3 and 91.7% in JG-62 and GPF-2 varieties, respectively). However, combination of bioantagonist with *rhizobia* further enhanced the seedling emergence in the present study (Table 6). Similar findings have been reported by Ashrafuzmann et al. (2009). Their studies also revealed that treatment with PGPR strains improved germination rate of the seeds as compared to negative control in the case of rice and maize, respectively. However, Hahm et al. (2012) reported 100% seed germination of pepper on treatment with PGPR culture alone. The increase in seed germination percentage may be due to modulation of hormone-linked phenomenon such as auxins and gibberellins production. Relative seedling emergence between different treatments and between both the varieties (GPF-2 and JG-62) has been shown in Figure 4 and Figure 7 respectively..

Effect of rhizobacteria on incidence of wilt

The pot studies, with 5 bioantagonists 2B, 7B, 28P, 34P and 38P exhibited similar results as *in vitro* tests, providing further confirmation of the efficacy of these isolates in suppressing wilt in chickpea. Symptoms of wilting observed were dry, brown and drooped plantlets whereas non-wilted plants were fresh and green. Previous studies also have reported antagonistic activity of *Pseudomonas* sp. against *Fusarium* sp. (Leon et al., 2009). The degree of disease suppression was significantly increased where dual culture treatment was used. However, overall more wilting was observed in susceptible variety JG-62 than resistant variety GPF-2. Hahm et al. (2012) also reported that the disease protection conferred by mixture of strains was not significantly different than that with the single strains. In both varieties (GPF-2 and JG-62), maximum reduction in wilt incidence was shown by 38P followed by 34P and their combination with *Rhizobium* as compared to fungicide (Table 6, Figure 5).

Variety JG-62 exhibited 85% wilt incidence in the negative control. However, seed treatment with fungicide

Figure 4. Relative seed emergence between different treatments.



Fungicide Control Negative control Isolate – 34 P

Figure 5. Relative symptoms of wilting between different treatments.

reduced the disease severity to 55.5% (Table 6). Treatment with bioantagonist 38P showed least disease incidence (44.6%) followed by 34P (47.7%), 7B (56.6%), 2B (60.3%) and 28P (68%). Isolates 2B (60.3%) and 28P (68%) showed disease severity as compared to other 3 isolates 38P, 34P and 7 B. Comparatively lesser disease severity was observed in the variety GPF-2 (Table 6).

However in variety GPF-2, least disease incidence was scored by 38P (38.1%) followed by 2B (39.6%) and 7B (40%). In both varieties (GPF-2 and JG-62), further reduction in wilt incidence was recorded in the case of co-inoculation with PGPR and *Rhizobium* due to their synergistic nitrogen fixation and antagonistic effect. Rhizobacterial isolates 38P, 34P and 7B exhibited maximum reduction of *Fusarium* wilt and this could be attributed to their ability to produce volatile and diffusible antifungal metabolites, phenolic compounds, cyanides and siderophores.

Effect of rhizobacteria on plant growth

Chickpea variety JG-62 treated with only PGPRs showed taller plants in the case of 4 isolates, that is, 38P (19.2 cm), 34P (18.8 cm), 2B (18.2 cm) and 28P (18.1 cm) which was at par with the plant height recorded with fungicide treatment but significantly higher than negative control (15 cm) (Table 6 and Figure 6). Co-inoculation of PGPR and *Rhizobium* resulted in further significant increase in shoot length as *Rhizobia* are reported to produce plant growth regulators such as auxins, cytokinins and gibberellins like substances that stimulate and enhance plant growth (Hemissi et al., 2011). Synergistic effect in the use of dual cultures is well documented (Hahm et al., 2012). Comparatively, in GPF-2 variety also, all the five PGPR, that is. 38P (19.1 cm), 34P (19 cm), 7B (18.4 cm), 28P (18.3cm) and 2B (18 cm) enhanced shoot length significantly, maximum being with 38P (19.1 cm) as compared to fungicide treatment (16.4 cm) and negative control (14.5 cm) (Table 6). In both varieties, that is, GPF-2 and JG-62, further increase in shoot length was recorded in the case of co-inoculation with PGPR and *rhizobia* as compared to PGPR isolates alone (Table 6) and in both varieties, rhizobial isolates 38P, 34P and 28P exhibited maximum enhancement of shoot length and this could be attributed to their ability to produce siderophore that help the plants not only to sequester iron from soil but also inhibit the fungal pathogen and thus enhance

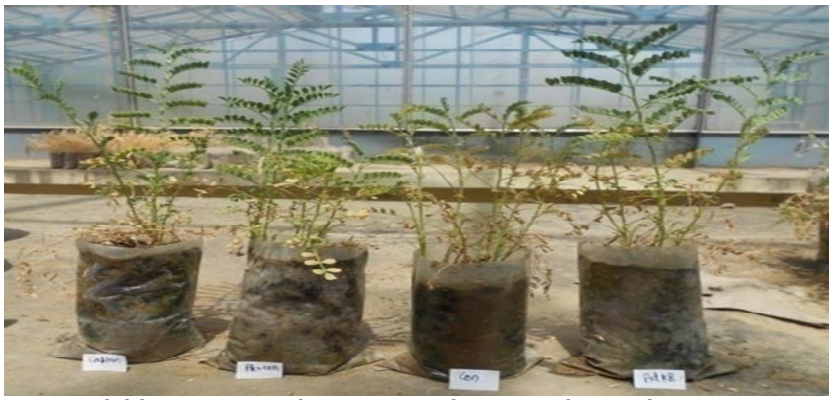


Figure 6. Relative growth between different treatments (GPF-2).

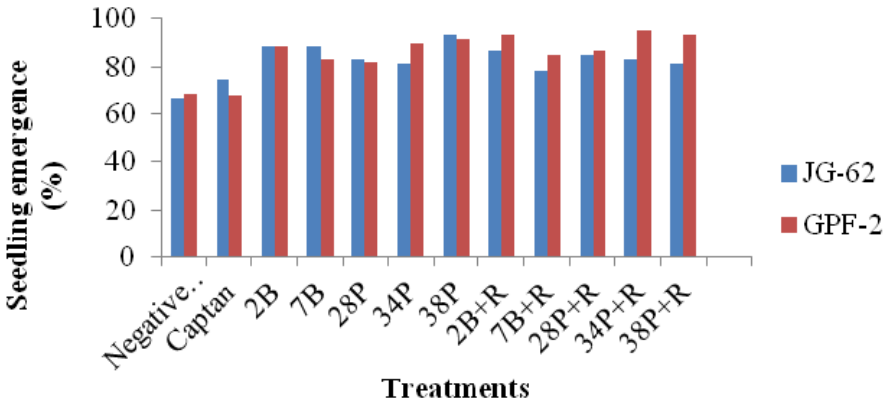


Figure 7. Relative seed emergence between different treatments in JG-62 and GPF-2 variety.

the growth of plants significantly. A relative comparison of plant growth is depicted in Plate 10. Hahm et al. (2012) also reported in his studies that PGPR treatments resulted in the taller peppers as compared to negative control treatments.

It has been reported that even if mixtures of PGPR strains do not always results in additive or synergistic effects of possible advantage, different strains may have different mechanisms and their combinations may provide a spectrum of activity which may have a beneficial effect on plant health and productivity (Raupach and Kloepper, 2000).

This study provided an initial assessment of the potential of rhizosphere bacteria associated with chickpea in Punjab, Uttar Pradesh and Palampur to control wilt of chickpea and promote plant growth individually and in combination with *Rhizobium* under controlled conditions in glass house. Three isolates, that is, 38P, 34P and 28P appeared to be the most promising biological control agents against chickpea wilt. But further study of these antagonists under field condition is needed for the more evident confirmation of their antagonistic trait against fusarium wilt in chickpea.

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