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Biological Control of Fusarium Wilt in Chickpea by Co-Inoculation of Antagonistic Plant Growth Promoting Rhizobacteria and *Mesorhizobium*

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The antagonistic activity of twenty five chickpea rhizobacteria were evaluated against *Fusarium oxysporum* f. sp. *ciceris* as potential biocontrol agents. Eleven potential bioantagonists, 7 belonging to *Pseudomonas* and 4 to *Bacillus* genera showed significant growth inhibition of test fungus. *In vitro* screening for antagonistic functionality traits showed significant difference between production of volatile and diffusible antifungal metabolites. Maximum fungal inhibition due to diffusible metabolites was exhibited by isolate 24P(87%) whereas it was (47%) in case of volatile metabolites. Inhibition due to diffusible metabolites was maximum with isolate 24P (87%) than in case of volatile metabolites as 24P (47%). Isolates 24P, 30B and 5P were also most promising for plant growth stimulation and wilt control. Greenhouse experiments on two varieties of chickpea JG-62 and GPF-2 showed that seed treatment with PGPR+*Mesorhizobium* had an advantage on disease control and plant growth promotion as compared to use of single bioinoculants, 30B reduced the wilt incidence to 48% which was at par to fungicide treatment where the observed wilt incidence was 55.6% whereas, in the pathogen control the severity of wilt was 82.4%. Same trend of wilt incidence was followed in GPF-2 variety. Rhizobacterial isolates 30B and 24P, co-inoculated with *Mesohizobium* enhanced the shoot length up to 16.2 cm and 15.6 cm as compared to fungicide treatment (14.4 cm), thus showing a plant growth stimulation effect of potential bioantagonists, isolated from chickpea rhizosphere.

Key words: Antagonism, Chickpea, *Fusarium*, PGPR and *Mesorhizobium*.

Application of plant growth promoting rhizobacteria as biocontrol agents of soil borne phytopathogens, has been steadily increasing in agriculture and offers an attractive alternative to chemical fertilizers, pesticides, and supplements (Ashrafuzzaman *et al.* 2009[1]). PGPR strains facilitate biocontrol and growth of plants by varied mechanisms. The direct mechanism of plant growth promotion involves the N_2 fixation (Wani *et al.* 2007[2]), solubilization of insoluble phosphorus (Khan *et al.* 2009[3]), sequestering of iron by siderophore (Rajkumar *et al.* 2006[4]), production of phytohormones such as, auxins, cytokinins and gibberellins, lowering of ethylene concentration (Liu *et al.* 2007[5]) and their transport to the developing plants or facilitating the uptake of nutrients from the recipient environment. The indirect mechanisms involves production of siderophore that chelate iron, making it unavailable to phytopathogens, antagonism by synthesis of volatile and diffusible antifungal metabolites such as phenazine and hydrogen cyanide (HCN) (Nelson 2004[6]). Rhizobacteria are reported to play an important role in biocontrol via production of volatile antifungal compounds such as ammonia, aldehydes, alcohals, ketones and sulfides (El-Katatany *et al.* 2003[7]) and via production of

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diffusible antifungal metabolite, Phenazine is a potent green-pigmented antimicrobial metabolite implicated in antagonism (Tjeerdvan *et al.* 2004[8]). Several bacterial strains such as *Bacillus*, *Pseudomonas* and the *Rhizobium* group are found to effectively control various soil-borne plant pathogenic fungi under green house and field conditions (Nelson 2004[6]). PGPR also mediate biological control indirectly by eliciting induced systemic resistance against a number of plant diseases (Jetiyanon and Kloepper 2002[9]).

Chickpea (*Cicer arietinum* L.) is an annual legume which is most effected by *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *ciceris*. PGPR strains particularly those belong to genera *Bacillus, Pseudomonas, Rhizobium, Azospirillum, Azotobacter* and *Serratia* have been reported to enhance plant growth, reduce pathogenic growth and disease development in various crops.

The research presented here investigated biocontrol activity and plant growth promotion of bacterial strains in which three *Pseudomonas* strains (5P, 21P and 24P) and two *Bacillus* strains (30B and 39B) were evaluated under greenhouse conditions, against control for determining the efficacy of suppression of *Fusarium* wilt. The specific objectives of the study was to evaluate the antagonistic potential of selected PGPRs alone and in combination with *Mesorhizobium* against Fusarium wilt and effect on plant growth under glass house conditions.

MATERIAL AND METHODS

Isolation of rhizobacterial and Biochemical characterization of rhizobacterial

A total of 25 isolates of rhizobacteria were isolated from soil samples taken from different locations. Out of these 16 isolates were selected from Kings B medium and 9 isolates from Nutrient Agar (King's *et al* 1954)[10]. Sixteen of the isolates from Kings B medium showed the characteristic fluorescent yellow green pigmentation, whereas the other nine isolates showed typical colony morphology which was predominantly off-white to creamish in colour. On the basis of cultural, morphological and biochemical appearance, these were tentatively assigned to genera *Pseudomonas* and *Bacillus* (Table 1). Biochemical characterization of these rhizobacterial isolates were conducted as per the standard methods (Cappuccino and Sherman 1992)[11].

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

Antagonistic activity

Antagonistic activity of the bacterial isolates against *Fusarium oxysporum* was evaluated based on dual culture technique (Lemessa and Zeller 2007[12]). Radial growth of the test fungus was measured and percentage growth inhibition was calculated using the formula:

$$\% \text{ Inhibition} = (R - r)\ D\ R \times 100$$

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

Fungal mycelium Proliferation inhibition

One ml 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 25p C on a rotary shaker. 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 70p C and weighed. The percent reduction in weight of the test fungus was calculated using formula:

$$\% \text{ reduction in weight} = (w1\text{-}w2)/\ w1 \times 100$$

Where, w1 represents the weight of the test fungus in control flasks and w2 with the bacterial antagonists.

Production of volatile antifungal compounds

The production of volatile antifungal compounds by the isolates was assayed by a sealed plate method by Fiddman and Rossal (1993)[13]. 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. The two plates were sealed together with parafilm and further incubated at 25p C and radial growth of the test fungus measured after 5 days.

Production of Diffusible antimetabolites

Production of diffusible antimetabolites was assayed by method of Montealegre *et al.* (2003)[14]. PDA plates covered with a cellophane membrane were overlaid with nutrient agar and inoculated with 100 µl of bacterial suspension. After incubation for 72 hrs at 28ÚC, the membrane along with the bacterial growth was removed and a 10

mm disc of *F. oxysporum* was placed in the centre of the plate and incubated at 28°C.

Evaluation of bioantagonistic potential under glass house condition

A pot culture experiment was conducted to study the influence of the selected antagonists as seed treatment on seedling emergence, growth and wilt control of chickpea. On the basis of the studies PGPR were selected for pot studies. Two genotype JG-62 (susceptible to *Fusarium* wilt) and GPF-2 (wilt tolerant) were selected. The seeds of both varieties of chickpea were dipped in PGPR cultures individually and in combination with *Mesorhizobium* for half an hour before sowing of seeds. After germination and seedling emergence the pots were watered regularly to maintain optimum moisture and other routine care was taken during the experimentation. Each treatment was replicated 5 times and parameters on plant growth in terms of percent germination, plant height and disease incidence in terms of wilting and yellowing of leaves were recorded upto 60 days after sowing.

RESULTS AND DISCUSSION

Twenty five PGPRs were isolated from chickpea rhizosphere and characterized as belonging to genera *Bacillus*[9] and *Pseudomonas*[16]. (Table 2). Evaluation of the antagonistic potential of these 25 isolates against *Fusarium oxysporum* sp. *ciceris* in dual culture conditions showed that 11 cultures effectively inhibited the growth of test fungus although variations in inhibiting effect was observed, out of positive antagonists 24P, 30B and 66B isolates showed maximum percent inhibition as these antagonists are major producers of inhibitory compounds. The growth of the fungus ranged from 3.9-4.7 cm (dia) in dual culture as

Table 1. Cultural, morphological and biochemical characteristics of rhizobacterial isolates

Characteristic of test organism	*Pseudomonas*	*Bacillus*
Gram's reaction	-ve	+ve
Shape	Rods	Rods
Pigment	+	-
Pigment colour	Flourescent green	White
Starch hydrolysis	+	-
Catalase production	+	+
Methyl red test	+	+
Citrate	-	-
Nitrate production	+	+

Table 2. Occurrence of rhizobacteria in Chickpea rhizosphere

Type of bacteria	No. of isolates
Pseudomonas	16
Bacillus	9

Table 3. Growth inhibition of *Fusarium oxysporum* in dual culture and biomass Inhibition in liquid medium by rhizobacterial isolates

Rhizobacterial Isolates	Dual culture antagonistic activity		Fungal mycelium inhibition in liquid media		
	Pathogen growth (cm)	Percent growth Inhibition (%)	Fungal biomass (g)	Fungal biomass with antagonist (g)	Reduction in dry weight (%)
Control	5.5	-	3.418	0.304	-
5P	4.2	23.7	2.758	0.089	70.7
12P	4.7	14.5	3.422	0.233	23.35
16P	4.6	16.4	1.916	0.129	56.6
17P	4.6	16.4	2.146	0.161	47.0
21P	4.1	25.5	3.267	0.208	31.6
22P	4.8	12.8	2.955	0.128	57.9
24P	3.8	30.9	1.605	0.056	81.6
18B	4.5	18.2	3.247	0.215	29.7
30B	4.4	29.1	3.287	0.120	60.5
39B	4.3	21.8	3.322	0.181	40.4
66B	3.9	29.1	1.698	0.078	74.3

compared to 5.5 cm in the control (Fig.1). Thus the potential antagonists were able to inhibit the growth of *Fusarium oxysporum* sp. *ciceris* by 0.8-1.6 cm as compared to control, zone of inhibition was clearly visible at 5th day after incubation. The percent growth inhibition was found to range between 12.8-30.9% (Table3). Growth inhibition of *Fusarium oxysporum* may be due to fungistatic effect or might be attributed to the secretion of antibiotics by the fungi or other inhibitory substances produced by the antagonists.

Quantitative evaluation of antagonism

In studies regarding fungal mycelial proliferation inhibitors the maximum percent biomass inhibition on dry weight basis was recorded after 5 days of incubation, 24P showed maximum inhibition (81.6%) followed by isolates 66B (74.3%), 5P (70.7%) and 30B (60.5%) (Table 3). All the isolates showed different capability 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. Similar studies were observed by Hassanein *et al* (2009)[15] in liquid media where 75%

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

Treatments	Volatile antifungal metabolites		Diffusible antifungal metabolites	
	Fungal growth (dia in cm)	Growth inhibition (%)	Fungal growth (dia in cm)	Growth inhibition (%)
5P	3.65	39.1	2.4	40
12P	3.45	42.5	2.7	32
16P	3.75	37.0	2.8	30
17P	3.60	40.0	2.6	35
18B	3.90	35.0	1.8	55
21P	3.30	45.0	0.8	80
22P	3.45	42.5	2.7	32
24P	3.35	44.1	0.5	87
30B	3.80	35.8	0.5	87
65B	3.70	38.3	2.3	42
66B	3.45	42.5	2.3	42
Control	6.00	_	4.0	_

Table 5. Effect of bioantagonists on germination, plant growth and disease control under glass conditions (JG-62)

Rhizobacterial Isolates	Seedling emergence (%)	Shoot length (cm)	Incidence of Wilt (%)
5P	76	13.8	62
21P	72	11.2	83
39B	68	11.7	85
24P	70	14.6	56
30B	72	15.3	53
5P + *Mesorhizobium*	78	15.1	64
21P + *Mesorhizobium*	72	14.9	82
39P + *Mesorhizobium*	72	14.8	76
24P + *Mesorhizobium*	78	15.6	59
30B + *Mesorhizobium*	74	16.2	48
Captan(fungicide)	72	14.4	55.6
Negative control(Pathogen)	68	10.4	82.4
Control	84	11.7	17.6
CD at 5%	NS	0.9	22.1

reduction in dry weight of *Fusarium oxysporum* by *Pseudomonas aeruginosa*.

Production of volatile antifungal metabolites

All the 11 isolates were found to produce toxic volatiles as was evident by reduction in the radial growth of the test fungus *Fusarium oxysporum* sp. *ciceris* (Fig.2). Growth inhibition ranged between 35-45% (Table 4) which was in accordance with findings of the dual culture studies where some isolates showed higher antagonistic potential due to production of volatile compounds such as HCN and ammonia. HCN acts as inhibitor of fungal growth.

Production of diffusible antifungal compounds

Similar trend was exhibited by the 11 potential antagonists, in terms of production of toxic diffusibles. Growth inhibition ranged between 30-87% (Fig.2). Fungal growth inhibition is maximum with diffusible metabolites as phenazine derivatives suach as phenazine-1-carboxyclic acid (PCA), 2- hydroxy phenazines and phenazine-1-carboxamide (PCN) are the metabolites which are responsible for fungal growth inhibition at lower concentrations. Maximum percentage of inhibition was observed in 24P and 30B (87%) after 5 days of inciubation (Table 4). Thus these isolates produced

Table 6. Effect of bioantagonists on germination, plant growth and disease control under glass house conditions GPF-2

Rhizobacterial Isolates	Seedling emergence (%)	Shoot length (cm)	Incidence of Wilt (%)
5P	68	15.4	21
21P	64	14.8	19
39B	74	16.6	33
24P	66	18.2	16
30B	66	18.3	13
5P + *Mesorhizobium*	70	16.7	15
21P + *Mesorhizobium*	68	16.8	14
39P + *Mesorhizobium*	70	17.1	31
24P + *Mesorhizobium*	88	18.2	13
30B + *Mesorhizobium*	76	18.6	13
Captan(fungicide)	64	12.2	19.8
Negative control (Pathogen)	70	12.9	31.2
Control	92	13.9	6.9
CD at 5%	17.1	1.0	NS

volatiles as well as diffusible antifungal metabolites which are known to play a major role in antagonism. Sharma and Parihar (2010)[16] reported in their investigation, the ability of extracellular antifungal metabolites of *Actinomycetes* against *Rhizopus stolonifer, Aspergillus flavus*, *F. oxysporum* and *Alternaria* sp.

Evaluation of bioantagonistic potential under glass house condition

Effect of rhizobacteria on seedling germination

The effects of seed treatment with PGPR and PGPR+ *Mesorhizobium* on two varieties of chickpea JG-62 and GPF-2 revealed that percent seedling emergence was enhanced in all the treatments. Most of the rhizobacterial isolates enhanced seedling emergence, compared to negative control as well as Captan treatment (Table 5). However, combination of bioantagonists with *Rhizobium* significantly enhanced the seedling emergence as compared to PGPR alone and were at par to the fungicide treatment. The increase in seed germination percentage may be due to modulation of hormone-linked phenomenon such as auxins and gibberellins production. Mia *et al* (2012)[17] reported in his studies that increament of seed germination percentage and seedling shoot length were considered typical gibberellins-like responses. They mimic the effect of exogenous GA3 application. Initially, inoculated plants showed higher emergence which might be due to the production of phytohormone as phytohormone influences seed germination.

Effect of rhizobacteria on plant growth

Chickpea variety JG-62 treated with only PGPR showed taller plants in case of 3 isolates i.e. 5P (13.8cm), 24P (14.6 cm) and 30B (15.3 cm) which

was at par to the plant height recorded with fungicide treatment but significantly higher than negative control (Table 5). Comparatively, in GPF-2 variety all the five PGPR, enhanced shoot length significantly maximum being with 30B (18.3 cm) as compared to its fungicide treatment (12.2 cm) and negative control (12.9 cm). Co-inoculation of PGPR and *Mesorhizobium* resulted in further significant increase in shoot length, that indicated a synergism between *Rhizobium* and the co-inoculant which may have asserted a cumulative effect. Synergistic effect in use of dual cultures is well documented. Rhizobia are reported to produce plant growth regulators such as auxins, cytokinins and gibberellins like substances that stimulate and enhance plant growth (Hahm *et al* 2012)[18]. Pathak

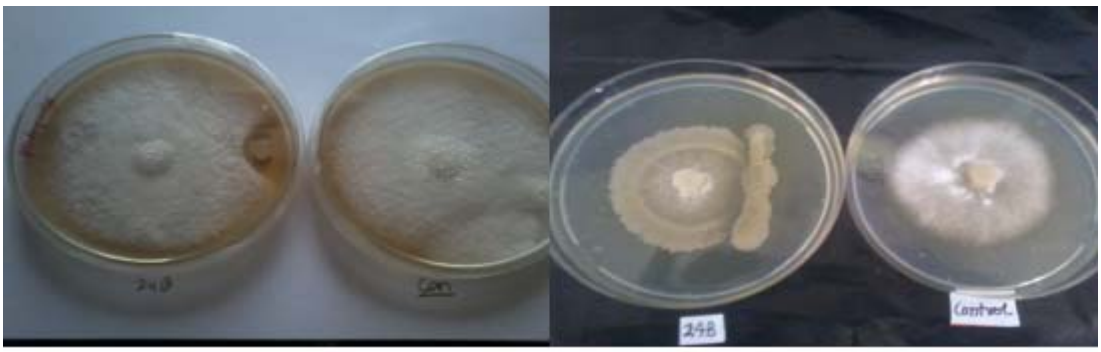

Fig. 1. Growth inhibition of *Fusarium oxysporum* by rhizobacterial isolates

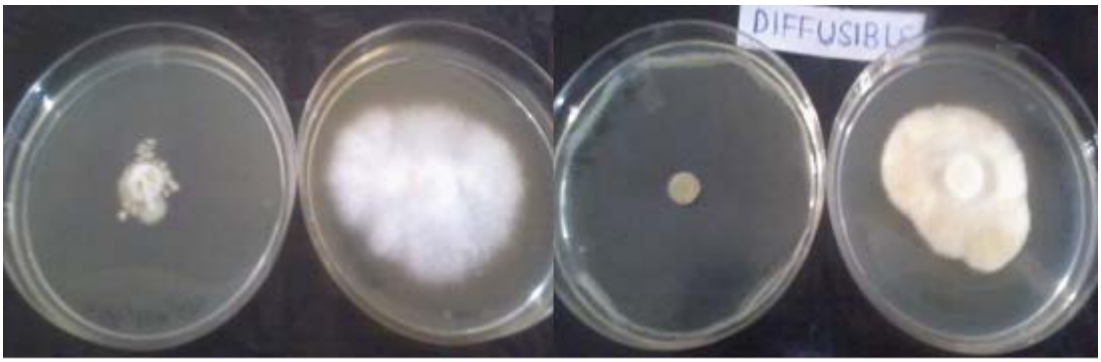

Fig.2. Antagonistic effect of volatile and diffusible antifungal compounds on the growth of *Fusarium oxysporum*

Fig. 3. Relative seed emergence between different treatments

et al (2001)[19] reported that seed inoculation with *Rhizobium* may have significantly increased the growth and yield of legume crops as it provided more nutrition to the plant.

Effect of rhizobacteria on percentage incidence of wilt

The pot studies, with 3 bioantagonists 5P, 24P and 30B exhibited similar results as *in vitro* tests, providing confirmation for the efficacy of these isolates in suppressing wilt in chickpea. Variety JG-62 exhibited 82.4% wilt incidence in the negative control as this variety is susceptible to disease (Fig. 3). However, seed treatment with fungicide reduced the disease severity to 55.6% (Table 5). Treatment with bioantagonist 30B showed least disease incidence (53%) followed by 24P (56%) and 5P (62%) which is due to the production of antifungal metabolites that inhibits fungus to grow. Isolates 39B and 21P showed disease severity comparable to the negative control. Comparatively lesser disease severity was observed in the variety GPF-2 (Table 6). Here too, least disease incidence was scored by 30B followed by 24P and 5P. Leon *et al* (2009)[20] reported similar trend of antagonistic activity among *Pseudomonas* sp. against *Pythium* sp. In variety GPF-2, a slight reduction in wilt incidence was recorded in the case of co-inoculation with PGPR and *Mesorhizobium*. Rhizobacterial isolates 5P, 24P and 30B exhibited maximum reduction of *Fusarium* wilt and this could be attributed to their ability to produce volatile and diffusible antifungal metabolites or due to the cumulative effect of different functionality traits. The degree of disease suppression did not increase significantly where dual culture treatment was used. Similar observations have been reported by Hahm *et al* (2012)[18].

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