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Biological control of Fusarium wilt of chickpea through seed treatment with the commercial formulation of *Trichoderma harzianum* and/or *Pseudomonas fluorescens*

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Summary. The effect of treating seed of chickpea (*Cicer arietinum* L.) cv. BG 256 with commercial formulations (2 g kg^{-1} seed) of *Trichoderma harzianum* and *Pseudomonas fluorescens,* singly and jointly, to control wilt caused by *Fusarium oxysporum* f. sp. *ciceri* was examined in chickpea plants growing in microplots under field conditions. On untreated control plants, the wilt fungus caused the characteristic symptoms of wilt and significantly (P=0.05) decreased dry weight and the yield of chickpea by 20 and 18% respectively (significant at P=0.05). On chickpea without wilt, treatment with *P. fluorescens* improved the yield by 36% and *T. harzianum+P. fluorescens* by 25%. Both biofungicides suppressed wilt severity (P=0.05), the most effective being *T. harzianum+P. fluorescens* (66%). Carbendazim reduced wilt severity by 51%. On chickpea inoculated with the wilt, yield increased by 39% with *P. fluorescens*, by 33% with *T. harzianum+P. fluorescens*, by 44% with *T. harzianum,* and by 20% with carbendazim as compared with the inoculated control. The soil population of the wilt fungus (cfu g^{-1} soil) in untreated plots increased during the first two months (P=0.05), but in the biofungicide/fungicide treated plots it gradually and significantly (P=0.05) decreased during the four months of the crop season. The greatest decrease in the soil population of the wilt fungus occurred with *T. harzianum* or *T. harzianum+P. fluorescens*, followed by *P. fluorescens* and carbendazim. The rhizosphere population of the bioagents increased significantly in those plots where wilt populations decreased. The greatest increase in the population of the bioagents was recorded for *T. harzianum* (108–120%), followed by *P. fluorescens* (65–119%) in the combined treatment, compared with the pre-plant control (December). When the bioagents were applied alone, the population of *T. harzianum* increased by 71–96% and that of *P. fluorescens* was by 46–103%.

Key words: antagonist, plant growth promoter, *Fusarium oxysporum* f. sp. *ciceri,* gram yield, rhizosphere population.

Introduction

Chickpea (*Cicer arietinum* L.) is an important pulse crop and India ranks first in the world in terms of the acreage cultivated with this crop (6.82 million ha) and the annual yield (3.87 million tons) (Venkataramani, 2002). Chickpea is frequently attacked by a wilt caused by *Fusarium oxysporum* f. sp. *ciceri* which is worldwide in distribution (Woltz and Jones, 1981). The fungus causes severe yield loss in chickpea: in India it is 10–15%, which in years of severe epidemics may rise to 60–70% (Jalali and Chand, 1992). Although several measures including chemical treatments are taken by growers, control is not satisfactory. Biological control is a potential alternative to chemical fungicides (Parker *et al*., 1985). Mycoparasites (Papavizas, 1985) and many bacteria (Weller, 1988) have shown promising results in managing phytopathogenic fun-

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gi. *P. fluorescens* has revolutionised the field of biological control of soil-borne plant pathogenic fungi (Burr *et al.,* 1998). That bacterium produces phenazin (Toohey *et al.*, 1965; Gurusiddaiah *et al.,* 1986), pyrolnintrin (Burkhead and Geoghegan, 1994), phloroglucinol (Howell and Stipanovic, 1980) and siderophores (Sakthivel *et al.,* 1986), which may be involved in the suppression of the wilt fungus (Fridlender *et al.,* 1993; Gamliel and Katan, 1993). Leeman *et al.* (1995) reported satisfactory control of Fusarium wilt of radish by treating the seed with *P. fluorescens*.. In addition, *P. fluorescens* produces auxins, gibberellins etc. (Glick, 1995) and solubilises phosphorus in the soil (Dube and Yeole, 1997), which helps plant growth. Among mycoparasites, the genus *Trichoderma* includes the most widely used biocontrol agent of soil-borne, seed-borne and other diseases (Chet *et al.,* 1979; Chet and Baker, 1981). *Trichoderma harzianum* and *T. virens* are active rhizosphere colonisers (Tronsmo and Harman, 1992) that produce antibiotics such as gliotoxin, viridin, and some cell wall degrading enzymes (Larito *et al.*, 1976; Bello *et al.,* 1997) and also certain biologically active heat-stable metabolites such as ethyl acetate (Claydown *et al.*, 1987). These substances may be involved in disease suppression or plant growth promotion. A field trial was conducted to examine the effectiveness of treating chickpea seed with recently developed commercial formulations of *T. harzianum, P. fluorescens,* and *T. harzianum+P. fluorescens* to control wilt caused in the plant by *F. oxysporum* f. sp. *ciceri*. Effects on biomass production and yield were also determined. The chemical fungicide carbendazim was used to compare the efficacy of the biofungicides.

Materials and methods

Pure culture of the wilt fungus *F. oxysporum* (f. sp. *ciceri*) was obtained from I.A.R.I., New Delhi, India. The fungus was mass cultured on sorghum seeds. The seeds were soaked overnight in a 5% sucrose and 30 mg l^{-1} chloramphenicol solution. The soaked seeds were transferred to 500 ml conical flasks and autoclaved twice at 15 Kg cm^{-2}, 121°C, for 15–20 minutes. Thereafter, the flasks were inoculated with pure culture of *F. oxysporum* f. sp. *ciceri* and incubated at 27±2°C for 8–10 days in an incubator. For soil inoculation, fungus colonised seeds (532 g) were ground in a mixer-grinder and suspended in 10 l tap water. The suspension (10 l) was spread uniformly on a microplot of 3×2 m to achieve an inoculum level of 1.5 g colonised seeds kg^{-1} soil. Soil inoculation was done two days before seed was sown. The biofungicides were based on *P. fluorescens* (MB-07) (Threvesan) Migula and *T. harzianum* (TO-14) Rifai and were prepared for the DBT project (Khan, 2003). Ingredients of these biopesticides are not described here to allow a patent to be applied for. The cfu load of *P. fluorescens* and *T. harzianum* in the biofungicides was $80\times10^{13}$and 11×10^{8} g^{-1} formulation respectively. For seed treatment, the formulations were applied at the rate of 2 g kg^{-1} seed along with the commercial rhizobium strain of chickpea (*Bradyrhizobium japonicum* Jordan). To compare the effectiveness of the biofungicides, treatment with an effective fungicide, carbendazim, was continued at 2 g kg^{-1} seed.

A field of 25×15 m was prepared in which 30 microplots (3×2 m) were demarcated by 25 cm wide, raised margins. Ten treatments were given, as shown in Table 1. Each treatment was applied on three microplots, distributed in a completely randomised block design in the field. Seeds of chickpea cv. BG 256 were sown in three rows (57 seeds/row) in the microplots, irrespective of whether the wilt fungus was added into the soil. The field was irrigated a week after sowing. At maturity, four months after sowing, twenty-five plants from each microplot were uprooted to determine dry-matter production and grain yield. Two-month-old plants were randomly uprooted from each microplot (10 plants/microplot) to count the root nodules. Pink and healthy nodules were counted as functional nodules, dark-brown and degenerated ones as nonfunctional nodules. Wilt incidence (%) and wilt severity were recorded on two and a half month old plants. Wilt severity (%) was scored on a 0–5 scale where: 0, no wilt; 1, 1–20%; 2, 21–40%; 3, 41–60%; 4, 61–80%; and 5, 81–100%. The rhizosphere population of the wilt fungus and the bioagents was estimated monthly from December to April using the dilution plate method. The formae specialis *F. oxysporum* f. sp. *ciceri* was identified on the basis of colony and conidial characteristics (Jalali and Chand, 1992).

Wilt incidence and severity were expressed according to the following formulae:

$$\text{Wilt incidence (\%)} = \frac{\text{No. of wilted plants in a microplot} \times 100}{\text{Total No. of plants in a microplot}}$$

$$\text{Wilt severity (\%)} = \frac{\text{No. of wilted branches in a plant} \times 100}{\text{Total No. of branches in a plant}}$$

The experiment was conducted for two consecutive growing seasons (2001–2002, 2002–2003) under identical conditions. The results between years were statistically similar. Hence only the data of the current year are presented. Observations from the twenty-five plants of each microplot were averaged and considered one replicate. Since three microplots were used for each treatment, there were therefore three replicates. Data on plant growth and yield were subjected to two-factor analysis of variance. Pathogens were considered one factor, and biofungicide treatments as the second factor. The data on wilt incidence and soil population were analyzed for single-factor ANOVA. Wilt incidence was angularly transformed before analysis. Least significance difference (LSD) was calculated at P=0.05 for all variables to compare individual treatments.

Results and discussion

Plants grown in plots soil-inoculated with *F. oxysporum* f. sp. *ciceri* showed the characteristic symptoms of Fusarium wilt with a wilt severity score of 3.7 and a wilt incidence of 62% (Table 1). Wilt appeared when plants were around 45 days old. Wilted leaves were mildly chlorotic and later turned brown (Woltz and Jones, 1981). Quite a few twigs/branches wilted around a month before maturity. Application of biofungicides decreased wilt severity to a varying extent (significant at P=0.05) (Table 1). In the plots where seed-treatment with

Table 1. Effect of seed treatment with biofungicides on dry matter, yield, nodulation, wilt incidence and severity of chickpea plants inoculated with *Fusarium oxysporum* f. sp. *ciceri* or not inoculated.

Treatment	Plant dry weight (g)	Yield/plant (g)	Nodules/root system		Wilt incidence (%)	Wilt severity (0–5 scale)
			Functional	Non functional		
Control	21.9	7.2	35	14	-	-
T. harzianum (Th)	25.9[a]	8.1[a]	46.7[a]	13	-	-
P. fluorescens (Pf)	30.5[a]	9.8[a]	49.0[a]	11.1[a]	-	-
Th + Pf	28.8[a]	9.0[a]	49.3[a]	10.8[a]	-	-
Carbendazim	23.7	7.6	29.0[a]	14.7[a]	-	-
Fusarium (FOC)	17.5[a]	5.0[a]	26.1[a]	12.2	61.8 (51.8)	3.7
FOC + Th	23.2	8.5[a]	30.3[a]	14.8[a]	24.9 (29.9)[a]	1.5
FOC + Pf	24.7[a]	9.0[a]	35.2[a]	12.8	27.1 (31.3)[a]	2.0
FOC + Th + Pf	24.3[a]	6.7[a]	35.9[a]	13.8	12.5 (20.7)[a]	1.2
FOC+ Carbendazim	21.3	7.1	29.7[a]	15.0[a]	36.4 (37.1)[a]	1.8
LSD (P=0.05)	1.9	0.63	3.7	1.2	2.3	
F-value						
Fusarium (df=1)	42[b]	30[b]	272[b]	40.5[b]		
Bioagent (df=4)	239[b]	131[b]	106[b]	21.5[b]		
Combined (df=4)	NS	10[b]	31[b]	NS		
Treatment(df=4)					897[b]	

Each value is mean of three replicates; values in parenthesis are angular transformed values.
[a] Significantly different from the respective control at P=0.05.
[b] Significant at P=0.05.
NS, not significant at P=0.05.

T. harzianum was given, the wilt severity score was 1.5; in plots with *P. fluorescens* or carbendazim it was 2.0 and 1.8 respectively. The lowest wilt severity score, 1.2, was recorded with *T. harzianum+P. fluorescens*. Biofungicides also influenced wilt incidence (Table 1). Treatment with only *P. fluorescens* or only *T. harzianum* reduced wilt incidence to 25–27%. In the plots with carbendazim seed-treatment, wilt incidence was 36%. A combination of the biofungicides decreased wilt incidence still further. The lowest number of plants with wilt symptoms (12–13%) was in plots with *T. harzianum+P. fluorescens* (Table 1).

T. harzianum, or *P. fluorescens*, or these two agents in combination significantly (P=0.05) increased the dry weight of chickpea (Table 1)**.** The greatest increase with a single biofungicide, 39%, was recorded with *P. fluorescens,* as compared with the uninoculated control. Combined treatment with *P. fluorescens+T. harzianum* led to a 32% increase in dry weight. Seed inoculation with *F. oxysporum* f. sp. *ciceri* caused a 20% decrease in dry weight (P=0.05). Application of the biofungicides together increased the suppressive effect of the pathogenic fungus, leading to a significant increase in the dry weight of the chickpea plants (Table 1). Application of *P. fluorescens* or of *T. harzianum* singly and of *P. fluorescens* and *T. harzianum* in combination increased dry matter production by 41, 33 and 39% respectively compared with the wilt-inoculated control (significant at P=0.05). Seed-treatment with carbendazim resulted in a 22% increase in the dry weight of fungus inoculated plants (Table 1).

Yield of chickpea, whether inoculated with the wilt fungus or not, was significantly (P=0.05) greater if the biofungicides were applied, singly or jointly (Table 1). The greatest increase in the yield of uninoculated plants was with *P. fluorescens* (36%), followed by *T. harzianum+P. fluorescens* (25%) and *T. harzianum* (13%), compared with the uninoculated control. The increase in the dry weight and yield of uninoculated plants treated with *P. fluorescens* was apparently due to the plant growth promoting activity of the biofungicide (Khan and Akram, 2000). This plant growth promoting effect of *P. fluorescens* may be due to the solubilisation of phosphorus in the soil (Dube and Yeole, 1997), or to auxins, gibberellins etc., produced by the fungus (Glick, 1995). *T. harzianum* also solubilises phosphorus (Sharma, 2003) and stimulates plant growth even in the absence of the pathogen (Windham *et al.,* 1986). That there is a significant decline in the yield of chickpea infected with *F. oxysporum* f. sp. *ciceri* has already been reported in India (Jalali and Chand, 1992). The micro-organisms applied antagonised *F. oxysporum* f. sp. *ciceri* and suppressed disease severity to a great extent.

Infection with *F. oxysporum* f. sp. *ciceri* suppressed the rhizobial nodules on the roots of chickpea plants (Table 1). A lower root mass due to wilt infection appeared to be largely responsible for this suppression of nodulation. The bioagents significantly (P=0.05) increased the number of functional nodules. Rhizobacteria (*Pseudomonas* spp.) and mycoparasites (*Trichoderma* spp.) are known to synergise *Bradyrhizobium* spp. (Khan *et al.,* 1998, Prabakaran, 1998). The number of functional nodules increased by 33% with *T. harzianum* and by 40% with *P. fluorescens* (Table 1). Treatments with both bioagents combined increased the functional nodule count by 41%. Carbendazim caused a significant (P=0.05) decline in the number of functional nodules. Seed treatment with *T. harzianum+P. fluorescens, P. fluorescens* and *T. harzianum* increased nodulation on the plants inoculated with the wilt fungus by 38, 35 and 16% respectively (Table 1). Carbendazim significantly (P=0.05) increased the number of functional nodules (14%) but the increase was much less than with *P. fluorescens,* alone or in combination with *T. harzianum*.

The rhizosphere population of the wilt fungus in the control plots was significantly (P=0.05) lower in January (18.2%), but increased by 25–34% as the season progressed, in comparison with the pre-plant rhizosphere population (December) (Table 2). Seed treatment with the bioagents or the fungicide significantly (P=0.05) decreased this population. *T. harzianum* caused a drastic decrease in the rhizosphere population of *F. oxysporum* f. sp. *ciceri* compared with the control, and *T. harzianum+P. fluorescens* decreased this population by 87–97%. Carbendazim decreased the rhizosphere population by 31–51% (Table 2). The rhizosphere population of *T. harzianum* increased by 71–96% during the experiment as compared with the pre-plant population, while the increase in the rhizosphere population of *P. fluorescens* was 46–103% during the experiment, except in January (Table 2). In the presence of *T. harzianum* this population increased further. In plots where *F. oxysporum* f. sp. *ciceri*

Table 2. Effect of seed treatment of biofungicides on the rhizosphere population of *Fusarium oxysporum* f. sp. *ciceri*, *Trichoderma harzianum* and *Pseudomonas fluorescens*.

Treatment	*Fusarium oxysporum* f. sp. *ciceri* (FOC) $\times 10^5$ cfu g^{-1} soil					LSD (P=0.05)	*Trichoderma harzianum* (Th) $\times 10^5$ cfu g^{-1} soil					LSD (P=0.05)	*Pseudomonas fluorescens* (Pf) $\times 10^7$ cfu g^{-1} soil					LSD (P=0.05)
	Dec.	Jan.	Feb.	Mar.	Apr.		Dec.	Jan.	Feb.	Mar.	Apr		Dec.	Jan.	Feb.	Mar.	Apr.	
Th	-	-	-	-	-		8.3	14.2[a]	14.9[a]	15.9[a]	16.3[a]	1.2	-	-	-	-		-
Pf	-	-	-	-	-		-	-	-	-	-		26	25	38[a]	45[a]	49[a]	3.2
Th + Pf	-	-	-	-	-		8.3	12.1[ab]	13.2[ab]	13.5[ab]	14.3[ab]	1.0	26	47[ab]	54[ab]	58[ab]	65[ab]	4.5
FOC	22	18[a]	27.4[a]	35.2[a]	29.5[a]	2.3	-	-	-	-	-		-	-	-	-		-
FOC + Th	22	6.2[ab]	3.2[ab]	1.9[ab]	1.2[ab]	0.6	8.3	12.9[ab]	13.5[ab]	15.8[ab]	17.2[ab]	1.0	-	-	-	-		-
FOC + Pf	22	7.2[ab]	4.9[ab]	5.2[ab]	2.9[ab]	0.7	-	-	-	-	-		26	38[ab]	45[ab]	52[ab]	53[a]	3.5
FOC+ Th + Pf	22	2.3[ab]	1.5[ab]	1.2[ab]	1.0[ab]	0.5	8.3	17.3[ab]	19.5[ab]	15.6[ab]	18.3[ab]	1.2	26	43[ab]	56[ab]	55[ab]	57[ab]	4.0
LSD (P=0.05)			0.8	1.0	1.0			0.8	1.1	1.1	1.1		1.6	3.2	5	3.9	5.6	

[a] Significantly different from the preplant (December) population at P=0.05.
[b] Significantly different from the data of the corresponding control month.

was inoculated, the increase in the population of *T. harzianum* or *P. fluorescens* was significantly greater than when no wilt fungus was present. The decrease with *T. harzianum* was significantly (P=0.05) greater than that with *P. fluorescens*. This indicates that a rhizosphere rich in spores/propagules of the wilt fungus is a better substrate for the multiplication of *T. harzianum*.

The study demonstrated that the biofungicides used controlled Fusarium wilt satisfactorily. *Pseudomonas* reduced wilt severity less than *Trichoderma* but the increase in yield enhancement was greater with the latter.

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