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Biological Control of *Fusarium* Wilt Disease of Pigeonpea

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Biological control of *Fusarium udum* causing wilt disease of pigeonpea was studied *in vitro,* as well as, *in vivo. Aspergillus flavus, Aspergillus niger, Bacillus licheniformis* (strain-2042), *Gliocladium virens, Penicillium citrinum,* and *Trichoderma harzianum,* which were found to be the most potent ones in inhibiting the radial colony growth of the test pathogen, were used as biological control by amending their inocula at different concentrations in pots and in pathogen-infested soil in the fields. Maximum reduction of the wilt disease was observed with *G. virens* both in pots and in the fields. The population of *F. udum* was found to be markedly reduced when the antagonists were applied in the soil. The study establishes that *G. virens* can be exploited for the biological control of wilt disease at field level.

Keywords : antagonists, biocontrol, pigeonpea, wilt disease.

Pigeonpea (*Cajanus cajan* L. Millsp.) is an important pulse crop cultivated in the tropics and sub-tropics. Crop yield is significantly reduced due to wilt disease caused by *Fusarium udum* Butler, with an estimated yield losses of US$36 million in India and $5 million in eastern Africa (Kannaiyan et al., 1984). Like any other soil-borne diseases, the wilt disease of pigeonpea is difficult to control. Some pesticides and chemicals have been recommended for the management of the disease, but none have been proven to give the desired success in controlling the disease (Singh, 1998). Pesticides are reported to cause adverse effects on treated soil ecosystem because of their non-biodegradable nature and also because they induce resistance in pathogens (Cohen and Leavy, 1990).

Biological pesticides have the potential to replace or augment conventional plant disease management which makes use of synthetic pesticides. Several studies have demonstrated reduced incidence of diseases in different crops after supplementing the soils with fungal or bacterial antagonists (Mukhopadhyay, 1987; Pusey, 1989; Smith et al., 1990; Bashar and Rai, 1994). This study was undertaken to assess the efficacy of certain biocontrol agents for the control of wilt disease of pigeonpea.

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Material and Methods

Isolation and *in vitro* screening of biocontrol agents. Dominant rhizosphere fungi of pigeonpea, namely, *Aspergillus flavus, Aspergillus luchuensis, Aspergillus niger, Aspergillus terreus, Alternaria alternata, Cladosporium cladosporioides, Penicillium citrinum, Gliocladium virens,* and *Trichoderma harzianum* were isolated from pigeonpea grown in the research farms of Banaras Hindu University, Varanasi, India where plants have been severely affected by the wilt disease caused by *Fusarium udum.* The research farms are situated at 25°18'N latitude and 85°1'E longitude. These dominant rhizosphere fungi were isolated by soil plate methods as described by Dhingra and Sinclair (1995) using Martin's agar medium. *Trichoderma* spp. and *Gliocladium virens* were, however, isolated on selective media of Elad and Chet (1983). A few bacteria, namely, *Bacillus licheniformis* (strains-2042 and 2044) and *Bacillus thuringiensis* (RSU-13) and two *Bacillus* spp. (RSU-62 and RSU-46) were obtained from Dr. R.S. Upadhyay of the Banaras Hindu University.

The rizosphere fungi and bacteria were screened for their antagonistic potential against *F. udum* by the method described by Upathyay and Rai (1987). Three blocks (5 mm each) of individual fungi, cut from the actively growing margins of a 5-day old culture, were inoculated separately in 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth. After 10 days of inoculation at 25±2°C, the culture filtrates were filtered first through Whatman filter paper no. 44 and then through a Seitz filter (G5) by vacuum pressure to obtain cell free culture filtrate. The metabolites of bacteria were obtained by inoculating a loopful of bacterial cells in nutrient broth and by incubating at 37±2°C for 10 days. After the incubation, the filtrates were obtained following the above method used for fungi. Four ml culture filtrate was poured on 16 ml autoclaved and cooled (40°C) potato dextrose agar (PDA) medium for fungi and on Thornton's agar medium for bacteria. The Petri plates containing the medium and culture filtrate were inoculated centrally with 5 mm agar block of 5-day-old culture of the pathogen. The experiment was performed in three replicates for each type of culture filtrate. For the control set, the same quantity of the sterilized and cooled distilled water was added on 16 ml of PDA medium. The radial colony growth was measured after 7 days of incubation. The percent inhibition was calculated by using the formula:

Percent growth inhibition = (C–T)/C × 100
Where, C = Growth in control; T = Growth in treatment.

Glasshouse experiments. Mass culture of the antagonists and the pathogen was prepared on wheat grains following the method of Singh et al. (1996). Sufficient soil samples from the pigeonpea field were collected and brought to the laboratory. The soil was air dried at 30°C for 24 h and then ground in a mechanical grinder and sieved through a 2-mm pore sieve. The soil was placed in containers and then autoclaved at 121°C for 30 minutes. The unsterilized and sterilized soil samples were well mixed separately with 1% (w/w) pure inoculum of the pathogen. The pathogen-infested soil was placed in plastic pots (25×20 cm) and kept at room temperature for 1 week to allow the pathogen to establish well in the soil.

The pure inocula [1, 2, and 3% (w/w)] of *A. flavus*, *A. niger*, *B. licheniformis* (strain-2042), *G. virens*, *P. citrinum*, and *T. harzianum* were mixed separately with the pathogen-infested soils. The pots containing the soil-pathogen inoculum mixture without the antagonists served as control. Four replicates were maintained for each inoculum concentration of the antagonists for both sets. Seeds of a susceptible variety of pigeonpea (var. Bahar) were surface sterilized with sodium hypochlorite for 1 minute and washed several times with tap water to remove traces of NaOCl, then the seeds were sown in soil at the rate of 10 seeds per pot. The soil moisture (20-25%) was maintained by adding sterilized distilled water from time to time. Disease development was observed regularly. However, the final record of the incidence of wilting in plants was done at 45 days after sowing. The percent seedling mortality and percent disease control were calculated using the following formula:

Percent mortality = (No. of seedlings in uninfested pot soil – No. of seedlings in infested pot soil)/No. of seedlings in infested pot soil × 100

Percent disease control = (Mortality (%) in check – Mortality (%) in treatment)/Mortality (%) in check × 100

To study the population of the test pathogen, rhizosphere soils samples were collected in sterilized polyethylene bags from pigeonpea plants treated with each antagonist and from the control set in triplicate. The population of the pathogen was estimated by soil plate method as described by Dhingra and Sinclair (1995). One g rhizospheric soil of pigeonpea from each pot was placed separately into 250 ml Erlenmeyer flask containing 100 ml sterilized distilled water. The flask was shaken on an electrical shaker (120 throws min^{-1} and 1.5 cm displacement per throw) to get a homogenous suspension and, thereafter, 1:10,000 (for fungi) and 1:100,000 (for bacteria) dilutions were prepared in sterilized distilled water. One ml aliquot of soil suspension was inoculated separately on melted and cooled Martin's and Thornton's agar media for fungi and bacteria, respectively. The experiment was done in three replicates for each sample and dilution. The plates were incubated at 25±2°C for the fungi and 37±2°C for the bacteria, and the colony forming units were recorded after 7 days of inoculation.

Field applications. To perform the experiment, a field 20×20 m^2 in size was selected in the Botanical Garden of the Banaras Hindu University where pigeonpea crop was regularly grown and the soil was already infested with *F. udum*. Sixteen plots 2×1.8 m^2 in size were made for four antagonists which were found to be potent against the test pathogen during glasshouse experiments. Each plot was infested with the pathogen by amending 150 g/m^2 of the inoculum on wheat grains, and leaving the plots for 20 days with light watering to establish the pathogen in the plots. One hundred fifty g of mass inoculum of individual antagonists was amended in the plots, then the plots were left as such for 10 days with sufficient soil moisture. The seeds of pigeonpea were sown in each plot at the rate of 40 seeds per plot. However, after germination, only 20 plants per plot were allowed to grow. The infested plot without the inoculation of the antagonists served as control. The plots were arranged in randomized block design with three replicates per treatment. The percent mortality and percent disease control were calculated by using the formula mentioned earlier after 60 days of sowing when the plants showed complete symptom of wilting.

Statistical analyses. The data from all the tests were analyzed statistically by applying Analysis of Variance (ANOVA) and Duncan's New Multiple Range Test (DNMRT) as described by Goon et al. (1986).

Results and Discussion

The culture filtrates of the test fungi and bacteria inhibited the radial colony growth of *F. udum* at varying degrees (Table 1). The effectiveness of the culture filtrates in inhibiting the pathogen was characterized as poor (<25%), moderate (25-49%), and high (>50%). The maximum inhibition (69%) was recorded in *G. virens*, *A. flavus*, and *B. licheniformis* (strain-2042). *P. citrinum* and *T. harzianum* also showed marked inhibition against the test pathogen (50.6-60.6%).

The growth inhibition of *F. udum* by culture filtrates of the test fungi is possibly attributed to the secretion of antibiotics by the fungi (Upadhyay & Rai, 1987) or other inhibitory substances produced by the antagonists such as geodin, terricin, terric acid, aspergillic acid, dermadin, etc. (Brian et al., 1945). The degree of effectiveness varies according to the nature, quality, and quantity of antibiotics/inhibitory substances secreted by the antagonists (Dennis and Webster, 1971a, 1971b; Skidmore and Dickinson, 1976). Wong and Hughes (1986) also recorded the inhibitory potential of *B. licheniformis* against plant pathogens. Weller (1988) reported that the inhibitory potential of bacteria is due to production of antibiotics, siderophores, and hydrogen cyanide. The inhibition of *F. udum* by culture filtrates of the test bacteria might be due to the production of such substances in the medium as reported by other workers in *Pseudomonas* spp. (Gurusiddaiah et al., 1986; Baker & Schippers, 1987; Jayaswal et al., 1993).

Table 1. Effect of culture filtrates of the isolated antagonistic fungi and bacteria on the radial colony growth of *Fusarium udum*

Antagonist	Isolate No.	Inhibition of *F. udum*[a] (%)
Fungi		
Aspergillus flavus	R-031	60.6x[b]
A. luchuensis	R-12	33.3s
A. niger	R-102	66.6y
A. terreus	R-21	38.8t
Alternaria alternata	R-49	29.7r
Cladosporium cladosporioides	R-5	28.8r
Gliocladium virens	R-32	69.4z
Penicillium citrinum	BR-14	50.6v
Rhizoctonia solani	BR-29	41.1u
Trichoderma harzianum	BK-021	55.5w
T. viride	BK-8	32.2s
Bacteria		
Bacillus licheniformis	2042	55.7v
Bacillus licheniformis	2044	16.0r
Bacillus thruingenises	RSU-13	18.8s
Bacillus sp. (isolate-I)	RSU-62	20.0t
Bacillus sp. (isolate-II)	RSU-46	26.0u

[a] Values represent mean of three replicates.
[b] Data followed by different letters in the column are significantly different (P=0.05) as per Duncan's new multiple range test (DNMRT). DNMRT has been applied separately for fungi and bacteria.

On the basis of screening results *in vitro*, when the selected antagonists were amended in unsterilized and sterilized soils at the rate of 3% (w/w), the disease was controlled significantly (disease control <66.6%). The effectiveness of the other antagonists was recorded in the following order: *G. virens* (100%), *A. niger* (88.4%), *P. citrinum* (86.3%), *T. harzianum* (80%), *A. flavus* (70.2%), and *B. licheniformis* strain-2042 (66.6%). The disease control was more pronounced in sterilized soil than in unsterilized soil. *G. virens* was found to be the most potent biological control agent against *F. udum*, with disease control of 96.6% in unsterilized soil and 100% in sterilized soil (Table 2).

Whipps and Mc Quilken (1993) reported that *A. niger, A. terreus, G. virens, P. citrinum, T. harzianum*, and species of *Bacillus* control soil-borne diseases. Bashar and Rai (1994) observed that *A. flavus, A. niger*, and *T. viride* amended in soil suppressed the growth of *F. oxysporum* f. sp. *ciceri* and exhibited strong fungistatic activity against germination of conidia of test pathogen. The antifungal activity of *B. licheniformis* against different plant diseases such as seed rot, seedlings rot, and seedling blight has also been reported by Pingle and Kshirsagar (1992).

It is evident from Table 3 that the population of *F. udum* declined in the treatments as compared to the control at all the inoculum concentrations of the antagonists amended in the soil. Minimum decline in the population of the pathogen was observed in *G. virens* at 3% of inoculum concentration. The fungistasis of soil is reported to cause such inhibition (Lingappa and Lockwood, 1961; Lockwood, 1977). Upadhyay (1992) reported that soil fungistasis plays an important role in the biological control of *F. udum* in the soil. Thus, due to inoculation of the antagonists, the fungistasic activity of the treated soil might have increased

Table 3. Effect of soil amendments with antagonists in unsterilized soil on the population dynamics of *Fusarium udum*

Antagonist	Control	Treatment[a]		
		1%	2%	3%
Aspergillus flavus	70w	45x[b]	40x	25y
A. niger	65w	38x	29y	18z
Bacillus licheniformis (strain-2042)	70w	52x	46x	38y
Gliocladium virens	65w	35x	24y	10z
Penicillium citrinum	68w	40x	30y	20z
Trichoderma harzianum	70w	45x	38x	22y

[a] Values represent the mean cfu g^{-1} rhizospheric soil.
[b] Values followed by different letters in the rows are statistically different (P=0.05) as per DNMRT.

Table 2. Effect of screened antagonists on the control of wilt disease of pigeonpea in unsterilized and sterilized soil under pot condition

Antagonist	Isolate No.	Disease control (%)					
		1%[a]		2%		3%	
		US	S	US	S	US	S
Aspergillus flavus	R-031	20.8x[b]	47.9x	37.5x	59.2x	66.1y	70.2y
A. niger	R-102	48.3x	52.9x	68.2y	74.0y	83.0y	88.4y
Bacillus licheniformis	2042	41.5x	45.5x	54.1x	55.3x	60.6x	66.6x
Gliocladium virens	R-32	50.0x	55.5x	70.0y	78.8y	94.6z	100.0z
Penicillium citrinum	BR-14	45.8x	51.8x	68.3y	72.3y	78.3y	86.3y
Trichoderma harzianum	BK-021	42.8x	50.8x	66.6y	70.3y	75.0y	80.0y

[a] Concentration of the inoculum of the antagonists; US=unsterilized soil; S=sterilized soil.
[b] Values followed by different letters in the rows are statistically different (P=0.05) as per DNMRT.

Table 4. Biological control effect of wilt disease of pigeonpea under field condition

Antagonists	% Disease control
Aspergillus niger	38.7y[a]
Gliocladium virens	50.5z
Penicillium citrinum	33.3x
Trichoderma harzianum	28.4x

[a] Values followed by different letters in the column are statistically different (P=0.05) as per DNMRT.

through time due to the production of certain substances of antibiotic/toxic nature in soil (Upadhyay, 1992). The inhibition of the pathogen in soil may be supported by the fact that the antagonists inhibited the radial growth of the pathogen *in vitro* (Table 1), as well as, *in vivo* (Table 3). Direct parasitism and competition also play an important role in antagonism (Sharma et al., 1999).

Under field conditions, maximum control of the disease was observed with *G. virens* (50%), followed by *A. niger* (38%), *P. citrinum* (33%), and *T. harzianum* (28%) (Table 4). *G. virens* and *T. harzianum* have been recognized as the most effective antagonists for biological control of several plant pathogens by many investigators (Mishra and Narain, 1994; Mukherjee et al., 1995; Papavizas and Collins, 1990; Smith et al., 1990). The mechanism of control of the disease through soil amendment with the antogonists was due to drastic suppression of the pathogen by the antagonists as observed in this study (Table 3).

From the present study, it is clear that *G. virens* is highly effective in controlling the wilt disease of pigeonpea both in the glasshouse and under field conditions. This antagonist can be exploited for biological control of the wilt disease of pigeonpea under field conditions. However, further research is needed to enhance the disease control capability of *G. virens* through biotechnological approaches, particularly through strain improvement, and also by developing their formulations in cheaper substrates to make them affordable to farmers.

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