SCREENING OF *BACILLUS* ISOLATES FOR POTENTIAL BIOCONTROL OF THE WILT DISEASE COMPLEX OF PIGEON PEA (*CAJANUS CAJAN*) UNDER GREENHOUSE AND SMALL-SCALE FIELD CONDITIONS

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SUMMARY

Twenty isolates of unidentified *Bacillus* species were obtained from pathogen-suppressive soils of pigeon pea (*Cajanus cajan*) fields for the biocontrol of a wilt disease complex caused by *Heterodera cajani*, *Meloidogyne incognita* and *Fusarium udum*. Five isolates (B602, B603, B605, B615 and B618) were considered to have biocontrol potential on the basis of antifungal activity, inhibitory effect on the hatching and penetration of nematodes and colonization of pigeon pea roots by these isolates. The five *Bacillus* isolates were evaluated for biocontrol potential under pot conditions. Isolates B615 and B603 were found to be the most promising and should be further studied in field experiments.

Key words: Fusarium udum, *Heterodera cajani*, *Meloidogyne incognita*, biocontrol, pigeon pea, *Bacillus*.

INTRODUCTION

Pigeon pea, *Cajanus cajan* (L.) Millsp., is an important pulse crop in India and is a major source of protein for most of the vegetarian population. Pigeon pea is susceptible to *Heterodera cajani* Koshy, *Meloidogyne incognita* (Kofoid and White) Chitwood and *Fusarium udum* Butler. These are deleterious parasites of pigeon pea and cause a wilt disease complex, which is a major constraint to pigeon pea cultivation in India (Hasan, 1984; Siddiqui and Mahmood, 1996, 1999a). There is an urgent need to develop a suitable biocontrol for this disease complex.

Numerous rhizobacteria have been used as biocontrol agents (Siddiqui and Mahmood, 1999b; Siddiqui *et al.*, 2005; Siddiqui, 2006) and *Bacillus* spp. are well known for their biocontrol of several plant pathogens (Siddiqui, 2006). Isolation of effective bacteria from pathogen- suppressive soils from the same environment in which they will be used may increase the chances of finding effective strains (Cook and Baker, 1983; Weller *et al.*, 1985). An attempt was made to isolate *Bacillus* from pathogen suppressive soils of pigeon pea fields. These isolates were screened against *F. udum*. Effects of these isolates on the hatching of *M. incognita*, penetration of *M. incognita* and *H. cajani* and colonization of pigeon pea roots were also studied. Later, these isolates were tested for their biocontrol potential in pots both in mono- and multi- pathogenic conditions.

MATERIALS AND METHODS

Four hundred forty soil and pigeon pea root samples were collected from 22 districts of Uttar Pradesh, India. The root and soil samples were examined for the presence of *Meloidogyne* spp. and *H. cajani* and populations of these nematodes were estimated (Southey, 1986). Moreover, isolation of *F. udum* was performed on PDA from infected roots and a pure culture was maintained. Suppressive soils became apparent because wilt incidence or severity was lower than expected for the prevailing environment as compared with the surrounding soils. Moreover, samples of these areas contained low numbers of the nematodes and were later used for the isolation of *Bacillus* spp. Rhizobacteria were isolated on nutrient agar and in nutrient broth. Twenty isolates of *Bacillus* were obtained in pure culture and identified, using Bergey's Manual of Determinative Bacteriology (Krieg *et al.*, 1994).

Effect of rhizobacteria on hatching of *M. incognita*. The effects of the 20 *Bacillus* isolates on hatching of *M. incognita* were studied in small Petri dishes at 30ºC. Twenty egg-masses of almost similar size were picked from the roots of *Solanum melongena* with sterilized forceps and placed in 20 ml bacterial suspension (1.5×10^7 bacterial cells ml^{-1}) to allow hatching. As a control, 20 egg-masses were placed in 20 ml double distilled water for hatching. Each set was replicated five times and the experiment was repeated. The effect of different isolates on hatching was determined.

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Effect of 20 *Bacillus* isolates from the rhizosphere on nematode penetration. Penetration into pigeon pea roots of *H. cajani* and *M. incognita* was studied, using root systems inoculated with rhizobacteria and uninoculated controls at 30ºC. For observation of nematode penetration, two pigeon pea seeds were sown in each ice cream cup with 100 g steam-sterilized soil. Before sowing ten ml (1.5×10^7 bacterial cells ml^{-1}) suspension were poured into 20 g soil and 50 seeds were wrapped in this soil to provide bacterial inoculum. One week after germination, thinning was done to maintain a single seedling per ice cream cup. Later, the soils around the seedlings were inoculated with 50 second-stage juveniles each of *H. cajani* and *M. incognita* separately. Each treatment was replicated five times. Roots were harvested one month after nematode inoculation, washed with distilled water and stained with lacto-phenol cotton blue. Roots were cut into small pieces and observed with a stereomicroscope. The number of nematodes per root system was counted.

Root colonization by rhizobacteria. Root colonization by the 20 *Bacillus* isolates was also used to screen for their effectiveness as biocontrol agent. Pigeon pea roots inoculated with the isolates were collected one month after sowing. Surface-sterilized 1 g samples of roots were crushed in sterilized normal saline solution (NSS) and 0.1 ml serially diluted extracts were plated on nutrient agar plates, incubated at 37ºC for 24 h and counted. Plates with colonies within the 30-300 range were selected and colony forming units (CFU) per g of root determined (Sharma, 2001).

Effect of rhizobacteria on the fungus. Antifungal activity of *Bacillus* isolates was determined by inoculating *F. udum* simultaneously with a *Bacillus* isolate on the same nutrient agar plate. Inhibition of growth of *F. udum* was observed and recorded.

Pot experiment. Sandy loam collected from a field belonging to the Botany Department, Aligarh Muslim University, was passed through a 10-mesh sieve and mixed with river sand in the ratio of 4:1 and the mixture placed in 15 cm diameter clay pots. Pots plus soil were steam-sterilized before use.

Pigeon pea seeds cultivar UPAAS-120 were sterilized with 0.01% mercuric chloride for 2 min and rinsed three times with sterilized water. Three seeds were sown in 15 cm clay pots in steam-sterilized soil mixture. One week after germination, thinning was done to maintain a single seedling per pot and seedlings were subjected to the treatments listed in Tables 1 and 3. Uninoculated plants served as controls and plants were kept in a glasshouse at 30-40ºC. Pots were arranged in a randomized block design and each treatment was replicated five times. Pots were watered as needed and the experiment was terminated 90 days after inoculation.

Large numbers of *M. incognita* egg-masses were hand picked, using sterilized forceps, from heavily infected *S. melongena* roots on which a pure culture of *M. incognita* was maintained. These egg-masses were washed in distilled water and then poured into a 10 cm diameter, 15-mesh coarse sieve, containing crossed layers of tissue paper and placed in Petri-plates containing water just deep enough to contact the egg-masses. The hatched juveniles were collected every 24 h, after which fresh water was added. The concentration of second-stage juveniles in the inoculum was adjusted to 200 ± 5 nematodes ml^{-1}. Five ml of this suspension (i.e. 1000 freshly hatched juveniles) were added to each pot containing a pigeon pea seedling.

For the *H. cajani* inoculum, soil and root samples were collected from a pigeon pea field. Roots were examined for the cysts, which were collected, but were also isolated from soil using a 100-mesh sieve and placed for hatching in pigeon pea root exudates. Juveniles collected were inoculated at the rate of 500 juveniles per plant.

Table 1. Effects of 5 *Bacillus* isolates on plant dry weight and nodulation against a single pathogen under pot conditions.

Bacillus isolates	Plant dry weight (g)				No. of *Rhizobium* nodules/root system			
	Control	H	M	F	Control	H	M	F
Control	25.34 d	18.76 mn	19.17klm	17.62 o	8 abc	4 cd	5 bcd	4 cd
B602	25.71 d	20.34 i	20.76 hi	18.53 n	9 ab	5 bcd	8 abc	4 cd
B603	26.26 b	21.77 g	22.41 f	19.34 kl	8 abc	3 d	5 bcd	4 cd
B605	26.16 bc	20.91 h	21.47 g	18.93 lmn	10 a	4 cd	6 abcd	6 abcd
B615	27.73 a	22.74 f	24.12 e	20.76 hi	7 abcd	4 cd	5 bcd	5 bcd
B618	25.72 cd	19.60 jk	19.84 j	18.05 o	9 ab	4 cd	4 cd	4 cd
L.S.D. P = 0.05	0.44				4			

H* = *H. cajani*; M* = *M. incognita*; F* = *F. udum*
*Different letters within one parameter are significantly different at P = 0.05.

Table 2. Effects of 5 *Bacillus* isolates against a single pathogen on cyst formation, galling and wilting index under pot conditions.

Bacillus isolates	Cysts/root system	Galls/root system	Wilting index
Control	102a	106a	3
B602	86c	90c	3
B603	64e	71e	3
B605	74d	79d	3
B615	51f	55f	2
B618	93b	98b	3
L.S.D. P = 0.05	5	7	-

*Different letters within one parameter are significantly different at P = 0.05.

F. udum was isolated from infected pigeon pea roots and maintained on potato dextrose agar (PDA). Inoculum was prepared by culturing the isolate in Richard's liquid medium (Riker and Riker, 1936) for 15 days at 25°C. Mycelium was collected on blotting paper and excess water and nutrients were removed by pressing between two folds of blotting paper. One hundred gram mycelium was macerated in 1 litre distilled water and 10 ml of this suspension containing 1 g of fungus was poured around the roots.

Bacterial isolates were cultured on nutrient agar and growth of each isolate was scraped and suspended in sterilized water to a concentration of 1.5×10^7 cells ml^{-1}. One hundred ml bacterial suspension of each isolate was poured in 200 g sterilized soil and 100 seeds of pigeon pea were planted yielding a 1.5×10^7 bacterial cells per seed.

For inoculation of nematodes and fungal suspension, soil around the roots was carefully removed without damaging the roots, inoculum poured around the roots and the soil replaced. Sterile water was used as a control suspension. The treatments were applied as shown in Tables 1 and 3. There were 24 treatments comprising 6 treatments of *Bacillus* (isolates B602, B603, B605, B615 and B618) each tested with the three pathogens and a control and each replicated five times (Table 1). Moreover, there were 30 treatments comprising 6 treatments of *Bacillus* isolates as shown in Table 3 (isolates B602, B603, B605, B615, B618) each tested with 4 combinations of pathogens and a control.

Plants were uprooted 90 days after inoculation. For dry weight determination, plants were kept at 60°C for 2-3 days before weighing. Number of nodules per root system, cysts per root system, galls per root system and wilting index were recorded. A wilting index was recorded by scoring disease severity on a 0-5 scale where 0 = no disease and 5 = severe wilting.

The entire data set was analyzed as a single two-factor experiment (pathogens × bacterial isolates) by the method of Dospekhov (1984). Least significant differences (L.S.D.) were calculated at P = 0.05 and Duncan's multiple range test was employed to test for significant differences between treatments.

Table 3. Effects of 5 *Bacillus* isolates on plant dry weight and nodulation against combined inoculation of pathogens under pot conditions.

Bacillus isolates	Plant dry weight (g)					No. of nodules /root system				
	Control	H+M	H+F	M+F	H+M+F	Control	H.+M	H+F	M+F	H+M+F
Control	25.34 d	14.24 no	12.55 q	13.73 p	10.42 s	8 abc	2 def	2 def	2 def	0 f
B602	25.71 cd	15.32 kl	14.44 mn	14.85 lm	12.13 qr	9 ab	3 cdef	3 cdef	2 def	1ef
B603	26.26 b	18.32 f	15.53 jk	16.05 i	14.28 n	8 abc	3 cdef	4 bcdef	2 def	2 def
B605	26.16 bc	15.92 ij	14.85 lm	15.54 jk	13.42 p	10 a	4 bcdef	3 cdef	3 cdef	2 def
B615	27.73 a	19.18 e	16.57 h	17.64 g	15.95 ij	7 abcd	4 bcdef	3 cdef	3 cdef	2 def
B618	25.72 c	15.27 kl	13.78 op	14.33 n	11.72 r	9 a b	6 abcde	5 abcdef	2 def	1 ef
L.S.D. P=0.05	0.47					5				

H*= *H. cajani*; M*= *M. incognita*; F*= *F. udum*

*Different letters within one parameter are significantly different at P = 0.05.

Table 4. Effects of 5 *Bacillus* isolates against combined inoculation of pathogens on cyst formation, galling and wilting index under pot conditions.

Bacillus isolates	Cysts / root system			Galls / root system			Wilting index		
	H+M	H+F	H+M+F	H+M	M+F	H+M+F	H+F	M+F	H+M+F
Control	92 a	84 ab	78 de	96 a	90 abc	85 cd	4	4	5
B602	81 cd	76 e	70 f	85 cd	81 de	76 ef	4	4	5
B603	60 g	57 gh	53 h	65 gh	62 hi	58 i	4	4	4
B605	70 f	66 f	61 g	76 ef	71 fg	66 gh	4	4	5
B615	46 i	42 ij	38 j	50 j	43 k	40 k	3	3	4
B618	88 ab	84 bc	80 cde	93 ab	89 bc	85 cd	4	4	5
L.S.D. P = 0.05	4			6			-		

H* = *H. cajani*; M* = *M. incognita*; F* = *F. udum*
* Different letters within one parameter are significantly different at P = 0.05.

RESULTS AND DISCUSSION

The twenty bacterial isolates isolated from pathogen suppressive soils of pigeon pea fields were tentatively placed in the genus *Bacillus* on the basis of all isolates being Gram-positive, growth on casein agar glucose medium, growth at 45°C at pH 7.5, and in 7% NaCl, endospore staining, utilization of citrate, acid and gas from glucose, starch hydrolysis and catalase test. We did not further identify the isolates.

Out of the 20 *Bacillus* isolates, 12 (B602, B604, B605, B607, B608, B609, B611, B612, B613, B615, B616 and B618) showed antifungal activity against *F. udum.* Moreover, 5 isolates (B602, B603, B605, B615 and B618) had adverse effects on hatching and penetration of both nematodes studied (Fig. 1). Finally, 5 isolates (B602, B603, B605, B615 and B618) were selected as potential biocontrol agents after greenhouse assay on the basis of antifungal activity, best root colonization and highest inhibitory effect on hatching and penetration of *M. incognita* and *H. cajani* (Fig. 1).

The five potential *Bacillus* isolates selected in the greenhouse assay were further tested for biocontrol of wilt under pot conditions. All 5 isolates increased growth of plants inoculated with pathogens and pathogen-free control plants (Table 1). Increases in plant growth caused by the isolates were higher in the pathogen-inoculated plants than in plants without pathogens. Isolate B615 showed the highest plant growth stimulating effect.

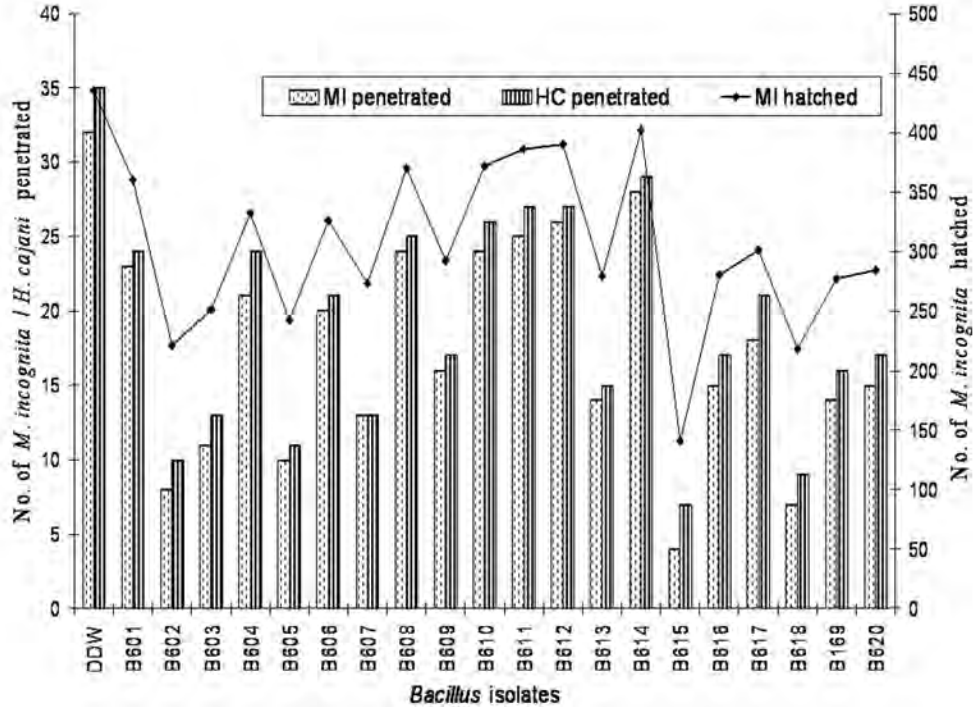

Fig. 1. Effects of 20 isolates of *Bacillus* on hatching of *M. incognita*, penetration of *M. incognita* and *H. cajani*.

Inoculation of pathogens had an adverse effect on nitrogen-nodulation of the plants, but inoculation of *Bacillus* isolates had no effect on nodulation. Isolate B615 also had highest adverse effect on cyst formation and root galling. Isolate B615 was the only one that reduced the wilting index (Table 2).

When the five *Bacillus* isolates were used with plants that were simultaneously inoculated with two or more pathogens, they also increased plant growth (Table 3). Isolate B615 again caused highest increase. Simultaneous inoculation of pathogens caused an adverse effect on nodulation, but inoculation of *Bacillus* isolates had no effect on nodulation. Again isolate B615 caused highest adverse effect on cysts formation and root galling. Isolates B615 and B603 reduced the wilting index in plants simultaneously inoculated with pathogens, while the other isolates had no effect (Table 4).

We suggest that isolates B615 and B603 are potential biocontrol agents to be further studied in field experiments.

ACKNOWLEDGEMENTS

The authors are grateful to D.S.T. New Delhi, for financial assistance through project No. SP/SO/A22/ 2001.

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Received October 10, 2005
Accepted February 12, 2007