Management of Bacterial Blight of Cotton Using a Mixture of *Pseudomonas fluorescens* and *Bacillus subtilis*

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Abstract

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The potential of antagonistic rhizobacteria in the management of bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) was evaluated under greenhouse and field conditions. In this study, 93 bacterial isolates from the rhizosphere of cotton were screened for their efficacy in inhibiting the growth of *Xam in vitro*. Among them, 21 isolates were found to inhibit the *in vitro* growth of *Xam*. These isolates were identified as *Pseudomonas fluorescens* and *Bacillus subtilis* based on phenotypic characteristics, biochemical properties and using 16S-23S intergenic transcribed spacer-Polymerase Chain Reaction (PCR). Among the 21 isolates, the isolates *P. fluorescens* Pf32 and *P. fluorescens* Pf93 and *B. subtilis* B49 exhibited the maximum inhibitory activity against *Xam*. Talc-based powder formulations of the effective antagonistic isolates of *P. fluorescens* (Pf32, Pf93) and *B. subtilis* (B49) were developed and evaluated individually and in combination for their efficacy in the management of bacterial blight of cotton under greenhouse and field conditions. The *P. fluorescens* isolates Pf32 and Pf93 and *Bacillus subtilis* isolate B49 survived well in the talc-based formulation for more than 90 days. The application of a mixture of Pf32, Pf93 and B49 to seed, soil and foliage significantly reduced the bacterial blight incidence and increased the plant height, number of branches and number of bolls under field conditions. The plots treated with a mixture of Pf32, Pf93 and B49 recorded the maximum yield of 1915 kg/ha and 1512 kg/ha in trial I and trial II compared to 1210 kg/ha and 987 kg/ha in the untreated control, respectively.

Keywords: biological control; *Pseudomonas fluorescens*; *Bacillus subtilis*; *Gossypium hirsutum*; bacterial blight

Bacterial blight of cotton (*Gossypium* spp.) caused by *Xanthomonas axonopodis* pv. *malvacearum* (E.F. Smith) (Vauterin) is a disease of economic significance throughout the world. The pathogen attacks host plants during all growth stages, infecting stems, leaves, bracts and bolls and causes seedling blight, black arm, angular leaf spot, and boll lesions (Verma 1986). Yield losses due to bacterial blight range between 1% and 27% depending on the cultivar and crop age (Mishra & Ashok Krishna 2001). Under natural bacterial blight infection, boll yield losses up to 35% have been reported (Sheo Raj & Verma 1988). Only a few sources of resistance to bacterial blight have been identified (Tyagi & Olngotie 1988). In the absence of resistant cultivars, antibiotics serve as the

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only means of controlling this disease (Zohurul Islam *et al.* 2003). Unfortunately, the application of antibiotics may result in undesirable effects. The greatest concern of antibiotic use in plant disease management is that spraying antibiotics in the open environment may lead to emergence of bacterial strains resistant to antibiotics and increase the frequency of transfer of antibiotic resistance from plant and soil-borne bacteria to bacteria that reside on or in humans (Levy 1998). Widespread resistance to streptomycin among isolates of *Pseudomonas cichorii* (Swingle) Stapp. and *E. amylovora* (Burrill) has been reported (Pohronezny *et al*. 1994). Biological control using antagonistic microorganisms could be a potential alternative or complementary approach to management of bacterial blight of cotton. Several strains of *Pseudomonas fluorescens*, *P. putida, P. cepacia, P. aeruginosa*, and *Bacillus subtilis* strains have been widely used for the biological control of several fungal, bacterial and viral pathogens (Raupach & Kloepper 1998). Some of the antagonistic *P. fluorescens* and *B. subtilis* also act as inducers of systemic resistance in plants (Vidhyasekaran *et al.* 2001; Jayaraj *et al*. 2004). Certain strains of fluorescent pseudomonas are also known as plant growth-promoting rhizobacteria (PGPR) as they promote the plant growth. The objective of this study was to evaluate the efficacy of selected isolates of *Pseudomonas fluorescens* and *Bacillus subtilis* either separately or in combination in controlling the bacterial blight of cotton. The effect of these treatments on cotton yield was also determined.

MATERIALS AND METHODS

Isolation of the pathogen and antagonistic bacteria. *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) isolated from infected cotton leaves (Salaheddin *et al.* 2005) was used in this study. Antagonistic bacteria were isolated from the cotton rhizosphere soil collected from different districts of Tamil Nadu with nutrient agar medium (NA) and King's medium B (KMB) (King *et al*. 1954). These bacterial isolates were identified by standard bacteriological tests (Schaad 1992) and 16S–23S ribosomal RNA gene sequencing (Ramesh Kumar *et al*. 2002).

In vitro ***screening of bacterial isolates against*** *Xam. Xam* was cultured in a 100 ml conical flask containing 40 ml of nutrient broth at 25°C for 2 days on a shaker at 150 rpm. One ml of bacterial suspension was mixed with 15 ml of molten nutrient agar medium and poured onto a sterile Petri dish (90 mm in diameter). A sterile filter paper disc (6 mm in diameter) was laid on the agar surface at the centre of the Petri dish and 5 µl of 48 h old broth cultures of *Pseudomonas* sp. or *Bacillus* sp. was applied to each disc. Then the plates were incubated at 25°C for 2 to 5 days. The diameter of the inhibition zone formed around the disc was measured. Each treatment was replicated 10 times and the results were averaged. The compatibility among the three selected bacterial isolates was tested by streaking the *B. subtilis* isolate B49 one side and streaking the *P. fluorescens* isolates Pf32 and Pf93 perpendicularly up to the test bacterium on a Petri dish containing NA medium. The growth of the bacteria was observed 2–3 days after incubation at room temperature (28 ± 2°C).

Development of talc-based powder formulations of *P. fluorescens* ***and*** *B. subtilis.* All manipulations were carried out under sterile conditions. Talc-based powder formulations of the effective isolates of *P. fluorescens* Pf32 and Pf93 and *B. subtilis* B49 selected based on their antagonistic potential against *Xam* under *in vitro* conditions were developed as described by Vidhyasekaran and Muthamilan (1995). Four hundred ml of 72-h-old bacterial culture in their respective medium with a population of 9×10^8 CFU/ml was mixed with 1 kg of talc containing 15 g of calcium carbonate and 10 g of carboxymethyl cellulose. Moisture content of the product was reduced to 20% by shade drying and it was packed in polythene bags. The population of bacteria was $2.5–3 \times 10^8$ CFU/g of talc powder at the time of application. To prepare a mixture of antagonistic bacteria, the bacterial antagonists were grown separately in their respective liquid media and equal volume (v/v) of liquid culture was mixed.

Shelf life of the formulation of biocontrol agents. The shelf life of the talc-based formulations of biocontrol agents stored for 3 months at room temperature (28 ± 2°C) was studied by a serial dilution technique. One gram of the sample drawn from each formulation periodically at 15, 30, 45, 60, 75, and 90 days of storage was mixed with 10 ml of sterile distilled water. From this serial dilutions were made. One ml aliquot of each dilution was pipetted out into sterilised Petri plates and 19 ml of NA or KB was added and incubated at room

temperature (28 ± 2°C). The number of colony forming units of bacteria was counted 3 days after plating and expressed as the number of CFU/g of formulation.

Efficacy of antagonistic bacterial isolates on plant growth promotion

Preparation of bacterial suspension. The bacterial isolates were grown in KB or NA liquid medium for 48 h at room temperature (28 ± 2°C) on a shaker. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min and bacterial cells were re-suspended in 0.01M phosphate buffer, pH 7.0. The concentration of bacterial cells was adjusted to 3×10^8 CFU/ml ($OD_{595} = 0.3$) using a spectrophotometer and used for inoculation (Thompson 1996).

Seed bacterisation. Acid delinted cotton seeds (cv. LRA 5166) were surface sterilised with 2% sodium hypochlorite for 30 s, rinsed in sterile distilled water and dried overnight under a sterile air stream. Seeds were then soaked in a bacterial suspension containing 3×10^8 CFU/ml for 2 h and shade dried.

Plant growth promotion. The plant growth promoting ability of bacterial isolates was assessed by a seedling vigour test using the standard roll towel method (ISTA 1999). Three replications were maintained for each treatment. The root and shoot length of individual seedlings were measured and the germination percentage of seeds was also calculated. The vigour index was calculated using the formula as described by Abdul Baki and Anderson (1973).

$$\text{Vigour index (VI)} = \text{germination percentage} \times \text{total seedling length}$$

where: total seedling length = root length + shoot length

Efficacy of biocontrol formulations in the control of bacterial blight of cotton

Greenhouse studies. The bacterial isolates either separately or as a mixture were assessed for their efficacy in the control of bacterial leaf blight of cotton under greenhouse conditions. The delinted seeds (cv. LRA 5166) were surface sterilised with 2% sodium hypochlorite and soaked overnight in double volume of sterile distilled water containing the talc-based formulation (10 g/l). The treated seeds were shade dried for 30 min and sown in pots (diameter 0.25 m; height 0.3 m) containing sterilised soil at the rate of 7 seeds per pot. For soil application, the talc-based formulation was applied 30 days after sowing at the rate of 5 g/kg of soil. For foliar spray (FS), the talc-based formulation was thoroughly mixed in water (20 g/l) and allowed to settle for 1 h, filtered through muslin cloth and sprayed 30 days after sowing (DAS) until run-off. Twenty-four hours after foliar application of biocontrol agents, the leaves of cotton were injured with sand paper and the injured plants were sprayed with *Xam* (0.45 OD) (Salaheddin *et al.* 2005). As a check the plants were sprayed with streptomycin (100 ppm). The plants sprayed with distilled water were kept as control. Growth parameters viz plant height, number of flowers, and number of bolls were recorded. The percentage of disease incidence was recorded 15 days after inoculation using a 0–7 scale (Santhanam 1967). The percent disease index (PDI) was calculated as described by Mc Kinney (1923) using the formula:

$$\text{PDI} = \frac{\text{Sum of all numerical ratings}}{\text{Total No. of leaves graded}} \times \frac{100}{\text{Maximum gradient}}$$

The trial was laid out in a completely randomised design (CRD) with three replications.

Field studies. Two field trials were conducted in 2005 (Trial I) and 2006 (Trial II) at the Cotton Breeding Station, Tamil Nadu Agricultural University, Coimbatore, where the disease occurs annually. Delinted cotton seeds (cv. LRA 5166) were soaked overnight in water containing talc-based formulations of *P. fluorescens* Pf32 and Pf93 and *B. subtilis* B49 (10 g/kg of seeds). The seeds soaked in distilled water served as control. Treated seeds were allowed to dry in shade and sown in plots 8×5 m^2 in size with a spacing of 70 × 35 cm. The talc-based powder product (2.5 kg/ha) was applied 30 days after sowing as soil application. For foliar application, the talc-based formulation was thoroughly mixed in water (20 g/l), allowed to settle for 1 h, filtered through muslin cloth and the filtrate was sprayed 45, 60, 75 and 90 DAS. An untreated control was also maintained. The trials were laid out in a randomised block design (RBD) with three replications. The incidence of bacterial blight was recorded 120 days after sowing. The growth parameters viz plant height, number of branches and number of bolls and yield were recorded at the time of harvest.

Statistical analysis. All the experiments were analysed independently. The treatment means were

compared by Duncan's multiple range test (DMRT) (Gomez & Gomez 1984). The IRRI STAT version 92-1 package developed by the International Rice Research Institute, Biometrics Unit, Philippines, was used for analysis.

RESULTS

Ninety-three isolates of bacteria were isolated from the cotton rhizosphere soils collected in different parts of Tamil Nadu. These isolates were identified as *Pseudomonas fluorescens* and *Bacillus subtilis* based on phenotypic characteristics, biochemical properties and using 16S–23S intergenic transcribed spacer-Polymerase Chain Reaction (PCR) (data not shown). These isolates were screened for their efficacy in inhibiting the growth of *Xam in vitro*. Among the bacterial isolates that exhibited the antagonistic activity against *Xam*, sixteen were *Pseudomonas fluorescens* and 5 were *Bacillus subtilis*. Among the 16 isolates of *P. fluorescens*, Pf32 was the most effective one in inhibiting the growth of *Xam* and an inhibition zone of 26.7 mm was recorded, whereas the isolates Pf34, Pf35, Pf84 and Pf93 produced inhibition zones of 22.6 mm, 21.8 mm, 22.4 mm and 25.4 mm, respectively (Table 1). Among the five isolates of *B. subtilis*, B49 exhibited the maximum inhibitory activity against *Xam* and an inhibition zone of 6.1 mm against *Xam* was recorded (Table 1). The *Pseudomonas fluorescens* isolates (Pf 32 and Pf 93) were found to be compatible with *Bacillus subtilis* isolate (B49) (data not shown).

Talc-based powder formulations of Pf32, Pf93 and B49 were prepared and the shelf life of bio-

Table 1. *In vitro* inhibition of growth of *Xanthomonas axonopodis* pv. *malvacearum* by various strains of *Pseudomonas fluorescens* and *Bacillus subtilis*

Sample No.	Rhizobacteria	Strains	Diameter of inhibition zone (mm)
1	*Pseudomonas* isolate	Pf5	3.2^{g}
2		Pf6	4.1^{e}
3		Pf8	1.2^{l}
4		Pf12	1.5^{k}
5		Pf19	2.3^{i}
6		Pf31	0.0
7		Pf32	26.7^{a}
8		Pf34	22.6^{c}
9		Pf35	21.8^{d}
10		Pf41	0.0
11		Pf84	22.4^{c}
12		Pf87	3.5^{f}
13		Pf93	25.4^{b}
14		PfV	2.7^{h}
15		Pf1	2.1^{j}
16		PfCHA0	2.6^{h}
17	*Bacillus* isolate	B49	6.1^{a}
18		B59	3.6^{c}
19		B75	4.3^{b}
20		B81	4.2^{b}
24		B90	3.1^{c}
Control		–	–

*Data are mean of three replications; in a column, means followed by a common letter are not significantly different at the 5% level by DMRT

Table 2. Shelf life of biocontrol agents in the talc-based formulation – Population (× 10^8 CFU/ml)*

Antagonistic bacteria	Days after storage						
	0	15	30	45	60	75	90
Pseudomonas fluorescens strain 32	72.9^c	51.5^c	40.1^a	15.3^c	9.1^a	3.6^a	1.30^b
Pseudomonas fluorescens strain 93	78.1^b	63.8^b	33.7^b	17.5^b	7.9^b	2.8^b	0.33^c
Bacillus subtilis strain 49	83.5^a	69.1^a	27.5^c	18.3^a	6.5^c	2.1^c	1.66^a

*Values are mean of three replications; in a column, means followed by a common letter are not significantly different at the 5% level by DMRT

control agents in the talc formulation was assessed at different time intervals. The results indicated that the shelf life of biocontrol agents in the talc formulation varied with the isolates during the storage period. There was a slight reduction in the population density during the first 30 days. However, more than 50% survival was observed on the 30th day (Table 2). The population of bacterial cells in the formulation declined later on. The formulations stored up to 30 days were used in greenhouse and field studies.

The effect of antagonistic bacteria on seed germination and seedling vigour was assessed by the paper towel method. The results indicated that the treatment of seeds with biocontrol agents significantly increased the seed germination and seedling vigour. The maximum increase in germination percentage (88.22%), root length (20.29 cm), shoot length (14.10 cm), dry matter production (0.110 mg), and vigour index (3034) was observed when the cotton seeds were treated with a mixture of Pf32, Pf93 and B49. The next best treatment was the treatment with a mixture of Pf 32 and B49, which recorded a vigour index of 2271 (Table 3).

The effect of biocontrol agents in the control of cotton bacterial blight was evaluated under greenhouse and field conditions. The results indicated that the application of the talc-based powder formulation of a mixture of Pf32, Pf93 and B49 effectively controlled BLB of cotton in both greenhouse and field trials (Table 4). In the greenhouse trial, the highest level of the disease control was observed in the treatment with streptomycin (58.7%) followed by the treatment with a mixture of Pf32, Pf93 and B49 (56.0%). Similar results were observed in both field trials. When the plants were sprayed with a

Table 3. Effect of *Pseudomonas fluorescens* and *Bacillus subtilis* on seed germination and seedling vigour of cotton

Sample No.	Treatment	Germination (%)	Root length (cm)	Shoot length (cm)	Dry matter (mg/plant)	Vigour index
1	Pf 32	70.15 (55.88)^c	15.83^{ef}	12.40^d	0.081^c	1980^e
2	Pf 93	75.11 (60.07)^b	16.99^d	11.60^e	0.092^b	2147^c
3	B49	73.18 (58.81)^b	16.11^e	13.70^b	0.061^e	2182^c
4	Pf 32 + Pf 93	75.15 (60.10)^b	17.89^c	10.10^g	0.054^f	2165^c
5	Pf 32 + B49	73.51 (59.02)^b	19.11^b	13.00^c	0.059^e	2271^b
6	Pf 93 + B49	72.81 (58.58)^b	15.93^e	12.10^d	0.044^g	2041^d
7	Pf 32 + Pf 93 + B49	88.22 (69.96)^a	20.29^a	14.10^a	0.110^a	3034^a
8	Streptomycin 0.1%	61.00 (51.35)^{de}	14.93^g	9.97^g	0.009^i	1519^g
9	Control (water)	59.37 (50.39)^e	14.00^h	8.13^h	0.067^d	1312^h

Values are mean of three replications; values in parentheses are arcsine transformed; in a column, means followed by a common letter are not significantly different at the 5% level by DMRT

Table 4. Effect of foliar application of talc-based powder formulation of *P. fluorescens* and *B. subtilis* in the management of bacterial blight of cotton (in %)

Treatments	Glasshouse condition		Trail I		Trail II	
	*BBC incidence	reduction over control	*PDI	reduction over control	*PDI	reduction over control
Pf32	45.0 (42.12)^{de}	39.1 (38.70)^{e}	13.4 (21.47)^{g}	37.6 (37.81)^{g}	15.5 (23.18)^{g}	35.1 (36.33)^{g}
Pf93	46.1 (42.76)^{e}	37.7 (37.87)^{f}	12.5 (20.70)^{f}	41.8 (40.28)^{f}	14.6 (22.46)^{f}	38.9 (38.58)^{f}
B49	45.1 (42.18)^{de}	38.9 (38.58)^{ef}	9.9 (18.33)^{d}	53.9 (47.23)^{d}	14.1 (22.05)^{e}	41.0 (39.81)^{e}
Pf32+ Pf93	44.4 (41.78)^{d}	39.9 (39.17)^{e}	10.7 (19.09)^{e}	50.2 (45.11)^{e}	13.8 (21.80)^{e}	42.25 (40.54)^{e}
Pf32 + B49	39.4 (38.87)^{c}	46.6 (43.05)^{d}	14.5 (22.38)^{h}	32.5 (34.75)^{h}	12.3 (20.52)^{d}	48.53 (44.15)^{d}
Pf93 + B49	38.3 (38.23)^{c}	48.1 (43.91)^{c}	8.3 (16.74)^{c}	61.3 (51.53)^{c}	8.5 (16.94)^{c}	64.43 (53.39)^{c}
Pf32 + Pf93 + B49	32.5 (34.75)^{b}	56.0 (48.44)^{b}	7.1 (15.45)^{b}	66.97 (54.92)^{b}	7.5 (15.67)^{b}	69.45 (56.44)^{b}
Streptomycin (100 ppm)	30.5 (33.52)^{a}	58.7 (50.01)^{a}	5.1 (13.05)^{a}	78.27 (65.85)^{a}	6.7 (15.00)^{a}	71.96 (58.03)^{a}
Control	73.9 (59.28)^{f}	–	21.5 (27.62)^{j}	–	23.9 (29.26)^{i}	–

*Mean of three replications; values in parentheses are arcsine transformed; in a column, mean followed by a common letter are not significantly different at the 5% level by DMRT

Table 5. Effect of biocontrol agents on the growth parameters* of cotton under glass house and field conditions)

Treatments	Glasshouse condition			Trial I				Trail II			
	plant height (cm)	No. of branches	No. of bolls	plant height (cm)	No. of branches	No. of bolls	yield	plant height (cm)	No. of branches	No. of bolls	yield
Pf32	40.1^{e}	6.3^{f}	6.0^{e}	91.04^{b}	14.2^{bc}	23.8^{de}	1632.5^{de}	105.25^{de}	14.67^{c}	24.5^{de}	1301.5^{d}
Pf93	45.3^{d}	6.5^{e}	6.0^{e}	79.16^{ef}	13.92^{cd}	23.1^{e}	1412.5^{f}	107.75^{d}	14.50^{c}	26.75^{c}	1190.3^{g}
B49	40.3^{e}	6.2^{f}	4.5^{g}	77.48^{f}	14.12^{bcd}	23.5^{de}	1698.5^{c}	105.58^{de}	14.47^{c}	24.7^{d}	1383.8^{c}
Pf32+ Pf93	40.3^{e}	6.9^{d}	7.0^{d}	82.48^{d}	13.96^{cd}	24.1^{d}	1607.3^{e}	108.58^{d}	15.58^{b}	29.25^{b}	1290.1^{e}
Pf32 + B49	59.0^{c}	7.3^{c}	8.0^{c}	81.12^{de}	13.88^{cd}	25.1^{c}	1750.5^{b}	112.42^{c}	15.22^{b}	28.83^{b}	1434.5^{b}
Pf93 + B49	63.0^{b}	8.0^{b}	8.5^{b}	85.24^{c}	14.50^{ab}	27.5^{b}	1890.3^{a}	118.08^{b}	16.17^{a}	29.63^{b}	1450.3^{b}
Pf32 + Pf93 + B49	72.3^{a}	8.6^{a}	10.5^{a}	100.45^{a}	14.76^{a}	31.5^{a}	1915.0^{a}	122.25^{a}	16.42^{a}	38.5^{a}	1512.1^{a}
Streptomycin (100 ppm)	37.3^{f}	5.0^{g}	5.0^{f}	77.48^{f}	13.28^{e}	22.00^{f}	1701.0^{c}	102.58^{ef}	14.01^{b}	23.75^{e}	1310.7^{d}
Control	40.6^{e}	5.1^{g}	4.0^{h}	72.2^{g}	13.68^{de}	19.3^{h}	1210.0^{g}	99.17^{g}	13.00^{d}	22.87^{f}	986.9^{h}

*Mean of three replications; values in parentheses are arcsine transformed. In a column, mean followed by a common letter are not significantly different at the 5% level by DMRT

mixture of Pf32, Pf93 and B49, the incidence of BLB was reduced by 66.9% and 69.4% in Trial I and Trial II, respectively, as compared to 78.2% and 71.9% in streptomycin treated plots. The control of bacterial blight by the application of a mixture of Pf32, Pf93 and B49 was statistically different from that obtained with streptomycin application.

The maximum height of the plant, number of branches and number of bolls were recorded in plants treated with the talc-based powder formulation of a mixture of Pf32, Pf93 and B49 under greenhouse conditions (Table 5). The highest boll yield of 1915 kg/ha and 1512 kg/ha in Trial I and Trial II, respectively, was recorded in the above treatments.

DISCUSSION

Biological control of crop diseases by antagonistic microorganisms has emerged as the most effective alternative to synthetic chemical pesticides in the last several years. Among the antagonistic bacteria, several species of *P. fluorescens, P. putida, P. cepacia,* and *P. aeruginosa* have also been successfully used for the biological control of plant diseases (Vidhyasekaran & Muthamilan 1999; Salaheddin 2002). The effectiveness of *P. fluorescens* in the control of bacterial diseases such as *Xanthomonas oryzae* pv. *oryzae* causing bacterial blight in rice (Vidhyasekaran *et al.* 2001), *Erwinia carotovora* ssp. *carotovora* (Kloepper *et al.* 1980), *Ralstonia solanacearum* in banana, egg plant and tomato (Anuratha & Gnanamanickam 1990) has been reported. *P. fluorescens* FPT9601, isolated from the tomato rhizosphere, was reported to significantly suppress the tomato bacterial wilt disease caused by *Ralstonia solanacearum* (Zhang *et al*. 2001). Thirukumaran (1999) reported that spraying of cotton plants with *P. fluorescens* recorded the least bacterial blight incidence compared to the control. Mondal (1999) found that five strains of *Pseudomonas* sp. were effective in controlling the bacterial blight of cotton. Sanjay and Parashar (2002) used *B. subtilis* to control the bacterial blight of cotton under glasshouse conditions. *Bacillus* species offer several advantages over fluorescent pseudomonads and other Gram-negative bacteria for the control of soil-borne pathogens, including longer shelf life, because of their ability to form endospores and the broad-spectrum activities of their antibiotics (Kim *et al*. 1997). In the present investigation a total of 93 bacterial isolates were isolated from different cotton rhizosphere soils and these isolates were tested for their antagonistic effect on *Xam* under *in vitro* conditions. Among them, *P. fluorescens* Pf32, Pf93 and *B. subtilis* B49 were found to be the most effective in inhibiting the growth of *Xam*, compared to other isolates. The bacterial isolates Pf32, Pf93 and B49 were found to be compatible with each other, which revealed the scope for the utilization of these two different genera of antagonistic bacteria in a mixture for the management of bacterial blight of cotton.

Talc-based powder formulations of the effective antagonistic isolates of *P. fluorescens* (Pf32, Pf93) and *B. subtilis* (B49) were developed for field application for the management of bacterial blight of cotton. The shelf life of the bacterial isolates in the talc formulation was evaluated at different time intervals. The results of the study revealed that the bacterial isolates (Pf32, Pf93 and B49) survived with the required cfu (10^8 CFU/ml) in the bioformulation up to 90 days of storage. The population of *Bacillus subtilis* was higher when compared to *P. fluorescens* after 3 months of storage. It may be due to the production of endospores by *Bacillus* spp. that offer great advantage for long-term storage (Powell *et al.* 1999).

The results of this study also indicated that the treatment with a mixture of biocontrol agents (Pf32, Pf93 and B49) effectively controlled bacterial blight of cotton both under greenhouse and field conditions. Some fluorescent pseudomonads are endophytes and can migrate to aerial parts of plants that are developed from seeds treated with the bacteria (Krishnamoorthy & Gnanamanickam 1998). Fluorescent pseudomonads and *Bacillus subtilis* are also known to induce disease resistance against foliar diseases (Liu *et al*. 1995; Jayaraj *et al*. 2004). Both direct inhibition of *Xam* and induced resistance might be involved in the control of bacterial blight of cotton by the application of a mixture of *P. fluorescens* and *B. subtilis*. Growth parameters like plant height, root length, number of branches and number of bolls were also increased when plants were treated with biocontrol agents compared to the untreated control. The increase in biomass production may be due to the production of plant growth promoters or to indirect stimulation of nutrient uptake and production of siderophores or antibiotics to protect plants from deleterious rhizosphere organisms. Van peer and

Schippers (1988) reported that the root length and shoot length of tomato, cucumber, lettuce and potato were increased as a result of bacterization with *Pseudomonas* strains. Salaheddin (2002) reported that there was a significant increase in seed germination, vigour index and dry weight of cotton when treated with *P. fluorescens* isolates (Pf1 and MMP) under glasshouse conditions. Safyazov *et al*. (1995) concluded that *P. fluorescens* stimulated the emergence and seedling growth and increased cotton yield besides reducing the disease intensity. The increase in plant growth and yield under field conditions might be due to the growth-promoting compounds such as gibberellins, cytokinins, auxin from tryptophan produced by biocontrol agents (Dubeikovsky *et al*. 1993; Pal *et al*. 2000;). Zhang *et al.* (2003) reported the PGPR-mediated growth promotion of tobacco by *P. fluorescens* 89B-61, *B. pumilis* SE 34, *B. pumilis* T4, *B. pasteurii* C-9 and *Serratia marcescens* 90-166. Ramamoorthy *et al*. (2002) demonstrated an increased vigour index of tomato and hot pepper by *P. fluorescens* isolates Pf1 ad Pf7 upon seed bacterisation. Ryu *et al*. (2003) demonstrated the involvement of the production of volatile compounds 2,3-butanediol and acetoin in plant growth promotion in *Arabidopsis thaliana* by *B. subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a.

Several approaches have been used to control crop diseases which include the combined application of two or more biocontrol strains to enhance the level and consistency in disease control (Raupach & Kloepper 1998). Guetsky *et al*. (2002) demonstrated that a mixture of yeast (*Pichia guilermondii*) and bacterium (*Bacillus mycoides*) resulted in additive activity compared with their separate application in the suppression of *Botrytis cinerea* on strawberry. Marimuthu *et al*. (2002) reported a synergistic effect of the combined application of *Azospirillum* and *Pseudomonas fluorescens* Pf1 in reduction of root rot incidence and enhancement of plant growth and cotton yield under field conditions. Biological control with multimechanisms may be achieved by using one biocontrol agent exhibiting several mechanisms or by applying more than one biocontrol agent in a mixture. Studies on the mode of action of *B. subtilis* have shown that the increase in crop growth is due to the release of bacterial metabolites having precursors of auxin (indole-3-pyruvic acid) or inducers (G3 fraction) for auxin synthesis (Bochow & Dolej 1999). Biosynthesis of antibiotics (Howie & Suslow 1991), production of lytic enzymes (Velazhahan *et al*. 1999), production of siderophores (Loper 1988), production of hydrogen cyanide (Ahl *et al*. 1986), competition for substrates (Elad & Chet 1987) and induced systemic resistance (Ongena *et a*l. 1999) are the major mechanisms proposed for the disease suppressive effects of various fluorescent pseudomonads. Mondal *et al.* (2001) demonstrated that 2,4-diacetylphloroglucinol was a key metabolite involved in the suppression of bacterial blight of cotton by strain CRb-26 of *P. fluorescens.* The biocontrol efficacy achieved by biocontrol agents viz *P. fluorescens* (Pf32 and Pf93) and *B. subtilis* (B49) exhibiting several distinct mechanisms of control in the present study might be due to additive or synergistic effects.

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