ISOLATION AND CHARACTERIZATION OF TWO NEW *BACILLUS SUBTILIS* STRAINS FROM THE RHIZOSPHERE OF EGGPLANT AS POTENTIAL BIOCONTROL AGENTS

D. Saha[1], G.D. Purkayastha[1], A. Ghosh[1], M. Isha[2] and A. Saha[2]

[1] *Plant Biotechnology Laboratory, Department of Biotechnology, North Bengal University, Siliguri 734013, India*
[2] *Molecular Plant Pathology and Fungal Biotechnology Laboratory, Department of Botany, North Bengal University, Siliguri 734013, India*

SUMMARY

Fusarium wilt is one of the most important diseases of eggplant and the cause of major crop losses. The present work was undertaken to evaluate the possibility of using antagonistic bacteria isolated from eggplant rhizosphere as biocontrol agents to reduce the incidence of wilt caused by *Fusarium solani*. Out of 141 isolates, two strains AI01 and AI03 which showed maximum antagonistic activity during initial screening against *F. solani* were selected. The strains were identified as *Bacillus subtilis* based on physiological and biochemical characters and analysis of the 16S rRNA gene sequence. Antagonistic activity was further tested against ten important fungal pathogens *in vitro*, which revealed that both strains inhibited these phytopathogens to a desirable extent. Scanning electron microscope observations disclosed the presence of deformity and lysis of fungal mycelia at the zone of pathogen/antagonistic bacteria interaction. Robust swarming motility was exhibited by strain AI01 in dual culture plates, which limited fungal growth. Studies on antifungal metabolites secreted by the bacteria confirmed the presence of siderophore and several hydrolytic enzymes like chitinase, protease, lipase and amylase. AI01 and AI03 were also capable of producing indole acetic acid (IAA), a plant growth-promoting trait, but exhibited no deleterious effects like production of hydrogen cyanide, cellulase and pectinase. AI01 possesses higher ability for biofilm formation on the wells of microtitre plate. Both bacterial isolates showed significant reduction of wilt incidence in eggplant seedlings pretreated with them under greenhouse conditions. Disease incidence was reduced upto 72% when the soil was sterilized before treatment. Treatment in unsterilized soil was less efficacious. Since both *B. subtilis* strains exhibited several traits beneficial to the host and showed promising results when applied as bioinoculants, they may be used to develop new, safer and effective formulations as an alternative to chemical fungicides.

Corresponding author: D. Saha
Fax: +91.353. 2699001
E-mail: dsahanbu@yahoo.com

Key words: antifungal metabolites, biofilm, biological control, *Fusarium solani*, siderophore, wilt disease.

INTRODUCTION

The eggplant (*Solanum melongena*) is one of the most widely grown vegetable crop all over the world, including the Indian sub-continent. This crop is prone to massive attacks by several species of *Fusarium* that cause wilt and root rot (Chakraborty and Chatterjee, 2007, 2008; Joseph *et al*., 2008; Akhtar *et al*., 2010). Because of the increasing restriction in the use of chemical fungicides due to concern for the environment and human health, microbial inoculants have been experimented extensively during the last decade to control wilt and other plant diseases (Siddiqui and Shakeel, 2006; Chakraborty and Chatterjee, 2008; Akhtar *et al*., 2010).

Bacteria have been explored as biocontrol agents for plant diseases (Gerhardson, 2002) and also as plant growth promoters and inducers of disease resistance (Catellan *et al*., 1999; Bargabus *et al*., 2002; Bais *et al*., 2004). Apart from improving plant health, they also meet the increasing demand for low-input agriculture. The use of antagonistic bacteria is reported as a powerful strategy to suppress soil-borne pathogens due to their ability to antagonize the pathogen by multiple modes and also to effectively colonize the rhizosphere. The widely recognized mechanisms of biocontrol action are competition for an ecological niche or substrate, as well as the production of inhibitory compounds and hydrolytic enzymes that are often active against a broad spectrum of fungal pathogens. Many microorganisms are known to produce multiple antibiotics which can suppress one or more pathogens (Haas and Defago, 2005; Stein, 2005; Ge *et al*., 2007). For instance, *Bacillus subtilis* produces several ribosomal and non-ribosomal peptides that act as antibiotics such as iturins, surfactins and zwittermycin (Asaka and Shoda, 1996; Stein, 2005) and it secretes also hydrolytic enzymes, i.e. protease, glucanase (Cazorla *et al*., 2007), chitinase (Manjula *et al*., 2004), lipase (Detry *et al*., 2006) and amylase (Konsoula and Liakopoulou-Kyriakides, 2006).

Extensive studies has been carried out to find proper

and effective means of application of biocontrol agents *in vivo* and their sustenance in soil environment (Jayaraj *et al*., 2007; Muller and Berg, 2008; Chakraborty *et al*., 2009). In many instances, *Pseudomonas* spp. and *Bacillus* spp. have been applied as biocontrol agents to suppress plant-pathogenic organisms (Joseph *et al*., 2008; Koumoutsi *et al*., 2007; Akhtar *et al*., 2010). *Bacillus* spp. in particular are gaining recognition as safe biocontrol agents in a variety of crops, specifically as seed protectants and antifungal agents (Asaka and Shoda, 1996; Stein, 2005). Moreover, they are spore-formers, which imparts a natural formulation advantage over other microorganisms (Emmert and Handelsman, 1999; Romero *et al*., 2007; Haas and Defago, 2005).

The present work aimed at evaluating the possibility of using antagonistic bacteria for controlling *Fusarium* wilt in eggplant. Therefore, two *Bacillus* spp. from eggplant rhizosphere that exhibited broad spectrum antagonistic activity against several fungal pathogens were isolated and characterized. The ability of these isolates to produce specific metabolites that might be involved in pathogen suppression, effective colonization and plant growth promotion was tested *in vitro*. Finally, their biocontrol potential in suppressing wilt incidence in eggplant seedlings caused by *F. solani* was evaluated *in vivo*.

MATERIALS AND METHODS

Microorganisms. The pathogens *Fusarium solani* (causing wilt), *F. oxysporum* (causing root rot) and *Sclerotinia sclerotiorum* (causing stem rot) were obtained from the Indian Type Culture Collection, IARI, New Delhi. *Colletotrichum gloeosporioides* (causing anthracnose disease) was isolated from infected eggplants. *Alternaria alternata*, *Macrophomina phaseolina*, *Pestalotiopsis theae*, *Colletotrichum camelliae*, *Lasiodiplodia theobromae*, *Curvularia eragrostidis* and *Rhizoctonia solani* had been isolated in our laboratory in previous studies (Dasgupta *et al*., 2005; Saha *et al*., 2005, 2008; Mandal *et al*., 2006; Choudhuri *et al*., 2008). The identity of *A. alternata*, *L. theobromae*, *C. eragrostidis* and *R. solani* were further confirmed from IARI, where these cultures were also deposited. Fungal cultures were maintained on potato dextrose agar (PDA) by subculturing at regular intervals.

Plant material. Eggplants cv. Pusa Purple Long (PPL) were used for *in vivo* experiments. Six- to eight-week-old eggplant seedlings, raised in seed beds were transplanted to pots (22 x 20 cm) containing 4.5 kg sterilized garden soil. Watering was with clean sterile water. The potted plants were maintained in screenhouses in the experimental garden of the Department of Botany, University of North Bengal (India) under normal light and temperature conditions.

Isolation of bacteria. Samples of rhizosphere soil from eggplant fields were collected from five different locations in the sub-Himalayan region of West Bengal, where biocontrol agents have never been applied. Plant roots with adherent soil were taken from healthy plants, placed in sterile bags and transported to the laboratory within 1 h. Before isolation, the roots were gently shaken to remove excess soil and vortexed for 10 min in sterile distilled water (1 g per 10 ml). Samples were serially diluted with sterile distilled water from 10^{-1} to 10^{-6} dilutions and 100 µl of each dilution was plated onto nutrient agar (Guha Roy *et al*., 2007). After incubation for 72 h at 28°C, 141 colonies were picked from dilution plates and maintained as pure cultures in nutrient agar slants with periodic transfers to fresh medium.

Test for antifungal activity. All the 141 bacterial isolates were initially screened by the dual culture technique for antagonism *in vitro* against *F. solani*. A 4 mm diameter mycelial disc of the fungal pathogen was inoculated at the centre of a 9 cm diameter Petri plate containing PDA and the bacterial isolate was streaked at a distance of 2-3 cm from the centre in a semi-circular pattern. The plates were incubated at 28°C and checked daily until the fungal growth on the control plate (inoculated only with *F. solani*) reached the edge of the plate.

For estimating antagonistic ability, the strains that tested positive by the initial screening were streaked circularly at a distance of 2 cm from the central 4 mm fungal inoculum in 9 cm PDA plates. The control plates were inoculated only with the fungal pathogens. Radial mycelial growth was recorded until the fungal growth in the control plates reached the edge of the plates. Percent inhibition was calculated as

$$[(9\text{-}d)/9] \times 100$$

where d is the diameter in cm of the indicator pathogen in the test plates. The tests were performed in five replications. Standard error was calculated using the statistical software SPSS version 11.0.

Characterization of bacterial strains. Phenotypic characterization was done with isolates AI01 and AI03 which showed maximum antagonism against all tested pathogens. The tests conducted were Gram staining, catalase activity, oxidase activity, nitrate reduction, production of hydrogen sulphide, hydrolysis of gelatin and starch, utilization of citrate and production of acid from glucose, L-arabinose, mannose, D-xylose, mannitol, inulin, raffinose, and cellobiose and growth at 42 and 4°C (Barrow and Feltham, 1993). Results of these tests were scored as either positive or negative.

Further characterization was done using 16S rRNA gene sequence. To this aim, total genomic DNA was isolated from strains AI01 and AI03 by the CTAB

method (Gomes *et al*., 2000). 16S rDNA primers fD1 and rP2 (Weisberg *et al.,* 1991) were used for PCR amplification of the 16S rRNA gene, which was performed in 25 µl reaction using the following conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min on a thermal cycler GeneAmp PCR 2400 (Applied Biosystems, USA). The amplified product was purified using PCR purification Kit (Bangalore Genei, India) and sequenced by Bangalore Genei. The obtained 16S rRNA gene sequences were deposited in GenBank under the accession Nos EU661865 (AI01) and EU661867 (AI03). Similarity searches of the sequences were carried out using the BLAST function of GenBank (Altschul *et al*., 1990).

Studies on the effect of biocontrol isolates on *F. solani* mycelium by scanning electron microscopy. Bacterial isolates and *F. solani* were co-inoculated on PDA plates as described in dual culture technique. Coverslips were placed at the bacterial-fungal interaction zone of dual culture plates and towards the edge of the Petri plate in the case of control. After fungal mycelia grew over the cover slip, it was removed and placed on a Petri plate. The mycelium was fixed with 2.5% glutaraldehyde solution for 1 h followed by dehydration through an ascending series of ethanol: 50% and 70% for 5 min, 90% ethanol for 30 min with two consecutive changes, and were finally dipped in 100% ethanol till microscopic observation. All ethanol dilutions were made with distilled, deionized water. After dehydration the samples were air-dried, adhered to stubs, coated with gold in a IB2-ion coater, and observed under a scanning electron microscope Hitachi S-530.

Test for hydrolytic activity and HCN production. Chitinase activity was determined by a modification of the method described by Bargabus *et al*. (2002). Bacterial cultures (24 h) in nutrient broth were centrifuged and the supernatants were filtered through 0.2 µm diameter cellulose acetate filter paper (Sartorius, USA). Culture filtrates (100 µl) were placed on agar cups containing 0.1% glycol chitin (Sigma-Aldrich, USA) and 1% agarose. The plates were incubated at 37°C for 24 h, then stained with fluorescent brightener 28 (Sigma-Aldrich, USA) and observed under UV light. Positive result was indicated by the presence non-fluorescent lytic regions. Cellulase and pectinase production by the antagonistic bacterial isolates were determined according to Cattelan *et al*. (1999) as modified by Kumar *et al*. (2005). For testing cellulase production, 9 cm Petri dishes containing M9 medium (Miller, 1974), supplemented with 10 g l^{-1} cellulose and 1.2 g l^{-1} yeast extract were inoculated and incubated for 8 days at 30°C. A clear halo surrounding bacterial colonies was considered as positive for cellulase production. Pectinase activity was tested in the same way but by amending M9 medium with 10 g l^{-1} pectin instead of cellulose. The plates were inoculated and incubated for 2 days at 30°C, then flooded with 2 M HCl. A positive result was indicated by visible clear halos around the colonies. Phosphatase activity was determined in Pikovskaya's agar medium (Pikovskaya, 1948) in which the development of a clear zone after 2-5 days of incubation at 30°C was considered as positive for phosphatase production. Proteolytic, amylolytic and lipolytic activities were determined as hydrolysing ability of the substrates casein, starch and Tween 80, respectively (Barrow and Feltham, 1993). HCN production test was performed as described (Askeland and Morrison, 1983; Bakker and Schippers, 1987). Briefly, bacteria were heavily inoculated in nutrient agar plates and incubated in an inverted position at 30°C with filter paper strips dipped in picric acid placed inside the lids. Change of colour of the indicator strip from yellow to brown was considered as a positive result.

Test for siderophore production. Siderophore production was determined using the Universal Chrome azurol S (CAS) assay (Schwyn and Neilands, 1987). Identification of catecholate type of siderophore was carried out according to Arnow (1937). Siderophore production was quantified following the method described by Sayyed *et al*. (2005). Siderophore content was calculated by using the formula:

$$\% \text{ siderophore units} = (A_r - A_s)/A_r \times 100,$$

where, A_r = absorbance of reference (uninoculated medium) at 630 nm and A_s = absorbance of the sample at 630 nm.

Test for production of plant growth promoting phytohormone IAA. Production of IAA (indole-3-acetic acid) was determined according to Patten and Glick (2002). Twenty-four -hour-old bacterial cultures grown on Luria-Bertani (LB) broth supplemented with L-tryptophan were centrifuged to obtain culture supernatants. The supernatants (1 ml) were mixed vigorously with 4 ml of Salkowski's reagent (Gordon and Weber, 1951) and the absorbance was measured after 20 min at 535 nm. The concentration of IAA was determined by comparison with the standard curve.

Assessment of biofilm formation by microtitre plate assay. Biofilm formation by the biocontrol bacterial isolates was assessed by the method of Harvey *et al*. (2007) with slight modification. Bacterial cultures (125 µl) grown in LB for 18 h were transferred to 5 ml of growth medium (LB) and vortexed for 1 min. Aliquots of 100 µl of the vortexed mixture were transferred to three wells of sterile polystyrene microtitre plates and incubated at 30°C for 24 or 48 h. Control wells contained

sterile growth medium only. Cell cultures were discarded after incubation and the plates were washed thrice with sterile distilled water and air-dried at 30°C for 30 min. Aqueous 1% crystal violet solution (150 µl) was added to each well and incubated at 30°C for 45 min. The crystal violet solution was then removed and the plates were washed thrice with sterile distilled water and air-dried as before. Next, 95% ethanol was added (100 µl) to the wells for destaining the biofilm and the concentration of crystal violet was determined by measuring the optical density (OD) at 595 nm (CV-OD_{595} value) in a micro-titre plate reader. The background error was corrected by subtracting the mean CV-OD_{595} value of the control wells from the mean CV-OD_{595} value of the strains AI01 and AI03.

Inoculum preparation for *in vivo* studies. The bacterial isolates AI01 and AI03 were grown separately in nutrient broth at 30°C for 48 h under constant shaking (120 rpm) and the culture thus obtained was centrifuged at 6,000 rpm for 10 min. Pelleted bacterial cells were suspended in 0.1 M phosphate buffer (pH 7.0) to obtain a final concentration of 10^8 CFU ml^{-1} and amended with carboxy methyl cellulose (1%) which acted as a binder (Nandakumar *et al*., 2001).

Fungal inoculum was prepared on wheat seeds. The seeds were washed and soaked in distilled water for 10-12 h in 500 ml Erlenmeyer flasks. Subsequently, excess water was drained off and the flasks, one-third filled with the imbibed seeds, were autoclaved twice in two consecutive days. Each sterilized flask was inoculated with five mycelial disks taken from 7-day-old PDB cultures of *F. solani* (Leslie *et al*., 2006). The inoculated flasks were incubated at 28°C for 20 days and shaken every three days to avoid lump formation and allow uniform growth of the fungus. For soil inoculation, 10 g of the infected seeds were mixed per kg soil in the experimental pots.

***In vivo* studies for the management of Fusarium wilt in eggplant by bacterial antagonists.** Six-week-old eggplant seedlings were transplanted to pots (20 cm diameter) containing sterilized garden soil and organic manure (1:1) pre-treated with fungal inoculum. The bacterial culture (25 ml) was poured carefully at the root level (Nandakumar *et al*., 2001). Disease severity was recorded by visual observation with reference to the untreated control where no biocontrol bacteria but the pathogen was applied. For each treatment 10 healthy plants were selected. All plants were kept in the experimental screenhouse under normal light and temperature conditions and watered with sterile water at regular intervals. Another set of treatment was performed replacing sterilized garden soil with unsterilized garden soil. In this case, unsterile tap water was used for watering the seedlings. All other experimental condition was similar to that of the sterilized set. Assessment of wilt incidence was done after 8 and 16 days following the method of Chen *et al*. (1995). The plants were uprooted carefully and symptom severity was graded into five disease classes, as follows: 0 = no disease; 1 = 0-25% of the leaves withered; 2 = 26-50% of the leaves withered; 3 = 61-75% of the leaves withered; 4 = 76-100% of the leaves withered. Based on the classes, the disease index was calculated using the following formula:

$$\text{Disease index} = \Sigma\,[(P \times DC) \times 100]/(T \times 4),$$

where P = plants per class, DC = disease class and T = total number of plants.

Percent efficacy of disease control (PEDC) was calculated using the formula (Purkayastha *et al*., 2010):

PEDC= [(Disease index in untreated control – Disease index in treated plants)/Disease index in untreated control]x100

Table 1. *In vitro* antifungal activity of two *Bacillus subtilis* strains against plant pathogens.

Pathogenic fungi (Host)	Strain identity	Percentage Inhibition over control[(a)]	
		AI01	AI03
Alternaria alternata (niger)	ITCC-6250.05	80.7±2.0	69.6±1.3
Lasiodiplodia theobromae (tea)	ITCC-5446.02	83.3±1.9	67.7±1.1
Colletotrichum camelliae (tea)	CC01	79.2±2.1	51.4±2.1
Colletotrichum gloeosporioides (eggplant)	ITCC-5446.02	74.4±1.9	58.1±2.1
Curvularia eragrostidis (tea)	ITCC-4150.2k	71.1±0.6	65.1±10
Fusarium oxysporum	ITCC-6246	66.7±0.8	65.2±0.6
Fusarium solani (eggplant)	ITCC-4999	66.7±1.6	63.3±0.7
Macrophomina phaeseolina (jute)	MP02	78.5±1.3	56.6±1.7
Pestalotiopsis theae (tea)	PT01	91.4±1.3	85.9±1.6
Rhizoctonia solani (tea)	ITCC-5995.05	87.4±0.6	85.1±1.0
Sclerotinia sclerotiorum	ITCC-6094	83.3±0.7	81.1±1.2

[(a)]: Inhibition zone was measured when fungal mycelia in the control plate reached the edge of petriplate. Data represents mean ± standard error value of five replicates.

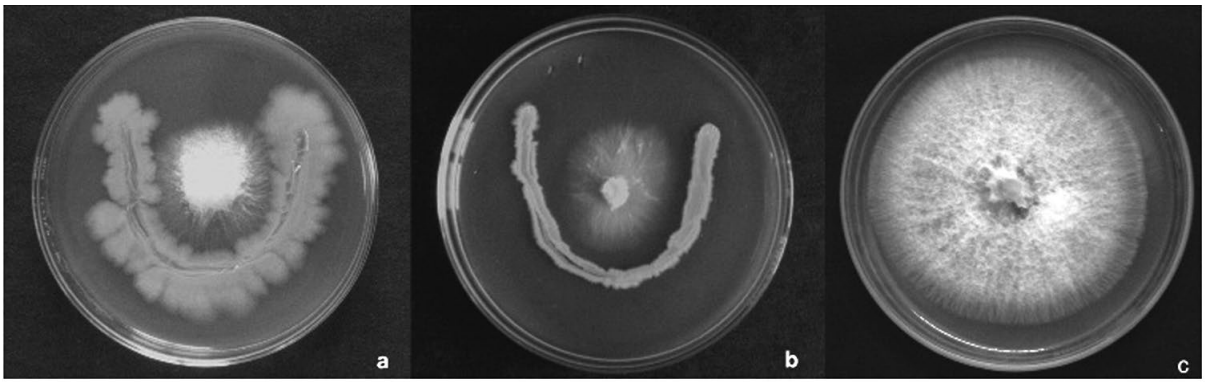

Fig. 1. Inhibition of *Fusarium solani* by antagonistic bacterial strain: (a) inhibition by *Bacillus subtilis* strain AI01; (b) inhibition by *Bacillus subtilis* strain AI03; (c) mycelial growth of *F. solani* in the control plate.

The experiment was replicated three times and data from three independent experiments were pooled and averaged. Standard error was calculated using the statistical software SPSS version 11.0.

RESULTS

Screening of antagonistic bacterial isolates against plant pathogens. Out of the 141 bacterial strains isolated from the rhizosphere, 33 produced inhibition zones against *F. solani* during the initial screening. Antagonistic activity of these 33 strains was assayed against 10 other phytopathogenic fungi by dual culture assay. Results showed that all tested bacterial strains produced inhibition zones against at least two of the tested pathogens (data not shown). Isolates AI01 and AI03 which showed highest antifungal activity against all the tested phytopathogens were selected for further studies on identification, electron microscopy and biocontrol of *Fusarium* wilt of eggplant. The level of antifungal activity exhibited by AI01 and AI03 in agar plates against individual fungi is summarized in Table 1. Both strains limited the growth of fungal mycelia to a considerable extent (Fig. 1a and 1b) as compared to the control (Fig. 1c). The strain AI01 showed swarming motility over agar surface and covered a major portion of the plates, thereby restricting the growth of the pathogen (Fig. 1a).

Identification and characterization of antagonistic bacteria. Bacterial characterization tests revealed that the strains AI01 and AI03 were Gram-positive, aerobic, endospore-forming, rod-shaped bacteria producing opaque colonies that were catalase positive and oxidase negative. The isolates were capable of hydrolyzing gelatin and starch. Both strains reduced nitrate to nitrite and tested positive for citrate utilization and H_2S production. Both bacteria were able to grow at 42°C and produced acid from all the tested carbon sources.

Amplification of 16S rRNA gene by PCR resulted in a product approximately 1.5 kb in size. Sequencing of the PCR product followed by BLAST searches revealed that AI01 and AI03 showed 98% similarity to *Bacillus subtilis* strains deposited in GenBank. The results of molecular analyses were consistent with the biochemical and physiological traits of the isolates as reported in Bergey's Manual of Systematic Bacteriology (1986). The findings from biochemical characterization studies as well as BLAST searches led to the conclusion that AI01 and AI03 are both *B. subtilis* isolates.

Fig. 2. Scanning electron microphotographs showing the antagonistic effect of biocontrol bacterial isolates on *Fusarium solani.* (a) Intact mycelia of *F. solani*; (b) Lysis of mycelia caused by isolate AI01 (indicated by arrows) and accumulation of bacterial cells; (c) Mycelial distortion caused by isolate AI03 (indicated by arrows).

Table 2. Production of hydrolytic enzymes and other substances related to biocontrol activity by the isolated *Bacillus subtilis* strains.

Production of	*Bacillus subtilis* strains	
	AI01	AI03
Lipase	+	+
Protease	+	+
Chitinase	-	+
Siderophore	+[(a)]	+[(a)]
HCN	-	-
Pectinase	-	-
Phosphatase	-	-
Cellulase	-	-
Indole acetic acid	+	+
Biofilm	+	+

+, tested positive; -, tested negative;
[(a)]: catecholate type of siderophore.

Studies on interaction of fungal pathogen and antagonistic bacteria by scanning electron microscopy. The zone of interaction between *F. solani* and *B. subtilis* revealed disintegration and bursting of the fungal mycelia and subsequent outflow of protoplasm. Bacterial cells accumulated around the burst sites and were often attached to the hyphal surface (Fig. 2b and 2c). Deformation of the hyphae with irregular cell surface morphology were also noticed. In the control, *F. solani* mycelium remained intact (Fig. 2a).

Production of hydrolytic enzymes and metabolites by bacterial antagonists. Both bacterial isolates showed positive results for lipase and protease production (Table 2). AI03 showed positive chitinase activity by producing clear halo under UV light. Both bacteria produced siderophore on CAS agar plates, and Arnow's test confirmed that the siderophore was catecholate type. However, AI03 produced a higher level (50%) of siderophore than AI01 (31%). IAA was produced by both bacteria while neither of them showed phosphate solubilizing ability in Pikovskaya's agar plates. AI03 produced 60 µg ml^{-1} of IAA under assay conditions while 35 µg ml^{-1} was the production by AI01.

Formation of biofilm by the bacterial antagonists. Both isolates formed biofilm in LB medium. AI01, however, was a more efficient producer than AI03. The level of adherence increased with increasing incubation period and almost doubled from 24 to 48 h.

***In vivo* biocontrol study for the management of wilt in eggplant.** *B. subtilis* AI01 and *B. subtilis* AI03 significantly reduced disease incidence as compared with the untreated controls (Fig. 3a) within 8 days of treatment, regardless of the soil condition. However, when sterile soil was used, the percentage efficacy of disease class (PEDC) was higher than in unsterile soil. The level of PEDC was slightly higher after 16 days of treatment (Table 3). The inhibition of *Fusarium* wilt afforded by the two isolates in sterilized soil was virtually the same: i.e. 72% of treated seedlings (AI01) (Fig 3b) and 70% of treated seedlings (AI03) (Fig. 3c).

DISCUSSION

The present study has shown that *Fusarium* wilt of eggplant can be efficiently controlled by *B. subtilis* strains AI01 and AI03 recovered from the host rhizosphere. Both strains were also effective in inhibiting the growth of a broad spectrum of other plant pathogenic fungi *in vitro*. As mentioned, biochemical characterization studies and phylogenetic analysis of 16S rRNA gene sequences revealed that both AI01 and AI03 were

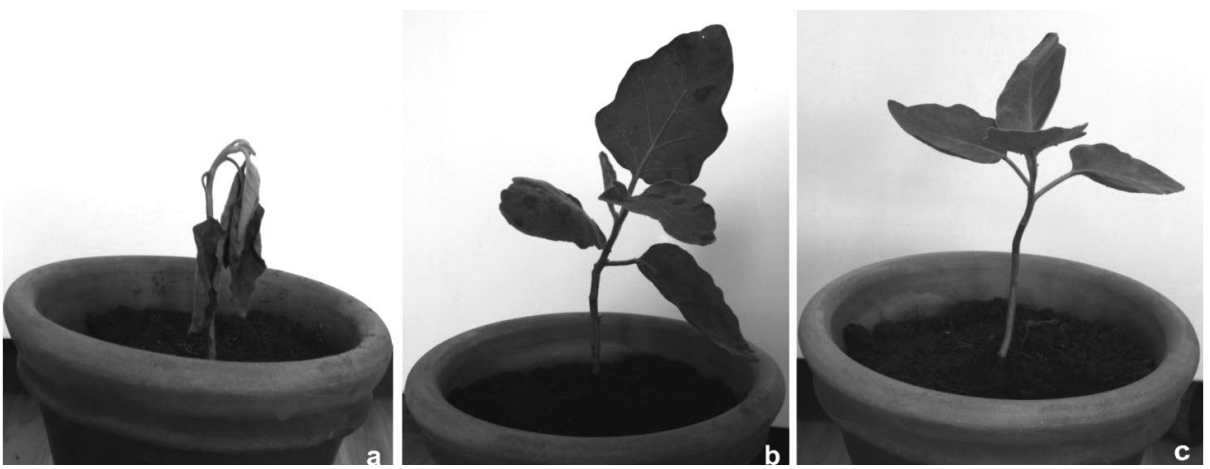

Fig. 3. Suppression of wilt disease in eggplant by the isolated *Bacillus subtilis* strains under sterile soil conditions: (a) severe wilt in untreated control set; (b) disease suppression by strain AI01; (c) disease suppression by strain AI03.

Table 3. Inhibition of wilt disease caused by *F. solani* in eggplant seedlings by antagonistic bacterial isolates AI01 and AI03.

Treatments	8 days after inoculation		16 days after inoculation	
	Disease index	PEDC [(a)]	Disease index	PEDC [(a)]
AI01 (sterilized soil)	24.7 ± 1.5	70.0 ± 1.5	23.9 ± 0.7	72.0 ± 1.2
AI01 (unsterilized soil)	31.3 ± 1.4	62.3 ± 1.2	31.1 ± 1.0	64.0 ± 0.9
AI03 (sterilized soil)	27.2 ± 1.3	67.0 ± 1.0	25.6 ± 0.9	70.0 ± 1.4
AI03 (unsterilized soil)	36.9 ± 1.2	55.5 ± 1.3	34.6 ± 2.0	60.0 ± 1.1
Untreated control (sterilized soil)	82.5 ± 1.3	0.0 ± 0.0	85.5 ± 1.3	0.0 ± 0.0
Untreated control (unsterilized soil)	83.0 ± 2.0	0.0 ± 0.0	86.5 ± 0.9	0.0 ± 0.0
C. D. at 5%	1.95	1.60	1.89	1.43

[(a)]PEDC: Percent efficacy of disease control. PEDC = [(Disease index in untreated control – Disease index in treated plants)/ Disease index in untreated control] x100. Data represent the means ± standard error.

Bacillus subtilis. Apart from *B. subtilis,* other species of the genus *Bacillus* that showed 98% similarity with the strains in question during the BLAST searches were *B. tequilensis* and *B. licheniformis*. However, *B. licheniformis* is capable of anaerobic growth (Claus and Berkeley, 1986) while AI01 and AI03 are strictly aerobic. *B. tequilensis* produces a yellow pigment, has colonies with a smooth appearance and is incapable of starch hydrolysis (Gatson *et al*., 2006). In contrast, AI01 and AI03 colonies are white to cream in colour with a rough surface (often wrinkled) and capable of starch hydrolysis, which matches with characters of *B. subtilis* (Claus and Berkeley, 1986). Additionally, *B. tequilensis* reduces nitrate to nitrogen whereas *B. subtilis* as well as our strains is capable of nitrate to nitrite reduction (Gatson *et al*., 2006). There are several phenotypic similarities between the two isolated strains and their ability to produce acid from carbon sources is the same. However, AI01 showed a better antagonistic activity *in-vitro*, although, unlike AI03, it did not produce chitinase.

Bacterial colonization and antagonistic activity in the environment is enabled by the production of bacterial allelochemicals that includes lytic enzymes, iron-chelating siderophores and antibiotics (Compant *et al*., 2005). In our case, isolate AI03 secreted chitinase, which allows the bacteria to degrade the fungal cell-wall. Extracellular chitinase production is considered crucial for the antagonistic activity of bacterial strains (Li *et al*., 2008; Shali *et al*., 2010). Application of a chitinase-producing strain of *Bacillus cereus* directly to soil significantly protected cotton seedlings from root rot disease caused by *Rhizoctonia solani* (Pleban *et al*., 1997). Cellulase and pectinase activity was not observed in any of our isolates which can be regarded as a desirable trait for the production of cellulase and pectinase is considered an undesirable characteristic of plant beneficial bacteria (Cattelan *et al*., 1999). Lack of hydrogen cyanide production is another beneficial trait present in our isolates for HCN is considered to inhibit plant growth and yield due to the interference with cytochrome oxidation (Bakker and Schippers, 1987). Biofertilizer traits like secretion of siderophore and IAA along with the ability to grow at 42°C may enhance the potential of AI01 and AI03 as bioinoculants. It has been documented that IAA production by bacteria associated with plants enhances the development of host plant root system, thereby favouring the growth of crop plants (Patten and Glick, 2002). Sequestration of iron by microbial siderophores were found to significantly increase crop yield and provide competitive inhibition to the growth of soil-borne pathogens (O'Sullivan and O'Gara, 1992). Our strains produce catecholate siderophores that may be involved in depriving phytopathogens of iron. *B. subtilis* is a well known producer of catecholate siderophore bacillibactin (Rowland and Taber, 1996) but its involvement in restricting phytopathogens is not well documented. Swarming is often linked with biofilm formation as a collective bacterial process and also with extracellular proteolytic activity (Connelly *et al*., 2004). Our isolates were found to form biofilm, showed proteolytic activity, and isolate AI01 also exhibited robust swarming in agar plates. Bais *et al*. (2004) reported that colonization of plant roots by *B. subtilis* is associated with surfactin production and biofilm formation and, interestingly, the biofilm protected the plant against infection by *Pseudomonas syringae*. Our strains exhibited properties found in good colonizers and showed biofertilizer traits *in vitro*. Further stud-

ies on the contribution of these factors to the *in vivo* biocontrol activity wouldbe of interest.

Scanning electron microscopic studies revealed the nature of antagonism involved in the interaction between the isolated *Bacillus* strains and *F. solani*. A clear distortion of fungal mycelium following lysis and bursting of the hyphae was observed at the interaction zone in dual cultures. The burst sites were surrounded by bacteria, some of which were found attached to the hyphae. Lim *et al.* (1991) reported abnormal hyphal swelling and lysis of hyphae in *F. solani* when co-cultured *in vitro* with the biocontrol strain YPL-1 of *Pseudomonas stutzeri*. Severe mycelial deformations of *Curvularia lunata* caused by *Bacillus* sp. strain BC121 was observed by Basha and Ulaganathan (2002). Senthilkumar *et al*. (2007) have also reported several structural deformities like hyphal lysis and bulging of the mycelium of *Rhizoctonia bataticola* caused by *Paenibacillus* sp. HKA-15. Enzymatic dissolution of cell walls leading to loss of fungal protoplasm is one of the main antagonistic mechanisms involved in the activity of biocontrol agents (Lim *et al*., 1991; Kim and Chung, 2004). Our strains are producers of several lytic enzymes, among which chitinase, a degrader of the fungal cell wall, by AI03 in particular. This is taken as an indication that cell wall degradation may be one of the mechanisms of antagonism exhibited by our bacterial isolates.

In screenhouse experiments, biocontrol bacteria and fungal pathogen were co-inoculated to assess the efficiency of the isolated bacteria in reducing wilt disease. Co-inoculation was successfully used for controlling *Macrophomina* and *Aspergillus* infecting chickpea with *Pseudomonas* M1P3 (Saraf *et al*., 2008). *Pseudomonas stutzeri* YPL-1 co-inoculated with the pathogen *F. solani* was also found to suppress root-rot of kidney bean (*Phaseolus vulgaris*) to a desirable extent (Lim and Kim, 1995). Thus, the application method is another aspect that contributes significantly towards achieving a good biocontrol efficiency and plant growth promotion (Xue *et al*., 2009). We have inoculated the bacteria in the soil as this method has been shown to produce better levels of colonization and biocontrol efficiency than other systems, like root dipping (Xue *et al*., 2009) or seed inoculation (Gotz *et al*., 2006). In our study, we have obtained about 70% reduction in disease incidence in the eggplant cv. Pusa Purple Long on direct soil application of *B. subtilis* isolates. Both AI01 and AI03 were equally competent in controlling pathogen infection *in vivo* although AI01 exhibited a better antagonistic activity *in vitro*. Others have previously reported suppression of root diseases by *Bacillus* spp. both in greenhouse and field conditions (Kim *et al*., 1994; Remadi *et al*., 2006; Nihorimbere *et al*., 2009; Akhtar *et al*., 2010). Remadi *et al*. (2006) observed 46% and 60% inhibition of *F. solani* and *F. graminearum,* respectively, infecting potato tubers following *in vivo* application of *Bacillus* sp. *B. subtilis* was also found to significantly reduce root rot disease caused by *F. solani* to a level of 76% (Kim *et al*., 1994). Akhtar *et al.* (2010) have successfully used mixture of *Pseudomonas alcaligens* and *Bacillus pumilis* to reduce wilt incidence of lentil plants.

In the present study, we have isolated two new *B. subtilis* strains from eggplant rhizosphere with broad-spectrum antagonistic action, which possessed several antifungal characters as well as plant growth promoting traits. These strains were found to be efficient in controlling *Fusarium* wilt of eggplant seedlings. *In vitro* evidences indicate a multiple mode of action including antibiosis, effective colonization and plant growth promotion which reveal their potentiality for field application and commercial use as biocontrol agents. Future studies on commercializing integrated stable bioformulations and their field applications for developing effective biocontrol strategies is in progress.

ACKNOWLEDGEMENTS

Financial assistance received from by the University Grants Commission (UGC), India and Department of Biotechnology, West Bengal is gratefully acknowledged.

REFERENCES

Akhtar M.S., Shakeel U., Siddiqui Z.A., 2010. Biocontrol of *Fusarium* wilt by *Bacillus pumilus, Pseudomonas alcaligenes,* and *Rhizobium* sp. on lentil. *Turkish Journal of Biology* **3**: 1-7.

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Arnow L.E., 1937. Colorimetric determination of the components of 3,4 dihydroxyphenylalanine-tyrosine mixtures. *Journal of Biological Chemistry* **118**: 531-537.

Asaka O., Shoda M., 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. *Applied and Environmental Microbiology* **62**: 4081-4085.

Askeland R.A., Morrison M., 1983. Cyanide production by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **45**: 1802-1807.

Bais H.P., Ray F., Vivanco J.M., 2004. Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiology* **134**: 307-319.

Bakker A.W., Schippers B., 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp.-mediated plant growth-stimulation. *Soil Biology and Biochemistry* **19**: 451-457.

Bargabus R.L., Zidack N.K., Sherwood J.E., Jacobsen B.J., 2002. Characterization of systemic resistance in sugarbeet elicited by a non pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. *Physiology and Molecular Plant Pathology* **61**: 289-298.

Barrow G.I., Feltham R.K.A., 1993. Cowan and Steel's Manual for the Identification of medical bacteria, 3rd edition. Cambridge University Press, Cambridge, UK.

Basha S., Ulaganathan K., 2002. Antagonism of *Bacillus* species (strain BC121) towards *Curvularia lunata*. *Current Science* **82**: 1457-1463.

Cattelan A.J., Hartel P.G., Fuhrmann J.J., 1999. Screening for plant growth–promoting rhizobacteria to promote early soybean growth. *Soil Science Society of American Journal* **63**: 1670-1680.

Cazorla F.M., Romero D., Pérez-García A., Lugtenberg B.J., Vicente A., Bloemberg G., 2007. Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *Journal of Applied Microbiology* **103**: 1950-1959.

Chakraborty M.R., Chatterjee N.C., 2007. Interaction of *Trichoderma harzianum* with *Fusarium solani* during its pathogenesis and the associated resistance of the host. *Asian Journal of Experimental Sciences* **21**: 351-355.

Chakraborty M.R., Chatterjee N.C., 2008. Control of *Fusarium* wilt of *Solanum melongena* by *Trichoderma* spp. *Biologia Plantarum* **52**: 582-586.

Chakraborty M.R., Chatterjee N.C., Quimio T.H., 2009. Integrated management of fusarial wilt of eggplant (*Solanum melongena*) with soil solarization. *Micologia Aplicada International* **21**: 25-36.

Chen C., Bauske E.M., Musson G., Rodriguez-Kabana R., Kloepper J.W., 1995. Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. *Biological Control* **5**: 83-91.

Choudhuri C., Saha D., Saha A., 2008. Screening of resistant varieties of niger (*Guizotia abyssinica*) against *Alternaria alternata* by serological techniques. *National Symposium on Diversity and Functionality of Plants and Microbes, Siliguri, India*: 26.

Claus D., Berkeley R.C.W., 1986. Genus *Bacillus*. Cohn 1872, 174AL. In: Sneath P.H.A., Mair N.S., Sharpe M.E., Holt J.G. (eds). Bergey's Manual of Systematic Bacteriology, Vol. 2, pp. 1105-1139. 1st Ed. Williams and Wilkins, Baltimore, MD, USA.

Compant S., Duffy B., Nowak J., Clément C., Barka E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology* **71**: 4951-4959.

Connelly M.B., Young G.M., Sloma A., 2004. Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. *Journal of Bacteriology* **186**: 4159-4167.

Dasgupta S., Saha D., Saha A., 2005. Levels of common antigens in determining pathogenicity of *Curvularia eragrostidis* in different tea varieties. *Journal of Applied Microbiology* **98**: 1084-1092.

Detry J., Rosenbaum T., Lütz S., Hahn D., Jaeger K.E., Müller M., Eggert T., 2006. Biocatalytic production of enantiopure cyclohexane-trans-1,2-diol using extracellular lipases from *Bacillus subtilis*. *Applied Microbiology and Biotechnology* **72**: 1107-1116.

Emmert E.A.B., Handelsman J., 1999. Biocontrol of plant disease: a Gram-positive perspective. *FEMS Microbiology Letters* **171:** 1-9.

Gatson J.W., Benz B.F., Chandrasekaran C., Satomi M., Venkateswaran K., Hart M.E., 2006. *Bacillus tequilensis* sp. nov., isolated from a 2000-year-old Mexican shaft-tomb, is closely related to *Bacillus subtilis*. *International Journal of Systematic and Evolutionary Microbiology* **56**: 1475-1484.

Ge Y.H., Pei D.L., Zhao Y.H., Li W.W., Wang S.F., Xu Y.Q., 2007. Correlation between antifungal agent phenazine-1-carboxylic acid and pyoluteorin biosynthesis in *Pseudomonas* sp. M18. *Current Microbiology* **54**: 277-281.

Gerhardson B., 2002. Biological substitutes for pesticides. *Trends in Biotechnology* **20**: 338-343.

Gomes L.H., Duarte K.M.R., Andrino F.G., Tavares F.C.A., 2000. A simple method for DNA isolation from *Xanthomonas* spp. *Scientia Agricola* **57**: 553-555.

Gordon S.A., Weber R.P., 1951. Colorimetric estimation of indoleacetic acid. *Plant Physiology* **26**: 192-195.

Götz M., Gomes N.C.M., Dratwinski A., Costa R., Berg G., Peixoto R., Mendonc L., Hagler H., Smalla K., 2006. Survival of GFP-tagged antagonistic bacteria in the rhizosphere of tomato plants and their effects on the indigenous bacterial community. *FEMS Microbiological Ecology* **56**: 207-218.

Guha Roy S., Chakraborty S., Mukherjee S.K., 2007. Biological control of *Phytophthora* spp. with a novel indigenous *Pseudomonas* isolate. *Journal of Mycopathology Research* **45**: 117-121.

Haas D., Defago G., 2005. Biological control of soil-borne pathogens by fluorescent Pseudomonads. *Nature Reviews of Microbiology* **3**: 307-319.

Harvey J., Keenan K.P., Gilmour A., 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. *Food Microbiology* **24**: 380-392.

Jayaraj J., Parthasarathi T., Radhakrishnan N.V., 2007. Characterization of a *Pseudomonas fluorescens* strain from tomato rhizosphere and its use for integrated management of tomato damping-off. *Biocontrol* **52**: 683-702.

Joseph B., Dar M.A., Kumar V., 2008. Bioefficacy of plant extracts to control *Fusarium solani* f.sp. *melongenae* incitant of brinjal wilt. *Global Journal of Biotechnology and Biochemistry* **3**: 56-59.

Kim Y.S., Lim H.S., Kim S.D., 1994. *Bacillus subtilis* YB-70 as a biocontrol agent of *Fusarium solani* causing plant root-rot. *Journal of Microbiology and Biotechnology* **4**: 68-74.

Kim P.I., Chung K.C., 2004. Production of an antifungal protein for control of *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens* MET 0908. *FEMS Microbiology Letters* **234**: 177-183.

Konsoula Z., Liakopoulou-Kyriakides M., 2006. Thermostable a-amylase production by *Bacillus subtilis* entrapped in calcium alginate gel capsules. *Enzyme and Microbial Technology* **39**: 690-696.

Koumoutsi A., Chen X.H., Vater J., Borriss R., 2007. DegU and YczE positively regulate the synthesis of bacillomycin D by *Bacillus amyloliquefaciens* strain FZB42. *Applied and Environmental Microbiology* **73**: 6953-6964.

Kumar R.S., Ayyadurai N., Pandiaraja P., Reddy A.V., Venkateswarlu Y., Prakash O., Sakthivel N., 2005. Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad-spectrum antifungal activity and biofertilizing traits. *Journal of Applied Microbiology* **98**: 145-154.

Leslie J.F., Summerell B.A., Bullock S., 2006. The *Fusarium* Laboratory Manual. Blackwell Publishing, Oxford, UK.

Li J.-G., Jiang Z.-Q., Xu L.-P., Sun F.-F., Guo J.-H. 2008. Characterization of chitinase secreted by *Bacillus cereus* strain CH2 and evaluation of its efficacy against Verticillium wilt of eggplant. *Biocontrol* **53**: 931-944.

Lim H.S., Kim Y.S., Kim S.D., 1991. *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. *Applied and Environmental Microbiology* **57**: 510-516.

Lim H.S., Kim S.D., 1995. The role and characterization of _-1,3-glucanase in biocontrol of *Fusarium solani* by *Pseudomonas stutzeri* YPL-1. *Journal of Microbiology* **33**: 295-301.

Mandal P., Das L., Saha D., Saha A., 2006. Screening, isolation and pathogenicity of *Rhizoctonia solani* associated with seeds of tea. *Indian Phytopathology* **59**: 384.

Manjula K., Krishna Kishore G., Podile A.R., 2004. Whole cells of *Bacillus subtilis* AF 1 proved more effective than cell-free and chitinase-based formulations in biological control of citrus fruit rot and groundnut rust. *Canadian Journal of Microbiology* **50**: 737-744.

Miller J.H., 1974. Experiments in Molecular Genetics, 2nd Ed. Cold Spring Harbor Laboratory Press, New York, NY, USA.

Muller H., Berg G., 2008. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on *Verticillium* wilt in oilseed rape. *Biocontrol* **53**: 905-916.

Nandakumar R., Basu S., Viswanathan R., Sheela J., Raguchander T., Samiyappan R., 2001. A new bio-formulation containing plant growth promoting rhizobacterial mixture for the management of sheath blight and enhanced grain yield in rice. *Biocontrol* **46**: 493-510.

Nihorimbere V., Ongena M., Cawoy H., Henry G., Brostaux Y., Kakana P., Thonart P., 2009. *Bacillus*-based biocontrol of *Fusarium* disease on tomato cultures in Burundi. *Communications in Agricultural and Applied Biological Sciences* **74**: 645-649.

O'Sullivan D.J., O'Gara F., 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens *Microbiological Reviews* **56**: 662-676.

Patten C.L., Glick B.R., 2002. Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology* **68**: 3795-3801.

Pikovskaya R.I., 1948. Mobilization of phosphorous in soil in connection with vital activity of some microbial species. *Mikrobiologiya* **17**: 363-370.

Pleban S., Chernin L., Chet I., 1997. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. *Letters in Applied Microbiology* **25**: 284-288.

Purkayastha G.D., Saha A., Saha D., 2010. Characterization of antagonistic bacteria isolated from tea rhizosphere in sub-Himalayan West Bengal as potential biocontrol agents in tea. *Journal of Mycology and Plant Pathology* **40**: 27-37.

Remadi M.D., Ayed F., Jaboun-Khiareddine H., Hibar, Mahjoub M.E., 2006. Effects of some *Bacillus* sp. isolates on *Fusarium* spp. *in vitro* and potato tuber dry rot development *in vivo*. *Plant Pathology Journal* **5**: 283-290.

Romero D., de Vicente A., Rakotoaly R.H., Dufour S.E., Veening J.W., Arrebola E., Cazorla F.M., Kuiper O.P., 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* towards *Podosphaera fusca*. *Molecular Plant-Microbe Interactions* **20**: 430-440.

Rowland B.M., Taber H.W., 1996. Duplicate isochorismate synthase genes of *Bacillus subtilis*: regulation and involvement in the biosyntheses of menaquinone and 2, 3-dihydroxybenzoate. *Journal of Bacteriology* **178**: 854-861.

Saha D., Dasgupta S., Saha A., 2005. Antifungal activity of some plant extracts against important fungal pathogens of tea (*Camellia sinensis*). *Pharmaceutical Biology* **43**: 87-91.

Saha A., Mandal P., Dasgupta S., Saha D., 2008. Influence of culture media and environmental factors on mycelial growth and sporulation of *Lasiodiplodia theobromae* (Pat.) Griffon et Maubl. *Journal of Environmental Biology* **29**: 407-410.

Saraf M., Thakker A., Patel B.V., 2008. Biocontrol activity of different species of *Pseudomonas* against phytopathogenic fungi *in vivo* and *in vitro* conditions. *International Journal of Biotechnology and Biochemistry* **4**: 223-232.

Sayyed R.Z., Badgujar M.D., Sonawane H.M., Mhaske M.M., Chincholkar S.B., 2005. Production of microbial iron chelators (siderophores) by fluorescent pseudomonads. *Indian Journal of Biotechnology* **4**: 484-490.

Schwyn B., Neilands J.B., 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**: 47-56.

Senthilkumar M., Govindasamy V., Annapurna K., 2007. Role of antibiosis in suppression of charcoal rot disease by soybean endophyte *Paenibacillus* sp. HKA-15. *Current Microbiology* **55**: 25-29.

Shali A., Ghasemi S., Ahmadian G., Ranjbar G., Dehestani A., Khalesi N., Motallebi E., Vahed M., 2010. *Bacillus pumilus* SG2 chitinases induced and regulated by chitin, show inhibitory activity against *Fusarium graminearum* and *Bipolaris sorokiniana*. *Phytoparasitica* **38**: 141–147.

Siddiqui Z.A., Shakeel U., 2006. Use of fluorescent *Pseudomonads* isolates for the biocontrol of wilt disease complex of pigeonpea in green house assay and under pot condition. *Plant Pathology Journal* **5**: 99-105.

Stein T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology* **56**: 845-857.

Weisburg W.G., Barns S.M., Lane D., 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.

Xue Q.Y., Chen Y., Li S.M., Chen L.F., Ding G.C., Guo D.W., Guo J.H., 2009. Evaluation of the strains of *Acinetobacter* and *Enterobacter* as potential biocontrol agents against Ralstonia wilt of tomato. *Biological Control* **48**: 252-258.

Received June 17, 2011
Accepted November 23, 2011