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Endophytic *Bacillus* Species Confer Increased Resistance in Cotton Against Damping off Disease Caused by *Rhizoctonia solani*

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Abstract: Endophytic bacterial strains were evaluated for their efficacy against the damping off disease caused by *Rhizoctonia solani* in cotton. Among hundred and three endophytic bacterial strains isolated, two strains (*Bacillus* sp. strains EPCO102 and EPCO16) significantly increased plant growth and inhibited the mycelial growth of *Rhizoctonia solani in vitro* conditions. The efficacy of talc-based bioformulation of endophytic bacterial strains, EPCO102, EPCO16 and *Pseudomonas fluorescens* strain Pf1 amended with and without chitin in inducing systemic resistance was tested against damping off disease under greenhouse conditions. The application of the bioformulation through seed, soil and foliar spray significantly reduced disease incidence under greenhouse conditions. The amendment of chitin in the formulation further reduced the disease incidence. EPCO102, EPCO16 and Pf1 strain along with chitin treatment was recorded 46.7, 53.3 and 40.0% damping-off incidence compared to control 83.3%. Treatment with the endophytic bacterial bioformulation increased the levels of the defence-related enzymes chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and phenol in cotton plants which had been challenged with *R. solani*. In addition to plant growth and antibiosis, endophytic bacterial strains enhanced the resistance in plants through the induction of defense enzymes in cotton plants.

Key words: Bioformulation, endophytes, induced systemic resistance, *Rhizoctonia solani*, *Bacillus*, damping-off

INTRODUCTION

Cotton (*Gossypium* sp.) is an important raw material for textile industry and plays a key role in economic and social affairs of the world engaging about 60 million people either through its cultivation, trade or processing (Mayee *et al*., 2002). Cotton is infected by a number of diseases, among which damping off caused by *Rhizoctonia solani* is important. The losses caused by this disease ranged from 3 to 90% under extreme conditions (Singh and Verma, 1988). Cotton crop suffers heavy losses due to damping off, particularly during the early stage of crop growth. Though effective fungicides are available to manage damping off incidence, they will not be reliable as a long term solution because of the concerns about exposure risks, health and environment hazards and residue persistence. Moreover, frequent applications of fungicides lead to the development of tolerance in the target pathogens. As a result, in recent years, the focus has been towards biological control of diseases using biocontrol agents (Saravanakumar *et al*., 2007), which is safe and promising alternative to fungicides. Among the bioagents, the utilization of the plants own defense mechanism induced by bacterial endophytes is the subject of current interest in the management of pests and diseases (Karban and Baldwin, 1997; Baker *et al*., 1997; Barka *et al*., 2002). Endophytic bacteria colonize the internal tissues of healthy plants without causing any disease symptoms (Wilson, 1995). There is evidence that endophytes contribute to the control of plant diseases (Kloepper *et al*., 1992; Kavino *et al*., 2007), insects (Dimock *et al*., 1988) and nematodes (Hallmann *et al*., 1995). In this study, we address the role of endophytic bacteria in increasing resistance of cotton to the damping off fungus *Rhizoctonia solani*.

Recently, researchers have reported that several bacterial endophytes promote plant growth, help the host to withstand against pathogen attack by competition, antibiosis and eliciting Induced Systemic Resistance (ISR). Mixing of chitin with the bioagents has also been found to increase the biocontrol efficacy (Manjula and Podile, 2001; Radjacommare *et al*., 2002; Bharathi *et al*., 2004).

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The present study has been carried out to evaluate the efficacy of endophytic bacterial strains with or without the addition of chitin against the damping-off pathogen *R. solani* under greenhouse conditions. The objectives are:

- To characterize and evaluate the endophytic bacteria isolated from different parts of cotton crop grown in different regions of South India against damping off disease under greenhouse conditions.
- To study the induction of various defense-related genes encoding proteins implicated in strengthening of plant cell walls by endophytic *Bacillus* sp. in response to infection by *Rhizoctonia solani.*

MATERIALS AND METHODS

Plant material, pathogen and endophytic bacterial strains: The experiments were conducted in the Rockefeller glasshouse, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India during 2002-2004. Susceptible cotton cultivar LRA 5166 obtained from Central Cotton Research Institute, Coimbatore, India and a virulent isolate of damping off pathogen *R. solani* were used in this experiment. The bacterial endophytes were isolated from different parts of cotton plants. For isolation, root and stem sections were made using a sterile scalpel. The root samples were taken just below the soil line for younger plants and 5-10 cm below the soil line for older plants. Stem sections were taken 1-2 cm above the soil line in younger plants and 10 cm above the soil line in older plants. Stem samples were first weighed and surface sterilized with 1% sodium hypochlorite (NaOCl) in 0.05% triton X-100 for 10 min and rinsed four times in 0.02 M sterile potassium phosphate buffer pH 7.0 (PB). A 0.1 mL aliquot was taken from the final buffer wash and transferred to 9.9 mL tryptic soy broth (TSB, Hi Media Code No. M 011) to serve as sterility check. Samples were discarded if growth was detected in the sterility check samples (agitating samples in TSB at 28±2°C) within 48 h.

Each tissue sample (0.5 g) was triturated with sterile mortar and pestle in 9.5 mL of the final buffer wash. Serial dilutions were made in phosphate buffer. Each dilution of every sample was plated (0.1 mL) on three plates each of three different media. (Tryptic soy agar; TSA-Hi Media, Code No. M290, Nutrient agar; NA g L^{-1}, peptone 5, beef extract 2 and agar 20, pH 5.0 and King's B (KB) medium, proteose peptone 20 g, K_2HPO_4 1.5 g, Mg SO_4. $7H_2O$ 1.5 g, glycerol 20 mL, water 1000 mL and agar 15, pH 7.2). The plates were incubated at 28±2°C for 48-72 h. At each sampling date and for each treatment one representative of each bacterium was transferred to fresh respective medium plates to establish pure cultures.

P. fluorescens isolate Pf1 (obtained from Dept. of Plant Pathology, TNAU, Coimbatore) and bacterial endophytes were cryopreserved at -80°C in 44% glycerol and cells from stocks were first grown in KB broth. Inoculum was produced by transferring two loopful from the culture to 100 mL of KB broth in a 250 mL Erlenmeyer flask and incubating at room temperature (28±2°C) on a shaker at 150 rpm for 48 h. These strains were sub-cultured once in a month and maintained until the end of experiment in KB and NA slants at 4°C for further studies.

Screening and selection of bacterial endophytes: The endophytic bacteria were grown in KB and NA broth with constant shaking at 150 rpm for 48 h at room temperature (28±2°C). The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min and bacterial cells were resuspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu mL^{-1} (OD_{595} = 0.3) and used as bacterial inoculum for treating cotton seeds (Thompson, 1996).

Plant growth promoting activities of bacterial endophytic strains was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). The vigour index was calculated by using the formula as described by Baki and Anderson (1973).

$$\text{Vigour index} = \text{Percent Germination} \times \text{seedling length (shoot length + root length)}$$

After screening for plant growth promotion, effective bacterial endophytic strains were tested for their inhibition on *R. solani* mycelial growth by following the dual culture technique (Dennis and Webster, 1971).

Identification of endophytic bacterial isolates: Various biochemical tests were carried out to identify the EPCO16 and EPCO102 endophytic bacterial strains (Aneja, 1993; Schaad, 1992). Endophytic *Bacillus* sp. was grown in nutrient broth or on nutrient agar plates at 28°C. Total DNA (including chromosomal and plasmid DNA) was extracted as described by Robertson *et al.* (1990) with slight modifications. To confirm strains as *Bacillus* sp., 16S rRNA intervening sequence specific BCF1 (CGGGAGGCAGCAGTAGGGAAT); BCR2 (CTCCCCAGGCGGAGTGCTTAAT) primers were used to get an amplicon size of 546 bp (Cano *et al.*, 1994). Further, clones were randomly selected and used as templates in

PCR to produce products of required size 546 bp in agarose gel. DNA sequencing was performed at Genei Pvt Ltd., Bangalore, India.

Talc-based formulation for endophytic bacterial strains: A loopful of bacterium was inoculated into KB and NA broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28±2°C). After 48 h of incubation, the broth containing 9×10^8 cfu mL^{-1} was used for the preparation of talc-based formulation. To 400 mL of bacterial suspension, 1 kg of the purified talc powder (sterilized at 105°C for 12 h), 15 g of calcium carbonate (to adjust the pH to neutral) and 10 g carboxymethyl cellulose (CMC) (adhesive) were mixed under sterile conditions, following the method described by Nandakumar *et al*. (2001). After shade drying over night, the formulation was packed in polypropylene bags and sealed. At the time of application, the population of bacteria in talc formulation was 2.5 to 3×10^8 cfu g^{-1}.

Chitin amendments of talc-based formulations: Five gram of crab shell chitin (Sigma, St. Louis, MO, USA) were slowly added into 100 mL of cold 0.25 N HCl with vigorous stirring and kept overnight at 4°C. The mixture was filtered through glasswool into 200 mL of ice cold ethanol at 4°C with rapid stirring. The resultant chitin suspension was centrifuged at 10000 rpm for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral. The concentration was adjusted to 10 mg mL^{-1} and added to KB and NA broth (1%, v/v). Bacterial endophytes were inoculated into NA broth containing chitin and after 48 h of incubation used for the preparation of talc-based formulation as described earlier.

Greenhouse study: The bacterial strains (with and without chitin) were assessed for their effectiveness in controlling damping off under greenhouse conditions. The trial was conducted in a completely randomized design. Potting medium (red soil: sand: cowdung manure at 1:1:1 w/w/w) was autoclaved for 1 h on two consecutive days. The virulent strain of *R. solani*, mass multiplied in the sand-maize medium, was mixed with the sterilized potting medium at the ratio of 19:1 w/w (sand: maize) (Riker and Riker, 1936). The soil was placed in pots (15 cm diameter; 30 cm height).

Endophytic bacteria treatment and challenge inoculation in greenhouse: Those endophytic bacterial strains showing enhanced seedling vigour and inhibition to *R. solani in vitro* conditions were selected and tested for their ability to reduce damping off on cotton under greenhouse conditions. For seed treatment, the cotton seeds were surface sterilized with 2% sodium hypochlorite, washed three times with sterile water and soaked in endophytic bacterial suspension (9×10^8 cfu mL^{-1}). After 24 h, the bacterial suspension was drained and the seeds shade dried. Bacterized seeds of cotton were thickly sown in the pots at twenty seeds per pot. Carbendazim as seed treatment at 4 g kg^{-1} of seed was included as a standard treatment for comparison. For soil application, 5 g of talc-based formulation per kg of soil was added 30 days after planting (40-45 cm height). Pathogen inoculated and pathogen un-inoculated control (healthy) was maintained. Watering was done and damping-off disease incidence was recorded regularly. Plant growth was measured by randomly selecting five plants in each pot. Four pots per replication were maintained. There were three replications and the pots were arranged in a randomized manner (CRD-Completely randomized design).

The percent disease incidence (PI) was assessed using the following formula:

$$PI = \frac{\text{No. of infected plants}}{\text{Total No. of seeds sown}} \times 100$$

Assay of bacterial endophytes induced proteins

Sample collection and enzyme extraction: The humidity in the greenhouse was maintained at around RH 80%. The temperature was adjusted to 26°C (day)/20°C (night). Plants were carefully uprooted without causing any damage to root tissues at different time intervals (0, 1, 2, 3, 4, 5, 7 and 10 days after the pathogen inoculation). Four plants were sampled from each replication of the treatment separately and were maintained separately for biochemical analysis. Fresh roots were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized root tissues were stored at -70°C.

Assay of peroxidase (PO): Root samples (1 g) were homogenized in 2 mL of 0.1 M phosphate buffer, pH 7.0 at 4°C. The homogenate was centrifuged at 16,000 g at 4°C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract and 0.5 mL of 1% H_2O_2. The reaction mixture was incubated at room temperature (28±2°C). The changes in absorbance at 420 nm were recorded at 30 sec intervals for 3 min. The enzyme activity was expressed as changes in the absorbance of the reaction mixture min^{-1} g^{-1} on fresh weight basis (Hammerschmidt *et al*., 1982).

Assay of polyphenol oxidase (PPO): Root samples (1 g) were homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was used as enzyme source. The reaction mixture consisted of 200 μL of the enzyme extract and 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 μL of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min^{-1} g^{-1} on fresh weight basis (Mayer *et al*., 1965).

Assay of Phenylalanine Ammonia-Lyase (PAL): The PAL assay was carried out as per the method described by Ross and Sederoff (1992). The assay mixture containing 100 μL of enzyme, 500 μL of 50 mM Tris HCl (pH 8.8) and 600 μL of 1 mM L-phenylalanine were incubated for 60 min. The reaction was arrested by adding 2 N HCl. Later 1.5 mL of toluene was added, vortexed for 30 sec, centrifuged (1000 rpm, 5 min) and toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. A standard curve was constructed using solutions of cinnamic acid in toluene of defined concentration.

Total phenolic content: Phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One gram of cotton root tissue was homogenized in 10 mL of 80% methanol with pestle and mortar and agitated for 15 min at 70°C. One milliliter of the methanolic extract was added to 5 mL of distilled water and 250 μL of Folin Ciocalteau reagent (1 N) and the solution was kept at 25°C. After 3 min 1 mL of saturated solution of sodium carbonate and 1 mL of distilled water were added and the reaction mixture was incubated for 1 h at 25°C. The absorption of the developed blue colour was measured using UV-Visible Spectrophotometer (Varian Cary 50, Victoria, Australia) at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reagent with a phenol solution (C_6H_6O) and expressed as catechol equivalents g^{-1} of tissue weight.

Assay of β-1,3 glucanase: β-1,3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method (Pan *et al*., 1991). The reaction mixture consisted of 62.5 μL of 4% laminarin and 62.5 μL of enzyme extract. The reaction was carried out at 40°C for 10 min. The reaction was stopped by adding 375 μL of dinitrosalicylic acid and heating for 5 min in boiling water, vortexed and absorbance measured at 500 nm. The enzyme activity was expressed as μg glucose released min^{-1} g^{-1} of sample.

Assay of chitinase: The colorimetric assay of chitinase (EC 3.2.1.14) was carried out according to the procedure developed by Boller and Mauch (1988). One gram of root tissue was extracted with 5 mL of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10000 rpm at 4°C and the supernatant was used as enzyme source.

Preparation of colloidal chitin: Colloidal chitin was prepared by treating 1 g of crab-shell chitin powder with acetone to form a paste and then slowly adding 20 mL of concentrated hydrochloric acid (HCl) while grinding in a mortar with the temperature maintained at 5°C. After several minutes, the syrupy liquid was filtered through glass wool and poured into vigorously stirred 50% aqueous ethanol to precipitate the chitin in a highly dispersed state. The residue was sedimented and resuspended in distilled water several times to remove excess acid and alcohol and then dialysed against tap water. Chitin content of the suspension was determined by drying a sample *in vacuo* and adjusted with distilled water to a final concentration of 10 mg mL^{-1} (dry weight/volume) and stored at 5°C for further use.

Preparation of snail gut enzyme: Six hundred milligram of the commercial lyophilized snail gut enzyme (Helicase, Sepracor, France) was dissolved in 10 mL of 20 mM potassium chloride (KCl) and chromatographed on a Sephadex G-25 column (38×1.5 cm) using a 10 mM KCl solution, containing 1 mM EDTA and adjusted to pH 6.8 for equilibration and elution. The first 20 mL eluted after the void volume was collected (Boller and Mauch, 1988).

Preparation of p-dimethyl aminobenzaldehyde (DMAB) reagent: Stock solution of DMAB was prepared by mixing 8 g of DMAB in 70 mL of glacial acetic acid along with 10 mL of concentrated HCl. One volume of stock solution was mixed with 9 volumes of glacial acetic acid immediately before use.

Assay: The reaction mixture consisted of 10 μL of 0.1 M sodium acetate buffer (pH 4.0), 0.4 mL enzyme solution and 0.1 mL colloidal chitin. After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 5000 rpm for 3 min. An aliquot of the supernatant (0.3 mL) was pipetted into a glass reagent tube containing 30 μL of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 μL of 3% (w/v) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 μL of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min. and then rapidly cooled in an ice-water bath. After

addition of 2 mL of DMAB, the mixture was incubated for 20 min. at 37°C. Immediately thereafter, the absorbance was measured at 585 nm. N-acetylglucosamine (GlcNAc) was used as a standard and the enzyme activity was expressed as n moles GlcNAc equivalents min^{-1} g^{-1} of fresh weight.

Activity gel electrophoresis

Peroxidase (PO): To study the expression pattern of different isoforms of peroxidases in different treatments, activity gel electrophoresis was carried out. The enzyme extract was prepared by homogenizing 1 g root tissue of cotton in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) at 4°C. The homogenate was centrifuged at 20,000 g for 15 min at 4°C and the supernatant was used as enzyme extract. For native anionic polyacrylamide gel electrophoresis, 8% resolving gel and 4% stacking gel were prepared. After electrophoresis, the gels were incubated in the solution containing 0.15% benzidine in 6% NH_4Cl for 30 min in the dark. Then drops of 30% H_2O_2 were added with constant shaking till the bands appeared. After staining, the gel was washed with distilled water and photographed.

Polyphenol oxidase (PPO): Enzyme was extracted by homogenizing 1 g of tissue in 0.01 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 20,000 g for 15 min at 4°C and the supernatant was used as enzyme source. After native electrophoresis, the gel was equilibrated for 30 min in 0.1% p-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in the appearance of dark brown discrete bands.

Glycol chitin preparation: Glycol chitin was obtained by acetylation of glycol chitosan by Trudel and Asselin (1989). Five grams of glycol chitosan were dissolved in 100 mL of 10% acetic acid by grinding in a mortar. The viscous solution was allowed to stand overnight at 22°C. Methanol (450 mL) was slowly added and the solution was vacuum filtered through a whatman No. 4 filter paper. The filtrate was transferred into a beaker and 7.5 mL of acetic anhydride was added with magnetic stirring. The resulting gel was allowed to stand for 30 min at room temperature and then cut into small pieces. The liquid extruding from the gel pieces was discarded. Gel pieces were transferred to a warring blender, covered with methanol and homogenized for 4 min at top speed. This suspension was centrifuged at 27,000 g for 15 min at 4°C. The gelatinous pellet was resuspended in about one volume of methanol, homogenized and centrifuged as in the preceding step. The pellet was resuspended in distilled water (500 mL) containing 0.02% (w/v) sodium azide and homogenized for 4 min. This was the final 1% (w/v) stock solution of glycol chitin.

Chitinase: Chitinase activity was detected in the PAGE according to Trudel and Asselin (1989) with modification. After electrophoresis, gels were incubated in 150 mM sodium acetate buffer at pH 5.0 for 5 min and then for 30 min at 37°C in 100 mM sodium acetate buffer at pH 5.0, containing 0.01% glycol chitin. The gels were finally transferred into a solution containing 0.01% (w/v) Calcofluor white M2R (Sigma) in 500 mM Tris HCl (pH 8.9). After 5 min the brightener solution was removed and the gels were rinsed with distilled water for more than 1 h. Lytic zones were visualized and photographed under UV light (Alpha innotech Corporation, UK).

Statistical analysis: All the experiments were repeated once with similar results. The data were statistically analysed (Rangasamy, 1995) and treatment means were compared by Duncans Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics Unit, The Philippines.

RESULTS

Isolation, screening and selection of endophytic bacterial strains: Bacterial endophytes were isolated from different parts of cotton plant. In total, 103 strains were isolated from stem, root, leaf and seed. Among them, EPCO16, EPCO29, EPCO74, EPCO102 were found to increase the vigour of cotton seedlings significantly compared to untreated control. EPCO102 showed high growth promotion compared to other strains. The efficiency of the strains varied in terms of vigour index of the treated seeds (Table 1). No external symptoms appeared after endophytic bacteria treatment. The strains showed high growth promotion and Pf1 were chosen for testing their efficacy against *R. solani* by the dual plate technique. The strain EPCO16 showed higher inhibition to *R. solani* followed by EPCO102 and Pf1 under *in vitro* conditions (Table 2, Fig. 1). EPCO102 (leaf isolate) showed high vigour index, EPCO16 (root isolate) showed high inhibition were choosen for further study.

Biochemical and molecular identification of effective bacterial endophytes: The isolated bacterial strains were identified by various biochemical tests. Based on these tests, the isolates obtained both from leaves and roots are *Bacillus* sp. (Table 3). The ITS primers amplified a fragment size of 546bp corresponding to the region of the

Fig. 1: *In vitro* antagonism of *R. solani* by bacterial endophytes. 1: EPCO29 vs *R. solani*, 2: EPCO16 vs *R. solani*, 3: EPCO102 vs *R. solani*, 4: EPCO 74 vs *R. solani*, 5: Pf1 vs *R. solani* and 6: Control (*R. solani*)

Table 1: Effect of bacterial endophytes on cotton seedling growth

	Vigour index	
Isolates	Roll towel	Pot study
EPCO1	435.0^{c-j}	334.7^{b-o}
EPCO2	2115.0^{a-B}	607.2^{e-x}
EPCO3	1939.0^{a-B}	746.1^{i-A}
EPCO4	1369.0^{g-x}	715.0^{g-A}
EPCO5	2169.7^{a-B}	715.0^{g-A}
EPCO6	2379.8^{A-D}	802.5^{a-B}
EPCO7	614.8^{c-m}	566.8^{d-x}
EPCO8	824.8^{f-r}	437.5^{c-t}
EPCO9	280.0^{hc}	382.2^{b-r}
EPCO10	2256.0^{a-D}	762.2^{i-B}
EPCO11	2770.0^{BCD}	660.0^{g-z}
EPCO12	340.0^{cd}	377.5^{b-p}
EPCO13	2104.0^{w-B}	684.85^{g-}
EPCO14	1962.0^{a-B}	1092.1^{a-A}
EPCO15	495.0^{c-k}	667.3^{g-z}
EPCO16	2710.0^{BCD}	1218.7^{y-C}
EPCO17	866.2^{i-z}	677.4^{g-y}
EPCO18	508.0^{c-k}	694.2^{g-y}
EPCO19	577.4^{c-l}	709.5^{g-A}
EPCO20	789.6^{e-q}	699.3^{h-A}
EPCO21	669.6^{c-v}	425.0^{b-s}
EPCO22	290.0^{hc}	335.0^{b-m}
EPCO23	1341.2^{q-w}	429.8^{c-t}
EPCO24	902.4^{j-z}	924.7^{n-C}
EPCO25	2286.0^{a-D}	0.5^{a}
EPCO26	2152.0^{a-C}	709.9^{g-A}
EPCO27	1970.0^{a-B}	627.2^{f-y}
EPCO28	0.5^{a}	919.8^{s-C}
EPCO29	2384.8^{A-D}	1296.9^{ABC}
EPCO30	889.5^{j-z}	952.29^{s-C}
EPCO31	2294.4^{a-D}	937.2^{t-C}
EPCO32	2720.0^{BCD}	0.5^{a}
EPCO33	709.8^{d-p}	667.1^{g-z}
EPCO34	854.4^{h-r}	595.0^{e-x}
EPCO35	709.8^{d-o}	590.0^{e-x}
EPCO36	1079.7^{m-t}	709.5^{g-A}
EPCO37	405.0^{cde}	365.0^{b-p}
EPCO38	145.0^{ab}	264.9^{b-g}
EPCO39	1217.4^{p-u}	687.3^{g-A}
EPCO40	865.0^{j-z}	482.4^{c-v}
EPCO41	1084.0^{m-t}	352.3^{b-q}

Table 1: Continued

	Vigour index	
Isolates	Roll towel	Pot study
EPCO42	350.0^{cd}	289.8^{b-i}
EPCO43	400.0^{c-h}	477.1^{c-t}
EPCO44	829.8^{g-r}	332.4^{b-n}
EPCO45	1785.0^{u-A}	197.5^{bcd}
EPCO46	1419.2^{r-y}	140.0^{bc}
EPCO47	380.0^{c-g}	354.7^{b-q}
EPCO48	840.0^{h-r}	364.6^{b-r}
EPCO49	639.6^{c-n}	322.4^{b-l}
EPCO50	719.8^{d-p}	587.1^{d-x}
EPCO51	619.9^{c-m}	574.7^{d-w}
EPCO52	75.0^{a}	529.9^{d-x}
EPCO53	405.0^{c-i}	509.5^{c-u}
EPCO54	1029.8^{l-s}	547.3^{d-x}
EPCO55	375.0^{c-f}	519.8^{d-w}
EPCO56	555.0^{c-m}	499.8^{d-w}
EPCO57	719.2^{d-p}	587.5^{e-x}
EPCO58	969.6^{k-s}	799.6^{n-B}
EPCO59	545.0^{c-l}	484.8^{d-v}
EPCO60	1309.4^{p-v}	689.7^{g-A}
EPCO61	1764.6^{t-v}	407.4^{b-r}
EPCO62	879.0^{j-s}	484.8^{d-v}
EPCO63	315.0^{cd}	267.4^{b-h}
EPCO64	515.0^{c-k}	612.2^{e-x}
EPCO65	1196.7^{n-u}	307.5^{b-k}
EPCO66	160.0^{ab}	392.4^{b-r}
EPCO67	2034.6^{v-B}	1052.3^{w-C}
EPCO68	0.5^{a}	369.9^{b-r}
EPCO69	0.5^{a}	0.5^{a}
EPCO70	2405.0^{A-D}	945.0^{t-C}
EPCO71	1999.7^{v-B}	1549.5^{C}
EPCO72	0.5^{a}	735.0^{g-A}
EPCO73	2502.8^{A-D}	547.2^{d-x}
EPCO74	2750.0^{BCD}	1582.0^{C}
EPCO75	0.5^{a}	342.5^{b-p}
EPCO76	0.5^{a}	819.7^{p-B}
EPCO77	1484.8^{p-z}	899.9^{s-C}
EPCO78	2497.8^{A-D}	307.5^{b-l}
EPCO79	0.5^{a}	795.0^{n-B}
EPCO80	2177.6^{y-C}	439.8^{c-t}
EPCO81	0.5^{a}	559.8^{d-x}
EPCO82	0.5^{a}	222.5^{b-e}
EPCO83	0.5^{a}	847.4^{r-B}
EPCO84	0.5^{a}	434.7^{c-t}
EPCO85	2116.8^{w-B}	837.3^{q-B}
EPCO86	1049.9^{l-s}	742.5^{i-A}
EPCO87	1972.9^{v-B}	772.5^{m-B}
EPCO88	0.5^{a}	367.4^{b-q}
EPCO89	1972.7^{v-B}	449.7^{c-u}
EPCO90	482.0^{c-j}	312.3^{b-j}
EPCO91	2109.6^{w-B}	627.3^{f-y}
EPCO92	2780.0^{BCD}	845.0^{r-B}
EPCO93	3090.0^{CD}	1012.4^{v-CC}
EPCO94	2388.0^{A-D}	1282.0^{z-C}
EPCO95	0.5^{a}	979.5^{u-C}
EPCO96	2740.0 ^{BCD}	1234.5^{y-C}
EPCO97	2420.0^{A-D}	832.3^{q-B}
EPCO98	2170.0^{y-C}	1010.5^{v-C}
EPCO99	0.5^{a}	178.0^{b}
EPCO100	2580.0^{A-D}	684.8^{g-A}
EPCO101	2172.8^{y-D}	997.2^{v-C}
EPCO102	3130.0^{D}	1404.5^{BC}
EPCO103	2363.0^{A-D}	755.0^{k-B}
Control	40.0^{a}	226.4^{b-f}

Vigour index = Percent Germination×seedling length (shoot length + root length). Values are means of three replications. Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at p = 0.05

Table 2: Antagonistic activity of bacterial endophytic isolates of cotton against *Rhizoctonia solani in vitro*

Isolates	Pathogen growth (mm)	Percent inhibition over control
EPCO16	43.3	41.9 (40.4)^{c}
EPCO29	44.3	40.6 (39.6)^{c}
EPCO74	51.7	30.7 (33.6)^{b}
EPCO96	45.3	40.4 (39.5)^{c}
EPCO102	53.7	28.1 (32.0)^{b}
Pf-1	52.7	29.4 (32.8)^{b}
Control	74.7	0.0 (0.0)^{a}

Values are means of three replicates. Data followed by the same letter(s) in a column are not significantly different from each other according to Duncan's multiple range test at p = 0.05. Values in parentheses are arcsine transformed

Table 3: Identification and characterization of bacterial endophytic strains by biochemical characteristics

	Isolate No.		
	EPCO16	EPCO102	Pf1
Biochemical tests	Cotton root	Cotton leaf	Black gram rhizosphere
Simple staining	short rod	rod	rod
Gram's staining	+ve	+ve	-ve
KOH test	-ve	-ve	+ve
Citrate utilization test	+ve	+ve	-ve
Catalase test	+ve	+ve	+ve
Starch hydrolysis	+ve	+ve	+ve
Gelatin hydrolysis	+ve	+ve	+ve
Methyl red test	-ve	-ve	+ve
Voges proskauer test	+ve	+ve	-ve
Growth in 7% NaCl	+ve	+ve	-ve
Tentatively identified as	*Bacillus* sp.	*Bacillus* sp.	*P. fluorescens*

16s-23s rRNA intervening sequence for *Bacillus* sp. The results of PCR amplification has further confirmed that these isolates EPCO16, EPCO102 were belong to *Bacillus* sp. group (Fig. 2). Complete sequence of one of the strain *Bacillus* ITS region (EPCO16) was obtained and submitted in the NCBI, GenBank, New York, USA. This isolate confirmed as *Bacillus subtilis* (Accession No. EF139864).

Damping off incidence under greenhouse conditions: The endophytic bacterial strains EPCO102, EPCO16 and Pf1 which effectively promoted plant growth in cotton and inhibited the mycelial growth of *R. solani* were used for the development of bioformulation. These endophytic strains (with and without chitin amendment) were evaluated against *R. solani* under greenhouse conditions. Seed soaking and soil application of talc-based formulation containing Pf1, EPCO102 and EPCO16 strains significantly reduced the damping off incidence under greenhouse conditions. However, Pf1 amended with chitin bioformulation significantly reduced the incidence of damping off (40%) when compared to untreated control (83.3%) and this was comparable with that of chemical treatment (36.7%) (Table 4).

Induction of defense enzymes: Bacterized plants expressed higher amounts of defense enzymes in cotton

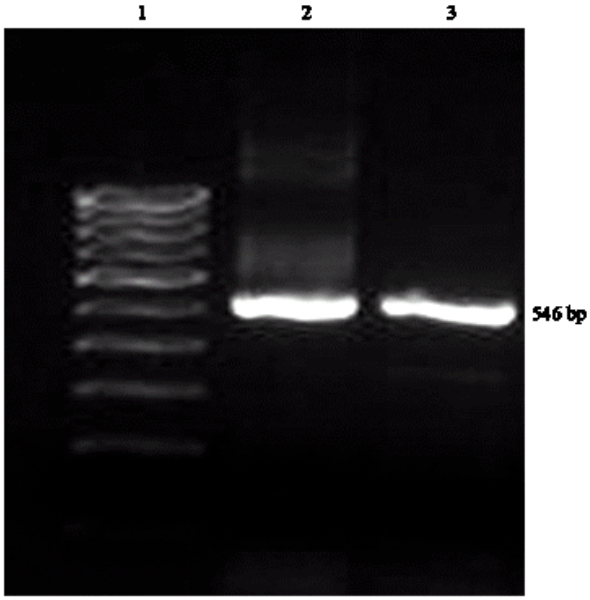

Fig. 2: Detection of endophytic *Bacillus* by gene specific primer. Lane 1: 100 bp ladder, Lane 2: EPCO102, Lane 3: EPCO16

Table 4: Effect of endophytic bacterial isolates against *Rhizoctonia* damping off disease of cotton under greenhouse conditions

Treatments	Mean Percent Disease Incidence (PDI)
EPCO102	43.3 (41.1)^{ab}
EPCO16	50.0 (44.9)^{ab}
Pf1	43.3 (41.1)^{ab}
EPCO102 + Chitin	46.7 (43.1)^{ab}
EPCO16 + Chitin	53.3 (46.9)^{b}
Pf1 + Chitin	40.0 (39.1)^{ab}
Chitin	76.7 (61.2)^{c}
Carbendazim	36.7 (36.9)^{a}
Control	83.3 (66.1)^{c}

Values are means of three replicates. Data followed by the same letter(s) in a column are not significantly different from each other according to Duncan's multiple range test at p = 0.05. Values in parentheses are arcsine transformed

plants challenge inoculated with *R. solani* when compared to plants without bacterization. Peroxidase activity was significantly higher in cotton root inoculated with *R. solani*, than the bacterized plants without challenge inoculation. In general, no significant difference of PO activity existed in the bacterized plant over time in uninoculated plants. Although there was increased PO activity in the *R. solani*, inoculated control plant, it was significantly greater when they were inoculated to the bacterized plant (Fig. 3) However, chitin amendment of endophytic bacterial strains enhanced the activity of defense enzymes compared to all other treatments. PPO accumulation reached maximum at 48 h after inoculation. Pf1, EPCO102 and EPCO16 strains significantly increased the PPO activity until 48 h after pathogen inoculation

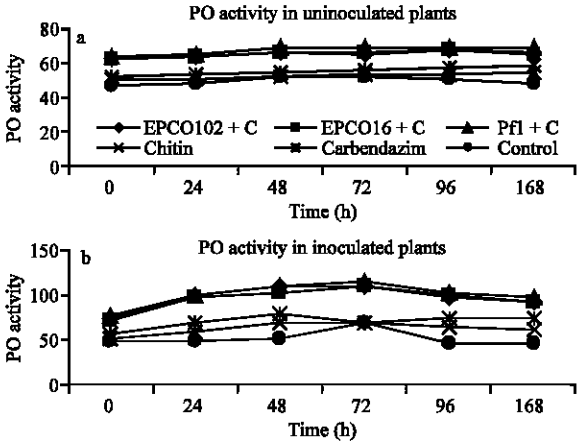

Fig. 3: Changes in peroxidase activity by seed soaking with endophytic bacterial strains in cotton challenged with or without *R. solani*

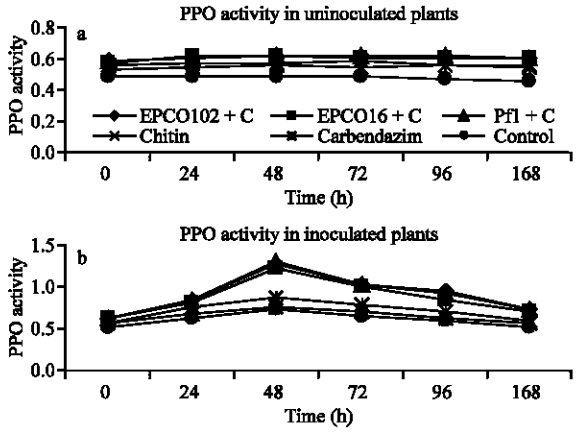

Fig. 4: Changes in polyphenol oxidase activity by seed soaking with endophytic bacterial strains in cotton challenged with or without *R. solani*

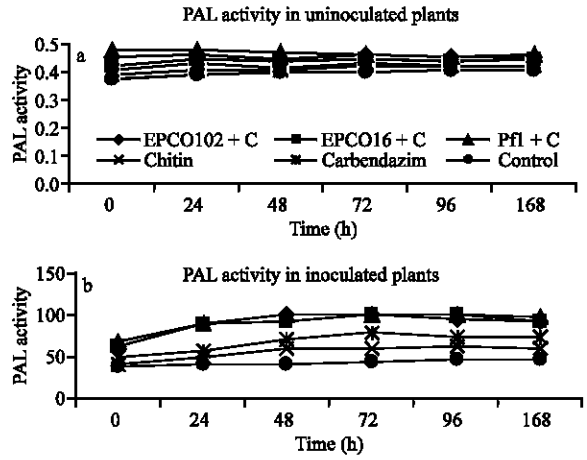

Fig. 5: Changes in phenylalanine ammonia lyase activity by seed soaking with endophytic bacterial strains in cotton challenged with or without *R. solani*

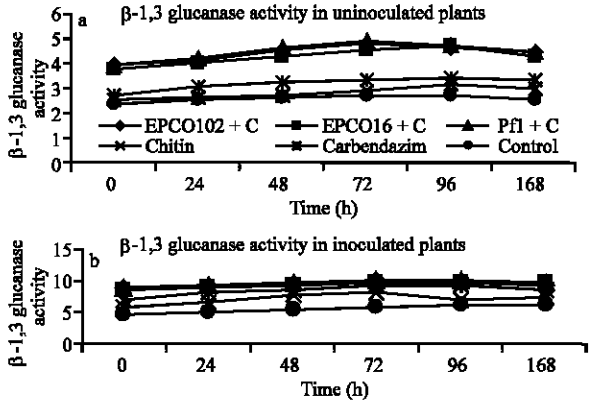

Fig. 6: Changes in β-1, 3 glucanase activity by seed soaking with endophytic bacterial strains in cotton challenged with or without *R. solani*

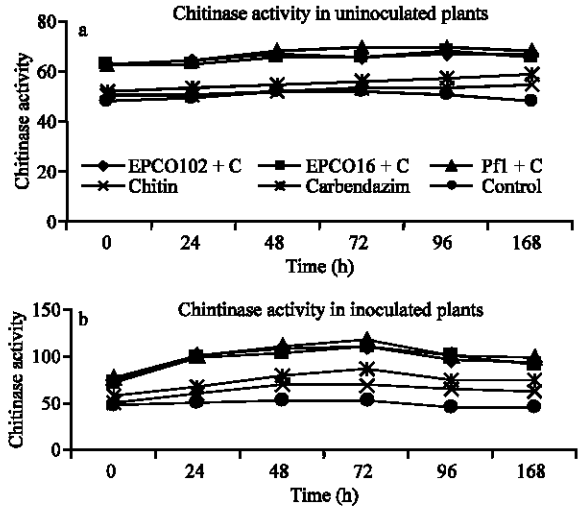

Fig. 7: Changes in Chitinase activity by seed soaking with endophytic bacterial strains in cotton challenged with or without *R. solani*

thereafter started to decline compared to other treatments. There was no difference in the chitin-amended treatments (Fig. 4). PAL activity began to increase by 24 h after pathogen inoculation. The increase in activity was transient in the root. No much difference in PAL activities were detected with and without chitin amendment. The induced activity of PAL in the root of bacterized cotton plants inoculated with pathogen was found to be at least 2 fold higher than the control plants (Fig. 5). Higher β-1,3 glucanase activity was noticed in Pf1 treated plants followed by EPCO16, EPCO102 treated plants up to 4 days thereafter started to decline. Bacterial strains amended with chitin showed enhanced levels which are on par with each other in inducing the β-1,3 glucanase activity compared to control plants (Fig. 6). During the

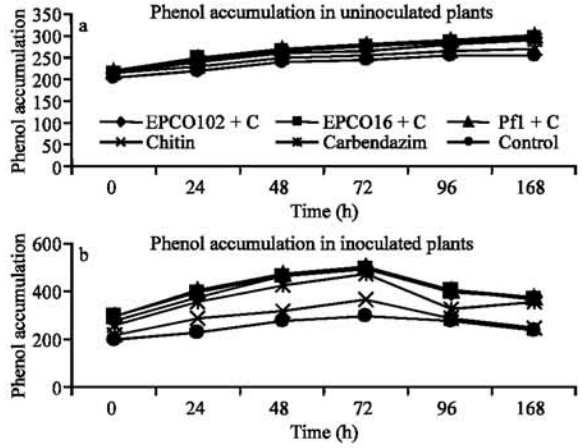

Fig. 8: Accumulation of phenolics by seed soaking with endophytic bacterial strains in cotton challenged with or without *R. solani*

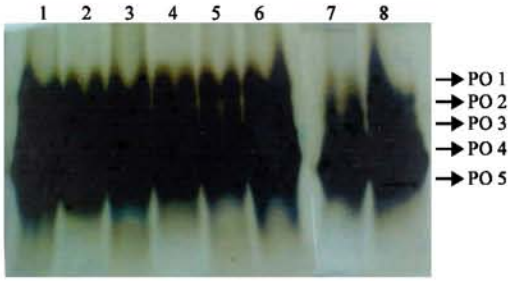

Fig. 9: Native PAGE analysis of peroxidase in cotton plants treated with endophytic bacterial strain against *R. solani*. Lane 1: EPCO102 + Chitin, Lane 2: EPCO102 + Chitin + *R. solani*, Lane 3: EPCO 16 + Chitin, Lane 4: EPCO 16 + Chitin + *R. solani*, Lane 5: Pf1 + Chitin, Lane 6: Pf1 + Chitin + *R. solani* Lane 7: Healthy control and Lane 8: Control inoculated with *R. solani*

development of symptoms by *R. solani,* chitinase in the infected root markedly accumulated with a maximum of 2 fold at 3 days after pathogen inoculation in the EPCO16 and Pf1 treatment amended with chitin than the control plants. In particular, the increase in chitinase activity occurred to a higher amount that persisted for longer time until 96 h after pathogen inoculation (Fig. 7). In addition, accumulation of phenols was higher in Pf1 amended with chitin followed by EPCO102 and EPCO16 plants challenged with pathogen and all were significantly different from control plants (Fig. 8).

Native PAGE analysis: Native PAGE analysis of peroxidase, polyphenol oxidase and chitinase revealed the more induction of defense enzymes in the endophytic

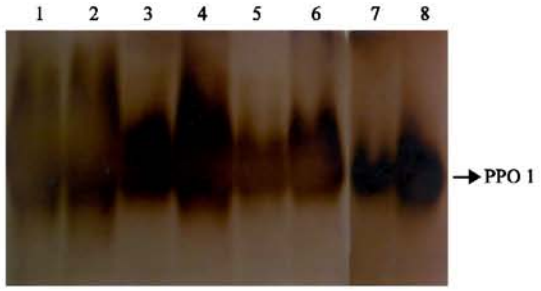

Fig. 10: Native PAGE analysis of polyphenol oxidase in cotton plants treated with endophytic bacterial strain against *R. solani*. Lane 1: EPCO102 + Chitin, Lane 2: EPCO102 + Chitin + *R. solani*, Lane 3: EPCO 16 + Chitin, Lane 4: EPCO 16 + Chitin + *R. solani*, Lane 5: Pf1 + Chitin, Lane 6: Pf1 + Chitin + *R. solani* Lane 7: Healthy control and Lane 8: Control inoculated with *R. solani*

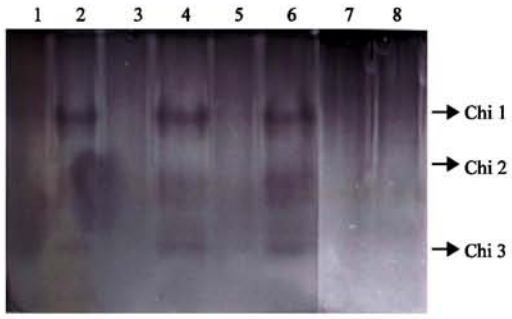

Fig. 11: Native PAGE analysis of chitinase in cotton plants treated with endophytic bacterial strain against *R. solani*. Lane 1: EPCO102 + Chitin, Lane 2: EPCO102 + Chitin + *R. solani*, Lane 3: EPCO 16 + Chitin, Lane 4: EPCO 16 + Chitin + *R. solani*, Lane 5: Pf1 + Chitin, Lane 6: Pf1 + Chitin + *R. solani* Lane 7: Healthy control and Lane 8: Control inoculated with *R. solani*

bacteria treated plants. Peroxidase isoform PO1 was induced in plants treated with endophytic bacterial strains in the absence of *R. solani*. This indicated the dual induction of defense enzymes in plants treated with endophytic bacterial strains alone (Fig. 9). In case of PPO, the endophytic bacteria treated plants showed the increased intensity of the isoform PPO1 compared to untreated control (Fig. 10). More induction of chitinase isoforms were noticed in EPCO16 and Pf1 amended with chitin treatment against *R. solani* infection. In case of non-bacterized plants, there was no induction of chitinase isoforms with or without challenge inoculation of pathogen (Fig. 11).

DISCUSSION

Damping off disease of cotton caused by *R. solani*, is a serious problem in cotton production. Biological control with endophytic bacteria offers an effective strategy for disease management (Kavino *et al*., 2007). Several endophytic bacterial strains have been reported to induce systemic resistance. As a result of ISR, plant growth promotion and disease reduction were observed in many crops (Adhikari *et al*., 2001; Bacon and Hinton, 2002). In the present study, the plants treated with endophytic bacterial strains EPCO102 and EPCO16 and Pf1 showed higher growth promotion and reduced disease severity under greenhouse conditions. Endophyte treatment played a dual role by promoting plant growth and reducing disease severity and it was comparable with chemical treatment. Several authors have reported that endophytic bacterial strains enhance the plant growth by producing plant growth regulators such as gibberellins, cytokinins and indole acetic acid which lead to direct or indirect plant growth and development (Barka *et al*., 2002; Holland, 1997). The present investigation confirms the earlier work. Endophytic bacterial strains were reported to contain the pathogen mycelial growth directly by producing antifungal or antibacterial agents (Maurhofer *et al*., 1998), antibiotic (Ramamoorthy and Samiyappan, 2001; Wulff *et al*., 2002). Similarly in present study, endophytic bacterial strain EPCO16, showed maximum mycelial inhibition of *R. solani* which may be attributed to the production of antifungal compounds. In the present study, individual strain amended with chitin was found to be effective in disease control. The role of chitin oligomers in eliciting antifungal chemicals have been well documented (Benhamou, 1996; El Ghaouth *et al*., 1994; Yuen *et al*., 2001). In this study, the chitin amendment considerably increased the biocontrol activity of endophytes against *R. solani*. It was used to improve the biocontrol activity of the bacterial formulation assuming that the chitin may induce (either alone or combination with bacterial cells) systemic resistance to pathogens. The number and activity of chitinolytic microorganisms capable of attacking the chitinaceous hyphal wall of the pathogen will increase in response to chitin amendment. Kokalis-Burelle *et al*. (1991) demonstrated that the insoluble polymer chitin could selectively enhance the growth and antagonism of a chitinolytic bacterium. The increased chitinase activity in chitin amended medium reveals that endophytic strains are able to degrade the complex chitin polymer the major component of fungal cell wall as evidenced by Viswanathan and Samiyappan (2002). The addition of chitin to talc-based formulation may enhance the effect of induced systemic resistance. The involvement of chitin in inducing systemic resistance either applied alone or in combination with biocontrol agents were well documented in many crops (Benhamou *et al*., 1998; Radjacommare *et al*., 2002; Vivekananthan *et al*., 2004; Bharathi *et al*., 2004).

Present study showed that an early and increased expression of chitinase, β-1.3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and phenol accumulation leads to significant disease suppression. The role of chitinases and peroxidases in plants against various pathogens has been demonstrated by Kandan *et al*. (2002), Chen *et al*. (2000) and Ramamoorthy *et al*. (2002) to be directly or indirectly involved in the induced systemic resistance of the plants against pathogens (Dalisay and Kuc, 1995). PO and PPO are involved in the formation of lignin to restrict the entry and movement of fungal pathogens in the plant. Increased PO and PPO activity has been shown in a number of incompatible interactions involving plant pathogenic fungi, bacteria and viruses (Chen *et al*., 2000; Kandan *et al*., 2002; Saravanakumar *et al*., 2007). Endophytic bacterial strains induce PAL which is the key biosynthetic enzyme for a large class of plant secondary metabolites including phenolic compounds (Zdor and Anderson, 1992). The present study also showed the higher induction and expression of defense enzymes in bacterized plants in response to pathogen infection.

In the present study, four chitinase isoforms and five peroxidase isoforms were induced in endophyte treated plants compared to untreated control. In control plants, the induction was strong only in initial stage of pathogen attack whereas in endophytic bacteria treated plants the activity persisted even after one week of challenge inoculation. Early and increased expression of these defense genes would have induced systemic resistance which may have resulted in reduced damping off incidence as shown by Dalisay and Kuc (1995), Chen *et al*. (2000) and Ramamoorthy *et al*. (2002).

The endophytic *Bacillus* strains EPCO102, EPCO16 and *P. fluorescens* strain Pf1 when applied as a talc-based formulation amended with chitin significantly reduced the damping off diseases in cotton through ISR possibly by enchancing the timely expression and induction of defense enzymes or by directly inhibiting the fungal growth by producing lytic enzymes or antibiotics. In the future, development of talc-based bioformulations would accelerate the large scale field use of plant growth promoting endophytic bacteria. However, development of bioformulation and their efficacy against plant disease under field conditions are to be exploited intensively in order to harness the approach of ecofriendly management of plant disease in the component of sustainable agriculture.

ABBREVIATIONS

EPCO = Endophytes cotton.
ISR = Induced systemic resistance.
KB = King's medium B.
PAGE = Polyacrylamide gel electrophoresis.
PAL = Phenylalanine ammonia-lyase.
PO = Peroxidase.
PPO = Polyphenol oxidase.

REFERENCES

Adhikari, T.B., C.M. Joseph, G. Yang, D.A. Phillips and L.M. Nelson, 2001. Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice. Can. J. Microbiol., 47: 916-924.

Aneja, K.R., 1993. Experiments in Microbiology, Plant Pathology and Tissue Culture. Wishwa Prakasham, New Delhi, pp: 117-195.

Bacon, W.C. and D.M. Hinton, 2002. Endophytic and biological control potential of *Bacillus mojavensis* and related species. Biol. Control, 23: 274-284.

Baker, B., P. Zambryski, B. Staskaawicz and S.P. Dineshkumar, 1997. Signaling in plant-microbe interaction. Science, 276: 726-733.

Baki, A.A.A. and J.D. Anderson, 1973. Vigour determination in soybean seed by multiple criteria. Crop Sci., 31: 630-633.

Barka, E.A., S. Gognies, J. Nowak, J.C. Audran and A. Belarbi, 2002. Inhibitory effect of endophyte bacteria on *Botrytis cinerea* and its influence to promote the grapevine growth. Biol. Control, 24: 135-142.

Benhamou, N., 1996. Elicitor-induced plant defense pathways. Trends. Plant Sci., 1: 233-240.

Benhamou, N., J.W. Kloepper and S. Tuzun, 1998. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: Ultra structure and cytochemistry of the host response. Planta, 204: 153-168.

Bharathi, R., R. Vivekananthan, S. Harish, A. Ramanathan and R. Samiyappan, 2004. Rhizobacteria-based bio-formulations for the management of fruit rot infection in chillies. Crop Prot., 23: 835-843.

Boller, T. and F. Mauch, 1988. Colorimetric assay for chitinase. Meth. Enzymol., 161: 430-435.

Cano, R.J., M.K. Borucki, M. Higby-Schweitzer, H.N. Poinar, G.O. Poinar and K.J. Pollard, 1994. *Bacillus* DNA in fossil bees: An ancient symbiosis? Applied Environ. Microbiol., 60: 2164-2167.

Chen, C., R.R. Belanger, N. Benhamou and T. Paulitz, 2000. Defence enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiol. Mol. Plant Pathol., 56: 13-23.

Dalisay, R.F. and J.A. Kuc, 1995. Persistence of reduced penetration by *Colletotrichum lagenarium* into cucumber leaves with induced systemic resistance and its relation to enhanced peroxidase and chitinase activity. Physiol. Mol. Plant Pathol., 47: 329-338.

Dennis, C. and J. Webster, 1971. Antagonistic properties of species groups of *Trichoderma* 1. Production of non-volatile antibiotics. Trans. Br. Mycol. Soc., 57: 25-39.

Dimock, M.B., R.M. Beach and P.S. Carlson, 1998. Endophytic Bacteria for the Delivery of Crop Protection Agents. In: Biotechnology, Biological Pesticides and Novel Plant Pest Resistance for Insect Pest Management, Roberts, D.W. and R.R. Grranados (Eds.). Boyce Thompson Institute for Plant Research. Ithaca, New York, pp: 88-92.

El Ghaouth, L.A., J. Arul, N. Benhamou, A. Assselin and R.R. Belanger, 1994. Effect of chitosan on cucumber plants: Suppression of *Pythium aphanidermatum* and induction of defense reactions. Phytopathology, 84: 313-320.

Hallmann, J., J.W. Kloepper, R. Rodriguezkabana and R.A. Sikora, 1995. Endophytic rhizobacteria as antagonists of *Meladogyne incognita* on cucumber. Phytopathology, 85: 1136.

Hammerschmidt, R., E.M. Nuckles and J. Kuc, 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol. Plant Pathol., 20: 73-82.

Holland, M.A., 1997. Occam's razor applied to hormonology: Are cytokinins produced by plants? Plant Physiol., 115: 865-868.

ISTA, 1993. Proceedings of the international seed testing association. International rules for seed testing. Seed Sci. Technol., 21: 25-30.

Kandan, A., R. Ramiah, R. Radja Commare, A. Nandakumar, T. Raguchander and R. Samiyappan, 2002. Induction of phenyl propanoid metabolism by *Pseudomonas* fluorescens against tomato spotted wilt virus in tomato. Folia Microbiol., 47: 121-129.

Karban, R. and I.T. Baldwin, 1997. Induced Responses to Herbivory (Univ. of Chicago Press, Chicago, IL).

Kavino, M., S. Harish, N. Kumar, D. Saravanakumar, T. Damodaran, K. Soorianathasundaram and R. Samiyappan, 2007. Rhizosphere and endophytic bacteria for induction of systemic resistance of banana plantlets against bunchy top virus. Soil Biol. Biochem., 39: 1087-1098.

Kloepper, J.W., B. Schippers and P.A.H.M. Bakker, 1992. Proposed elimination of the term endorhizosphere. Phytopathology, 82: 726-727.

Kokalis-Burelle, N., P.A. Backman, R. Rodriguez-Kabana and D.L. Ploper, 1991. Chitin as a foliar amendment to modify microbial ecology and control disease. Phytopathology, 81: 1152 (abstract).

Manjula, K. and A.R. Podile, 2001. Chitin ?Supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. Can. J. Microbiol. (In Press).

Maurhofer, M., C. Reimmann, S.P. Sacherer, S. Heeb, D. Haas and G. Defago, 1998. Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. Phytopathology, 88: 678-684.

Mayee, C.D., T.P. Rajendran and M.V. Venugopalan, 2002. Surviving under pressurised trade. The Hindu, Survey of Indian Agric., 19: 129-132.

Mayer, A.M., E. Harel and R.B. Shaul, 1965. Assay of catechol oxidase, a critical comparison of methods. Phytochemistry, 5: 783-789.

Nandakumar, R., S. Babu, R. Viswanathan, T. Raguchander and R. Samiyappan, 2001. Induction of systemic resistance in rice against sheath blight disease by plant growth promoting rhizobacteria. Soil Biol. Biochem., 33: 603-612.

Pan, S.Q., X.S. Ye and J. Kuc, 1991. Association of β-1,3-glucanase activity and isoform pattern with systemic resistance to blue mold in tobacco induced by stem injection with *Pernospora tabacina* or leaf inoculation with tobacco mosaic virus. Physiol. Mol. Plant Pathol., 39: 25-39.

Radjacommare, R., R. Nandakumar, A. Kandan, S. Suresh, M. Bharathi, T. Raguchander and R. Samiyappan 2002. *Pseudomonas fluorescens* based bioformulation for the management of sheath blight and leaf folder in rice. Crop Protect., 21: 671-677.

Ramamoorthy, V. and R. Samiyappan, 2001. Induction of defense-related genes in *Pseudomonas fluorescens* treated chilli plants in response to infection by *Colletotrichum capsici.* J. Mycol. Plant Pathol., 31: 146-155.

Ramamoorthy, V., T. Raguchander and R. Samiyappan, 2002. Induction of defense related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. *lycopersici*. Plant Soil, 239: 55-68.

Rangasamy, R., 1995. A Text Book of Agricultural Statistics. New Delhi, India: New Age International publisher Ltd. ISBN 81-224-0758-7.

Riker, A.J. and R.S. Riker, 1936. Introduction to Research on Plant Diseases. John Swift, New York, pp: 117.

Robertson, D.L., T.S. Bragg, S. Simpson, R. Kaspar, W. Xie and M.T. Tippets, 1990. Mapping and characterization of the *Bacillus anthracis* plasmid pX01 and pX02. Salisbury Med. Bull., 68: 55-58.

Ross, W.W. and R.R. Sederoff, 1992. Phenylalanine ammonia lyase from loblolly pine: Purification of the enzyme and isolation of complementary DNA clones. Plant Physiol., 98: 380-386.

Saravanakumar, D., Charles Vijayakumar, N. Kumar and R. Samiyappan, 2007. PGPR-induced defense responses in the tea plant against blister blight disease. Crop Prot., 26: 556-565.

Schaad, N.W., 1992. Laboratory Guide for Identification of Plant Pathogen Bacteria. 2nd Edn. International Book Distributing Co., Lucknow, pp: 44-58.

Singh, R.P. and J.P. Verma, 1988. Cotton Disease Problems in India. In: Perspectives in Mycology and Plant Pathology, Agnihotri, V.P., A.K. Sarbhoy and D. Kumar (Eds.). Malhotra Publishing House, Delhi, pp: 266-281.

Thompson, D.C., 1996. Evaluation of bacterial antagonist for reduction of summer patch symptoms in Kentucky blue grass. Plant Dis., 80: 856-862.

Trudel, J. and A. Asselin, 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem., 105: 673-678.

Viswanathan, R. and R. Samiyappan, 2002. Induced systemic resistance by fluorescent pseudomonads against red rot disease of sugar cane caused by *Colletotrichum falcatum*. Crop Prot., 21: 1-10.

Vivekananthan, R., M. Ravi, D. Saravanakumar, N. Kumar, V. Prakasam and R. Samiyappan, 2004. Microbially induced defense related proteins against post harvest anthracnose infection in Mango. Crop Prot., 23: 1061-1067.

Wilson, D., 1995. Endophyte-the evolution of a term and clarification of its use and definition. Oikos, 73: 274-276.

Wulff, E.G., C.M. Mguni, K. Mansfeld-Giese, J. Fels, M. Lubeck and J. Hockenhull, 2002. Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris*. Plant Pathol., 51: 574-584.

Yuen, G.Y., J.R. Steadman, D.T. Lindgren, D. Schaff and C. Jochum, 2001. Bean rust biological control using bacterial agents. Crop Prot., 20: 395-402.

Zdor, R.E. and A.J. Anderson, 1992. Influence of root colonizing bacteria on the defense responses in bean. Plant Soil, 140: 99-107.

Zieslin, N. and R. Ben-Zaken, 1993. Peroxidase activity and presence of phenolic substances in peduncles of rose flowers. Plant Physiol. Biochem., 31: 333-339.