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Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3

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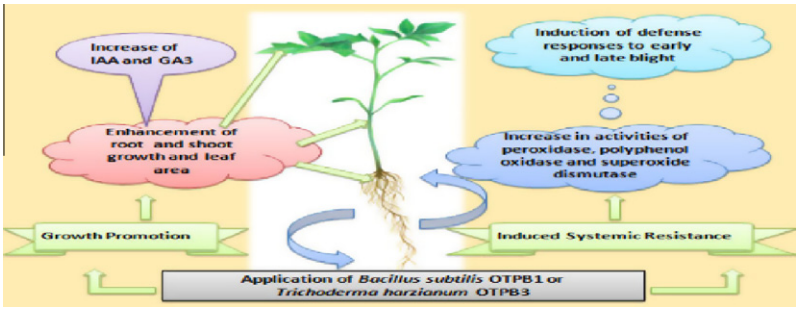
HIGHLIGHTS

- Antagonistic bacterial or fungal isolate exhibited antibiosis to early and late blight pathogens of tomato.
- Promoted plant growth in tomato seedlings.
- Induced systemic resistance against early and late blight in tomato seedlings.
- Assumed significance in production of disease free quality tomato transplants.

GRAPHICAL ABSTRACT



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ABSTRACT

The plant growth -promoting rhizobacteria and fungi are known to enhance growth and induce systemic defense responses in plants. The efficacy of *Bacillus subtilis* OTPB1 and *Trichoderma harzianum* OTPB3 were evaluated for *in vitro* antibiosis to *Alternaria solani* and *Phytophthora infestans*, growth stimulation, and induction of systemic resistance in tomato seedlings against early and late blight. Cell suspensions of OTPB1 or spore suspensions of OTPB3 were incorporated into plastic pots containing tomato seed var. Arka vikas and data were recorded 30 days after inoculation. Both isolates inhibited mycelium growth of *A. solani* and *P. infestans* under *in vitro* conditions and significantly increased root and shoot growth, leaf area and seedling vigour index in tomato. The levels of indole-3-acetic acid (IAA) and gibberellic acid (GA_3) were increased significantly in roots of seedlings treated by OTPB1 or OTPB3 by 29.12% and 45.82% or 54.34% and 67.59%, respectively, as compared to uninoculated controls. Treatment with OTPB1 or OTPB3 enhanced the levels of defense–related enzymes including peroxidase, polyphenol oxidase and superoxide dismutase in tomato plants. This study also showed that in addition to plant growth and antibiosis, OTPB1 and OTPB3 enhanced systemic resistance in tomato seedlings through induction of growth hormones and defense enzymes. The use of OTPB1 or OTPB3 in raising disease-free and quality tomato seedlings in pot trays is discussed.

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1. Introduction

Tomato *(Lycopersicon esculentum* Mill.) is grown in 865,000 hectares with an average productivity of 19.5 metric tonnes/ha in India (http://nhb.gov.in/area-pro/database-2011.pdf). Early blight, caused by *Alternaria solani* (Ellis & Martin) Sorauer, (Chaerani and Voorrips, 2006) and late blight, incited by *Phytophthora infestans* (Mont.) deBary (Deahl et al., 2008) are economically important diseases of tomato worldwide including India causing crop losses up to 100% (Fry et al., 1993; Chaerani and Voorrips, 2006). Since commercial cultivars do not have sufficient resistance to leaf blights, cultural practices and fungicides applied at 5–7 days intervals form the basis for leaf blight management programs (Tumwine et al., 2002). However, development of fungicide resistance, accumulation of residues in fruits, reduction of beneficial phylloplane and

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soil microbes and environmental pollution are associated problems (Akinnifesi et al., 2005).

Plant growth-promoting rhizobacterium (PGPR) such as *Bacillus subtilis* (Ehrenberg) Cohn and the plant-growth promoting fungus (PGPF) *Trichoderma harzianum* Rifai are used in a wide range of crop plants as biocontrol agents for management of different pathogens (Murphy et al., 2003; Harman, 2011; Woo et al., 2006). Induced systemic defense responses in plants have been reported as one of the mechanisms by which these organisms reduce the diseases in plants in conjunction with other mechanisms including direct antagonism, antibiosis and siderophore production. Induction of defense responses by *Bacillus* spp. and *Trichoderma* spp. is largely associated with production of pathogenesis related proteins like β-1,3-glucanase and the defense enzyme phenylalanine ammonia-lyase and oxidative enzymes like peroxidase, polyphenol oxidase and superoxide dismutase (Yedidia et al., 1999; Ahmed et al., 2000; Compant et al., 2005; Elad, 2000; Yang et al., 2009; Babitha et al., 2002). Apart from controlling diseases, these biocontrol organisms also promote plant growth by production of plant growth hormones like IAA and GA_3 coupled with increased availability of nutrients (Cattelan et al., 1999; Chen et al., 2007; Harman, 2011). The objectives of the present study were to examine whether isolates of two potential biocontrol agents *B. subtilis* OTPB1 and *T. harzianum* OTPB3 could enhance the growth of tomato and induce systemic protection against early and late blight in tomato.

2. Material and methods

2.1. Isolation and identification of biocontrol strains

B. subtilis (isolate OTPB1) and *T. harzianum* (isolate OTPB3) were isolated from the phylloplane of tomato and field beans (*Phaseolus vulgaris* L.), respectively, were used. *T. harzianum* (isolate OTPB3) and *B. subtilis* (isolate OTPB1) were isolated from phylloplane following leaf wash method (Gould et al., 1996) using *Trichoderma* selective medium(TSM) (Elad et al., 1981) and King's B Medium (King et al., 1954) respectively. Ten grams of leaf sample was taken in 90 ml sterile water and mixed well in a rotary shaker at 140 rpm for 30 min. Then 0.1 ml of sample was spreaded on the plate having selective media. These plates were incubated at 26 °C ± 1 °C for 5–7 days. Morphological identity of *Trichoderma* was done as outlined by Rifai (1969) and Bissett (1991). For molecular identification, isolate OTPB3 was grown in potato dextrose broth at 26 °C ± 1 °C for 5 days. DNA was extracted from five days old culture according to method of Raeder and Broda (1985) with slight modifications made by Chowdappa et al., (2003). PCR amplification of ITS region of r DNA of fungal isolates was carried out with universal primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′ (White et al., 1990). PCR was performed in 50 µl reaction volumes. Each reaction consisted of approximately 1 µl of template DNA, 5 µl 10× PCR buffer, 40.75 µl sterile distilled water, 1 µl 2.0 mM dNTPs, 1 µl each of 50 pM primers ITS4 and ITS5 and 0.25 µl Taq polymerase (5U/µl) (Merck Bio Sciences, India). Thermal cycling conditions were initial denaturation at 95 °C for 3 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. Negative controls (no template DNA) were used in every experiment to test for the presence of contamination in reagents. PCR products were analysed by electrophoresis in 2% (w/v) agarose gel in 1× Tris Borate-EDTA buffer and stained with ethidium bromide (5 µg/ml) and visualized by Alpha imager EP (Alpha Innotech Corporation, USA). The amplified PCR product were ligated into pTZ57R/T, transformed into *Escherichia coli* DH5α. The plasmid was purified from *E. coli* and the cloned DNA fragment was sequenced to confirm that it has homology identical to the previously reported rDNA sequence of *T. harzianum* available in NCBI.

Bacterial DNA was isolated from 36 h old cultures, grown in nutrient broth at 26 °C ± 1 °C, using bacterial DNA isolation kit (Zymo Research Bacterial DNA Mini Prep.,USA). PCR amplification of 16S rDNA was carried out using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) Weisberg et al., 1991). and 1492R-5́GGTTACCTTACGACTT 3́ (Reysenbach et al., 1992). PCR was carried out in 50 µl reaction volumes. Each reaction consisted of approximately 1 µl of template DNA, 5 µl 10x PCR buffer, 40.75 µl sterile distilled water, 1 µl 2.0 mM dNTPs, 1 µl each of 50 pM primers 27F and 1492R and 0.25 µl Taq polymerase (5U/µl) (Merck Bio Sciences, India). Thermocycling conditions consisted of initial one denaturation step of 94 °C for 5 min followed by 32 amplification cycles of 94 °C for 30 s, 55 °C for 40 s, 72 °C for 40 s followed by a final extension at 72 °C for 5 min. PCR products were analysed by electrophoresis in 2% (w/v) agarose gel in 1× Tris Borate-EDTA buffer and stained with ethidium bromide (5 µg/ml) and visualized by Alpha imager EP (Alpha Innotech Corporation, USA). PCR products were sequenced to confirm that it has homology identical to the previously reported rDNA sequence of *B. subtilis* available in NCBI.

The phylogentic analysis was inferred using the Maximum Parsimony method (MP). The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 303 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Both biocontrol strains, *B. subtilis* OTPB1 and *T. harzianum* OTPB3 were deposited at National Bureau of Agriculturally Important Microorganisms, Mau, India as accession numbers NAIMCC-B-01339 and NAIMCC-F-03065, respectively.

2.2. Preparation of Inoculum of biocontrol strains

Inoculum of OTPB1 *B. subtilis* was prepared for all experiments by harvesting cells from nutrient broth cultures grown at 28° ± 1 °C for 48 h followed by centrifugation at 6000 rpm for 15 min. The inoculum was re-suspended in sterile distilled water and then the concentration was adjusted using spectrophotometer to 10^8 CFU/ml (Thompson, 1996; Yan et al., 2002). Spore suspensions of *T. harzianum* OTPB3 were prepared by scraping the spores from cultures grown on potato dextrose agar plates placed under cool-white fluorescent light with a 12 h alternating light and dark cycle at 25 ± 1 °C for 7 days. Spores were suspended in sterile distilled water and the number of colony forming units (CFU) that developed from spore suspensions was assayed on a *Trichoderma* selective medium (Elad et al., 1981) and adjusted the values to 10^8 CFU/ml. For long term storage, *B. subtilis* OTPB1 was preserved in 30% glycerol stock at −80 °C and *T. harzianum* OTPB3 was stored on potato dextrose agar slopes under paraffin oil at 8 °C.

2.3. Isolation and identity of pathogens

Isolates of *A. solani* OTA 22 (NCBI accession No. HQ270459) and *P. infestans* PIT 30 (GenBank accession JF834691) were obtained from naturally infected tomato foliage samples collected from Hesaraghatta and Aalur (Bangalore rural district, Karnataka) respectively. For isolation of *P. infestans* PIT 30, late blight affected tomato leaf tissue was rinsed in sterilized distilled water, surface-sterilized in 0.5% sodium hypochlorite for 30 s and washed twice in distilled water. Tissue pieces were dipped in a carbendazim solution (50 mg 50% carbendazim WP per 100 ml) for 30 s and small areas of infected leaf tissue near the advancing margin of the lesion were placed on rye A agar (Caten and Jinks, 1968) amended with 20 mg of rifamycin, 200 mg of vancomycin, 200 mg of ampicillin, 68 mg of PCNB and 50 mg 50% carbendazim WP per liter. After 10–15 days at 18 °C, isolates were derived by transferring a hyphal tip to unamended rye agar A. Isolates were maintained on rye agar A at 18 °C in darkness and were transferred every 4–5 weeks. The identity of the *P. infestans* was confirmed based on morphology, ITS sequencing and blast search at NCBI and species-specific PCR (Chowdappa et al., 2013). For isolation of *A. solani*, tissue pieces of tomato foliage with typical early blight symptoms were surface sterilized in a 1% sodium hypochlorite solution for 1 min, rinsed thrice in sterile distilled water, placed on potato dextrose agar (PDA) medium in 80 mm Petri plates and incubated at 25 °C with 12 h photoperiod. Fungal hypal tips from mycelium growing from diseased tomato leaf tissue were transferred onto 80 mm petriplates containing V8 agar. The resulting isolates were maintained on V8 agar slants at 8 °C for short term storage and were preserved in V8 agar slopes in paraffin oil at 8 °C for long term storage. *A. solani* was deposited at National Bureau of Agriculturally Important Microorganisms, Mau, India as an accession number NAIMCC-F-02671 and *P. infestans* was stored at IIHR culture repository and The James Hutton Institute, U.K. Pathogenicity tests for the pathogens were carried out on detached tomato leaves in artificial inoculated conditions (Chaerani and Voorrips, 2006; Chowdappa et al., 2013). The pathogens were re-isolated from the artificial inoculated tomato leaves to confirm Koch's postulates.

2.4. Pathogen culture and inoculum preparation

P. infestans PIT 30 was grown on Rye agar B medium at 18 °C under light (16 h cool white fluorescent light and 8 h dark) for 14 days. Inoculum was obtained from rye agar plates that were gently washed with cold sterile distilled water to liberate sporangia. The sporangial suspension was placed in a refrigerator for 2 h to induce zoospore release. Zoospores were separated from sporangia by filtration through a 12-μm mesh filter and diluted to a concentration of 3×10^5/ml. Highly aggressive *A. solani* OTA 22 isolate was grown on V8 juice agar at 25 °C for 7 days at 12 h photoperiod and 5 mm diameter disk containing mycelia was used as inoculum.

2.5. Agar plate-based pathogen inhibition assays

Antagonistic effect of *B. subtilis* OTPB1 or *T. harzianum* OTPB3 were evaluated against *A. solani* OTA22 and *P. infestans* PIT 30 by adopting dual culture method (Webber and Hedger, 1986). For inhibition assays by *B. subtilis* OTPB1, a 5 mm-diameter agar plug of a 7-day-old culture of *A. solani* OTA22 or *P. infestans* PIT 30 was transferred to the center of a plate of PDA or Rye agar A and incubated at 25 ± 1 or 19 ± 1 °C, respectively, in darkness. Then, 5 μl from an exponentially growing bacterial culture in nutrient broth at OD600 of 0.1 was spotted 1 cm from the edge of the PDA or rye agar plate on one side of the fungal plug. Controls consisted of a 5 mm-diameter agar plug of each fungus without *B. subtilis* OTPB1 or *T. harzianum* OTPB3. For inhibition assays by *T. harzianum* OTPB3, mycelial agar plugs (5 mm diameter) of *A. solani* OTA22 or *P. infestans* PIT 30 and *T. harzianum* OTPB3 were placed at diametrically opposite points 1 cm from the edge of the petridish containing PDA or rye agar. The radial growth of the pathogens were measured after every 24 h till the fungus reached the perimeter of the control plate (up to 7 days) and percent inhibition was calculated after 7 days of inoculation using the formula. $PI = C - T/C \times 100$, where PI = percent inhibition of mycelial growth, C = radial growth of the pathogen in control plates (mm) and T = radial growth of pathogen in dual culture (mm). Assays were conducted nine times, with each time pathogen replicated thrice. Each replicate contained six plates.

2.6. Induction of systemic resistance

Tomato cv. Arka vikas, which is highly susceptible to *P. infestans* and *A. solani* were used in all experiments. Treatments included *B. subtilis* OTPB1 and *T. harzianum* OTPB3 and untreated control. Plastic pot trays containing ninety six cavities with 10 cm diameter, filled with well composted and sterilized coco peat (soilless growth media), were used to produce the tomato seedlings. At the time of seeding, the cell suspension of *B. subtilis* (OTPB1) (1×10^8 CFU/ml), the spore suspension (1×10^8spores/ml) of *T. harzianum* (OTPB3) and sterile distilled water were incorporated into plastic pots at the rate of 1 ml/cavity (Yan et al., 2002) and one tomato seed was placed in each cavity. Seed receiving only sterile distilled water served as untreated controls. Seedlings were allowed to grow for 30 days at 25 ± 2 °C under natural light.

2.7. Efficacy of antagonists on plant growth promotion

Plastic pot tray experiments as described under Section 2.6, were carried out in a greenhouse with three replicates, each consisting of 96 plants, thereby, making a total of 288 plants per treatment. The experiment was repeated 12 times. The germination percentage was calculated on the 14 day after sowing as the most of the seeds germinate within this period. Seeds were considered as germinated when their two cotyledons leaves were visible above the coco peat. About 3456 seeds (12 independent experiment with three replicates) were scored for determination of germination percentage. Seedling growth parameters including root length, shoot length, root fresh weight, shoot fresh weight and leaf area were determined for 3180 seedlings 30 days after sowing. A Seedling vigour index was calculated using the following formula as described by Baki and Anderson (1973) i.e.

$$\text{Seedling vigour index} = \text{seedling length(cm)} \times \text{germination percentage}.$$

The data of all the 3180 seedlings were pooled together and analyzed after no block effects were noted.

2.8. Inoculation of tomato plants with A. solani and P. infestans

Thirty day old tomato seedlings were placed in plastic chambers (45 cm height × 40 length × 15 cm width) containing water to maintain humidity. Then, the third intact leaf from bottom of the seedlings were kept on wire mesh in horizontal position and inoculated with 10 μl droplets of a zoospore suspension containing 2.5×10^5 spores/ml of *P. infestans* at the centre of the leaf as point inocultion. Seedlings, which received 10 μl droplets of sterile distilled water served as control. The inoculated plants were then incubated at 18 ± 1 °C under 16 h cool white fluorescent light and 8 h dark. For inoculation of *A. solani*, fully colonized 5-mm-diameter V8 juice agar disk, mycelium side down, was placed on the

adaxial side of the leaf and the plants were then incubated at 25 ± 1 °C under 16 h light and 8 h dark cycle. The control plants received sterilized, uncolonized block of agar. Each experiment was replicated thrice using 25 plants per replicate. Lesion size was measured after 5 days of incubation. The length and width of each lesion was measured separately. The lesion area was calculated by multiflying length and breadth of lesion and expressed as cm^2. The lesion areas on all 225 plants (resulted from three replications and repeated thrice) were pooled together to obtain means.The percent protection offered was calculated by formula, (control − treated)/control × 100.

2.9. Determination of growth hormones induced by inoculations with biocontrol strains

2.9.1. Culture broth assays

Estimation of IAA and GA_3 levels in culture broth produced by *B. subtilis* OTPB1 and *T. harzianum* OTPB3 were carried out following Kelen et al. (2004) with a few modifications by growing *B. subtilis* OTPB1 in nutrient broth (Himedia, Mumbai, India) at 25 ± 1 °C for 36 h at 120 rpm till an optical density of 1.0 was attained and *T. harzianum* was cultured in potato dextrose broth (Himedia, Mumbai, India) at 25 ± 1 °C for seven days. The cell free conditioned medium(a fluid left after centrifuging cells down to a pellet) was collected by spinning down the cells at 10,000 rpm at 4 °C for 15 min. Uninoculated potato dextrose broth was maintained as control for *T. harzianum* (OTPB3) and non-inoculated nutrient broth for *B. subtilis* (OTPB1). The experiment was repeated five times with six culture flasks at each time.

2.9.2. Tomato root assays

IAA and GA_3 levels were determined in the roots of the tomato seedlings treated with bio-control agents and untreated control according to the method of Kelen et al., (2004) with a few modifications. Tomato root samples (10 g) from 30 day old seedlings were macerated in 80% chilled methanol (50 ml) and centrifuged at 4000 rpm for 10 min after leaving the extract overnight at 4 °C in refrigerator. The supernatant was evaporated *in vacuo* at 40 °C, residue dissolved in water and adjusted to pH 8.0. The alkaline extract was partitioned two times with ethyl acetate and the ethyl acetate portions were discarded. The pH of aqueous phase of the extracts was adjusted to pH 2.5 using 0.5 N hydrochloric acid. The acidic extract was then partitioned twice with equal volumes of diethyl ether. The diethyl ether portion of extract, after drying over anhydrous Na_2SO_4, was filtered through Whatman No. 1 filter paper,and the ether was removed *in vacuo*. The residue was dissolved in 0.5 ml of 100% methanol for GA_3 and IAA analyses as described below.

2.9.3. High performance liquid chromatography (HPLC) conditions

IAA and GA_3 were assessed by HPLC (Model-Prominence, Make-Schimadzu, Japan) as described by Kelen et al., (2004) with a few modifications. A C_{18} reverse phase column (Synergi, 250 × 4.6 mm, 4 μm, Phenomenex, USA) and photodiode array (PDA) detector (Model SPD-M20A, Schimadzu, Japan) were used in the HPLC system. The solvent system included 70% water at pH 4.0 [adjusted with ortho phosphoric acid (5%)] (B) in acetonitrile (A) at a flow rate of 0.8 ml/min to resolve GA_3 and IAA. The quantification of these phytohormones was carried out at 205 and 220 nm using external standards. The experiment was repeated 12 times with five plants each time.

2.10. Assay of proteins induced by biocontrol strains

2.10.1. Sample collection, enzyme extraction and assays

Induction of defense enzymes was assessed in the leaves of the treated and control tomato plants. For each treatment, three leaf samples of one each (1 g) per replication were collected after 30 days of biocontrol agents inoculation by carefully removing the leaves without causing any damage. The modified method of Argandona et al., (2001) was adopted for the extraction of anti-oxidative enzymes. The freshly collected leaf samples (1 g) were washed in running tap water and homogenized in 10 ml of icecold 0.1 M phosphate buffer pH 7.0 in pre-chilled pestle and mortar. After filtration through cheese cloth, the homogenate was centrifuged at 10,000 rpm at 4 °C for 15 min. The, final supernatants were immediately used for determination of activities of peroxidase, polyphenol oxidase and superoxide dismutase. All steps, in preparation of the extract, were carried out at 0–4 °C. Enzyme activities were expressed as unit/g fresh weight (fw). Each enzyme assay consisted of eight replications (leaves) and two spectrophotometric readings per replication using a Biomate 3 spectrophotometer (Thermospectronic, USA). Each replication consists of five plants.

2.10.2. Peroxidase

Peroxidase activity was determined by pipetting out 2.9 ml of 0.05 M phosphate buffer, pH 6.8, 0.05 ml of 0.05 M freshly prepared enzyme substrate pyrogallol, 0.05 ml of enzyme extract and 0.1 ml of 0.3% freshly prepared solution of hydrogen peroxide. The reaction mixture was incubated at 25 ± 1 °C. The change in absorbance was read at 420 nm spectrophotometrically at 60 s interval for 3 min (Manoranjan and Dinabandhu, 1976). The enzyme activity was expressed as changes in absorbance of the reaction mixture for min^{-1} g^{-1}.

2.10.3. Polyphenol oxidase

Polyphenol oxidase activity was also carried out by as described by Manoranjan and Dinabandhu (1976) by pipetting out of 2.9 ml 0.05 M citrate buffer, pH 6.8, 0.05 ml of enzyme extract and 0.1 ml of 0.05 M freshly prepared enzyme substrate pyrogallol and incubated reaction mixture at 25 ± 1 °C. The change in absorbance was read at 450 nm spectrophotometrically at 60 s interval for 3 min. The enzyme activity was expressed as change in absorbance of the reaction mixture as units/min^{-1} g^{-1} fresh weight.

2.10.4. Superoxide dismutase

The assay mixture contained 3 ml of 50 mM phosphate buffer solution (pH 7.8), 0.25 ml of 0.5 mM EDTA and 0.65 ml of 65 mM methionine, 1 ml of 20 μM riboflavin and 0.3 ml of enzyme extract and mixed well gently. Added 0.1 ml of 150 μM of Nitroblue tetrazolium chloride and mixed thoroughly (Zhanyuan and Bramlage, 1994). Identical tubes with the reaction mixture were kept in the dark and termed as blank. The absorbance was read at 560 nm. The enzyme activity was expressed as units/min^{-1} g^{-1} fresh weight.

2.11. Statistical analysis

All data were statistically analyzed using oneway analysis of variance (ANOVA) to identify the origin of significance and followed up with a Fishers test to separate means and treatments using Graphpad Prism V.5.00 for windows (Graph pad software,San Diego, California, USA).Means were compared between treatments by LSD (least significant difference) at the 1% level ($p < 0.01$). Percentage data were arcsin-transformed before analysis according to y = arcsin [sqr (×/100)].

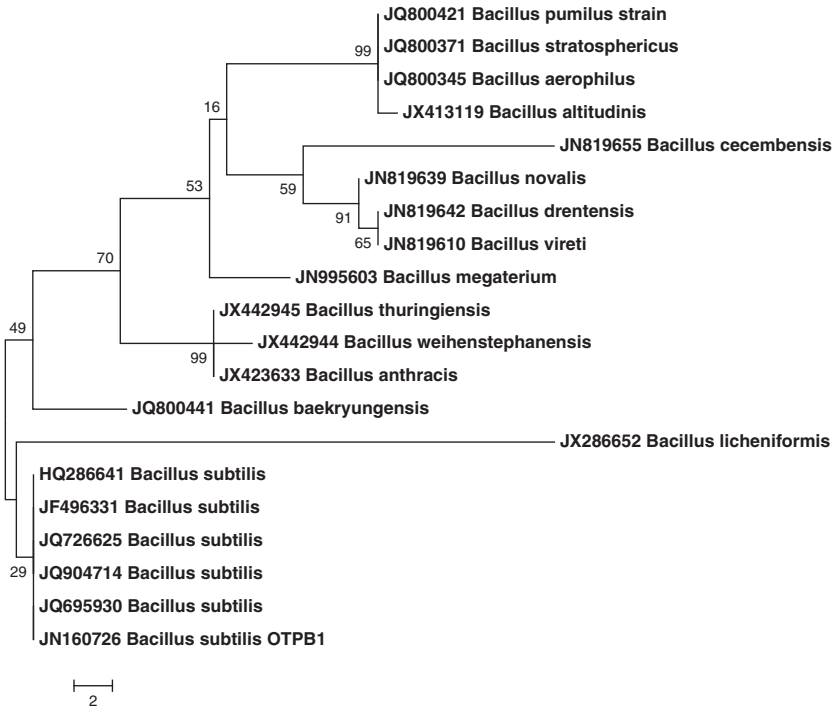

Fig. 1. Phylogenetic tree of the *Bacillus subtilis* OTPB1 based on the 16s rDNA gene sequences.

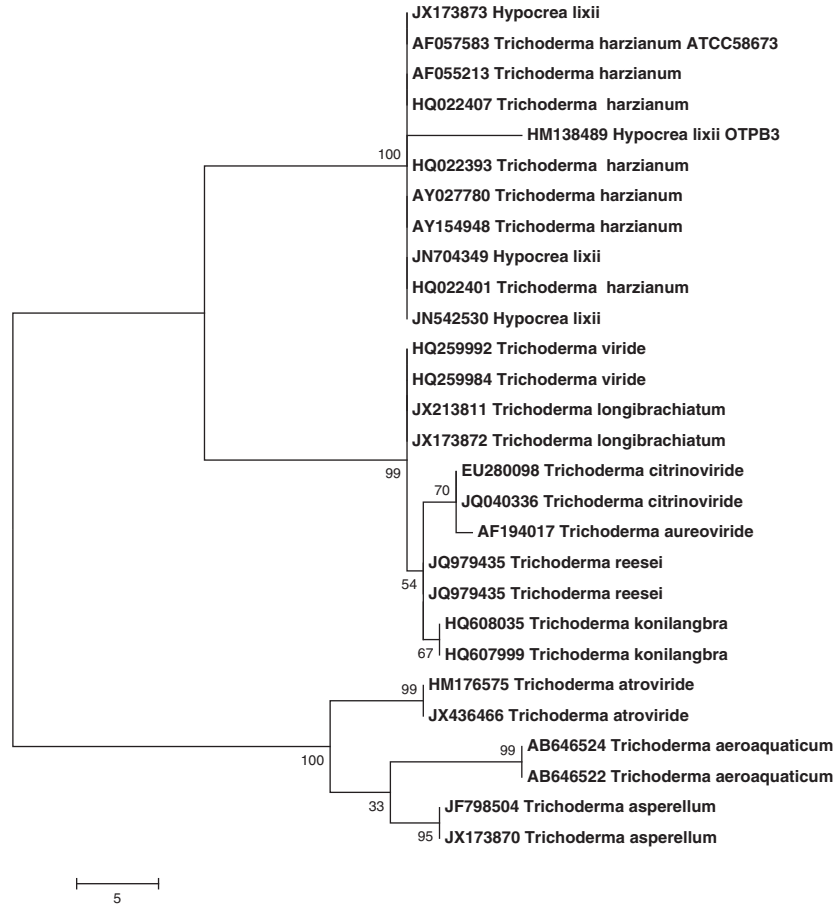

Fig. 2. Phylogenetic tree of the *Trichoderma harzianum* OTPB3 based on the ITS – rDNA gene sequences.

Table 1
In vitro inhibition of *A. solani* (OTA 22) and *P. infestans* (OTA 30) by *T. harzianum* (OTPB3) or *B. subtilis* (OTPB1)[A].

Isolate	Pathogen	Pathogen growth (mm)
T. harzianum (OTPB3)	*A. solani* (OTA22)	15.3 ± 0.5 (78.7)
	P. infestans (PIT30)	10.0 ± 1.0 (86.0)
B. subtilis (OTPB1)	*A. solani* (OTA22)	21.7 ± 1.1 (69.3)
	P. infestans (PIT30)	18.0 ± 1.0 (74.0)
Control	*A. solani* (OTA22)	70.3 ± 1.5
	P. infestans (PIT30)	69.7 ± 0.5

Values in parentheses indicate percent inhibition of pathogen growth over control. Percentage of inhibition was calculated based on data collected after seven days of inoculation. Inhibition percentage defined as [*C*-*T*/*C*](100)], where *C* is the colony diameter of pathogen on control plate and *T* is the colony diameter of pathogen against test antagonist(mm). Percentage data were arcsin-transformed before analysis according to y = arcsin [sqr. (×/100)].
Data are the means and standard deviation of nine independent experiments. Each experiment contained three replicates. Each replicate contained six Petri plates.
[A] *Alternaria solani* inhibition assay on potato dextrose agar (PDA) at 25 ± 1 °C and *Phytophthora infestans* inhibition assay on rye A agar 19 ± 1 °C were performed. The radial growth of the pathogens were measured after every 24 h till the fungus reached the perimeter of the control plate (up to 7 days).

3. Results

3.1. Identification of B. subtilis OTPB1

A 1464 bp PCR product of the 16S rDNA gene was amplified from the genomic DNA of *B. subtilis* OTPB1. A sequence similarity search showed that the *B. subtilis* OTPB1 16S rDNA gene sequence had 100% similarity to the 16S rDNA gene sequences of the *B. subtilis* strains (JF496331, HQ286641) and 99% to the JQ726625, JQ904714 in the NCBI. A phylogenetic analysis based on the 16S rDNA gene sequences revealed that *B. subtilis* OTPB1 was closely related to *B. subtilis* (Fig. 1). Based on the consistent results of the sequence analyses of the 16S rDNA gene, the OTPB1 was identified as *B. subtilis*. The sequence was deposited in NCBI with accession no. JN160726.

3.2. Identification of T. harzianum OTPB3

When amplified the ITS region of rDNA of *T. harzianum* OTPB3, the primers ITS1 and ITS 4 produced the expected size PCR product showing band at 560 bp .A sequence similarity search showed that the resulting *T. harzianum* (*Hypocrea lixii*) OTPB3 PCR product for the ITS region shared 100% homology with their respective corresponding gene sequences of the *T. harzianum* (JX 173873, AFO 57583,AFO 555213, HQ022407, HQ022393) in the NCBI database, confirming the identity. A phylogenetic analysis based on the ITS region of rDNA gene sequences revealed that *T. harzianum* (*H. lixii*) OTPB3 was closely related to *T. harzianum* (JX 173873,AFO 57583,AFO 555213, HQ022407, HQ022393) (Fig. 2).The sequence of OTPB3 was submitted in the GenBank with the accession No. HM138489.

Table 3
Ability of *B. subtilis* OTPB1and *T. harzianum* OTPB3 to produce growth hormones in culture broth.[A]

Treatments[B]	IAA (nmol/ml) [B]	GA3(nmol/ml) [B]
T. harzianum OTPB3	58.8 ± 1.1 (90.8)a	20.0 ± 0.9 (85.3)a
B. subtilis OTPB1	42.9 ± 1.8 (86.4)b	15.2 ± 0.8(82.0)b
Potato dextrose broth	7.9 ± 1.6c	2.9 ± 0.4e
Nutrient broth	4.1 ± 1.9d	2.7 ± 0.1e
CD1%	3.2	1.4

[A] Values are mean of five independent experiments ± standard deviation. Each experiment consists of six cultures. Values in parentheses indicate percentage increase over control. For each row values are followed by a different lower case letter. a,b,c,d indicates significantly different at $p < 0.01$ for each of pair of treatment according to Fishers LSD test. e indicates non significance for each of pair of treatment.
[B] Indole-3-Acetic acid (IAA) and Gibberlic acid (GA3) levels in culture broth produced by *B. subtilis* OTPB1 and *T. harzianum* OTPB3 was estimated and quantified using HPLC with C_{18} reverse phase column by growing *B. subtilis* OTPB1 in nutrient broth at 25 ± 1 °C for 36 h at 120 rpm till an optical density of 1.0 was attained and *T. harzianum* was cultured in potato dextrose broth at 25 ± 1 °C for seven days. The solvent system included 70% water at pH 4.0 in acetonitrile. The quantification of these phytohormones was carried out at 205 and 220 nm using external standards. The experiment was repeated 12 times with five plants each time.

Table 4
Ability of *B. subtilis* OTPB1and *T. harzianum* OTPB3 to induce growth hormones in tomato roots[A].

Treatment[B]	IAA(nmol/g)[B]	GA_3(nmol/g)[B]
T. harzianum OTPB3	33.9 ± 1.3 (54.5)a	10.1 ± 1.3 (67.6)a
B. subtilis OTPB1	21.8 ± 2.4 (29.1)b	6.5 ± 0.9 (45.8)b
Control	15.5 ± 4.9c	3.5 ± 1.6c
CD1%	3.3	1.3

[A] Values are mean of six plants ± standard deviation. Five plants each were drawn from 12 independent experiments.Values in parentheses indicate percentage increase over control. For each row values followed by a different lower case letter are significantly different at $p < 0.01$, according to Fishers LSD test.
[B] Indole-3-Acetic acid (IAA) and Gibberlic acid (GA3)levels were determined in the roots of the tomato seedlings treated with bio-control agents and untreated control using HPLC method by macerating tomato root samples (10 g) from 30 day old seedlings in 80% chilled methanol (50 ml). The quantification of these phytohormones was carried out at 205 and 220 nm using external standards.

3.3. In vitro evaluation of antagonists

The *B. subtilis* OTPB1 and *T. harzianum* OTPB3 significantly reduced mycelia growth of *A. solani* and *P. infestans* under *in vitro* conditions (Table 1).

Table 2
Effect of *Bacillus subtilis* OTPB1 and *Trichoderma harzianum* OTPB3 on growth of tomato seedlings.[A]

Treatment[B]	Root Length (cm)	Shoot Length (cm)	Seedling vigor index[C]	Root weight (g)	Shoot weight (g)	Leaf area (cm^2)
OTPB3	17.0 ± 3.3 (38.5)a	22.5 ± 1.3 (32.0)a	3883.4 ± 320.6 (44.7)a	1.2 ± 0.4 (36.2)a	2.9 ± 0.6 (28.9)a	9.1 ± 1.6 (62.7)a
OTPB1	14.9 ± 3.0 (32.2)b	18.6 ± 1.2 (17.7)b	3240.6 ± 321.0 (34.9)b	1.0 ± 0.3 (15.9)a	2.1 ± 0.3 (8.8)b	6.5 ± 0.55 (37.7)b
Control	10.6 ± 2.8c	15.3 ± 2.0c	2162.3 ± 217.3c	0.50 ± 0.2b	1.4 ± 0.4c	3.4 ± 1.7c
CD1%	3.8	1.8	359.8	0.4	0.6	2.8

[A] Values are mean of 12 independent experiments ± standard deviation. Each experiment consists of 3 pot trays with 96 plants/tray, totaling 288 plants. Total number of plants used for experiments are 3180 seedlings. Seedling growth parameters like root length, shoot length, root fresh weight, shoot fresh weight and leaf area were determined for 3180 seedlings 30 days after sowing. Values in parentheses indicates percentage increase over control. For each row values followed by a different lower case letter are significantly different at $p < 0.01$, according to Fishers LSD test.
[B] Cell suspension of 48 h old *B. subtilis* (OTPB1) (1×10^8 CFU/ml) culture grown at 28 ± 1 °C in nutrient broth and the spore suspension (1×10^8spores/ml) prepared from seven days old *T .harzianum* (OTPB3) grown on potato dextrose agar at 25 ± 1 °C and sterile distilled water were incorporated into plastic pots at the rate of 1 ml /cavity and one tomato seed var. Arka vikas was placed in each cavity. Seed receiving only sterile distilled water served as untreated control and growth parameters were recorded after 30 days of sowing.
[C] Seedling vigour index = seedling length (cm) × germination percentage .Vigor indices were calculated after 4 weeks.

Table 5
Induction of defense enzyme activities in tomato seedlings treated with *T. harzianum* OTPB3 or *B. subtilis* OTPB1[A].

Treatments[B]	Polyphenoloxidase (U/g/min)[C]	Peroxidase (U/g/min)[D]	Superoxide dismutase(U/g/min)[E]
T. harzianum OTPB3	2.4 ± 0.2 (67.8)a	10.7 ± 1.3 (59.9)a	1.6 ± 0.2 (46.7)a
B. subtilis OTPB1	2.2 ± 0.3 (65.0)a	7.6 ± 2.1 (43.7)b	1.3 ± 0.2 (42.2)a
Control	1.3 ± 0.3b	4.3 ± 1.0c	0.74 ± 0.3b
CD1%	0.4	2.3	0.4

[A] Values are mean of eight replications ± standard deviation. Each replication consists of five plants. Values in parentheses indicate percentage increase over control. For each row values are followed by a different lower case letter. a indicates non significance each of pair of treatment, b,c indicates significantly different at $p < 0.01$,according to Fishers LSD test.
[B] Anti-oxidative enzymes was extracted according to Argandona et al. (2001) from freshly collected 30 day old tomato leaf samples (1 g) treated with *B. subtilis* OTPB1 and *T. harzianum* OTPB3 along with untreated control.
[C] Polyphenol oxidase activity was performed by using 0.05 M freshly prepared enzyme substrate pyrogallol and the change in absorbance was read at 450 nm spectrophotometrically at 60sec interval for 3 minutes. The enzyme activity was expressed as change in absorbance of the reaction mixture as units/min^{-1} g^{-1} fresh weight.
[D] Peroxidase activity was performed by using 0.05 M freshly prepared enzyme substrate pyrogallol and 0.1 ml of 0.3% freshly prepared solution of hydrogen peroxide. The change in absorbance was read at 420 nm spectrophotometrically at 60 sec interval for 3 minutes. The activity was expressed as change in absorbance of the reaction mixture as units/min^{-1} g^{-1} fresh weight.
[E] Superoxide Dismutase activity was performed by using 0.1 ml of 150 μM of Nitroblue tetrazolium chloride and The absorbance was read at 560 nm. The activity was expressed as change in absorbance of the reaction mixture as units/min^{-1} g^{-1} fresh weight.

3.4. Growth parameters

The inoculation of pot trays containing tomato seeds with *B. subtilis* OTPB1 or *T. harzianum* (OTPB3) lead to a significant increase ($p < 0.01$) in all growth parameters of tomato seedlings (Table 2). The OTPB3 increased root length, shoot length, leaf area, fresh weight of shoots and roots by 38.53%, 32.04%, 62.68%, 28.87% and 36.21%, respectively as compared to the control seedlings (Table 2). The results also showed that *T. harzianum* (OTPB3) stimulated higher growth than *B. subtilis* OTPB1.

3.5. Growth hormones in culture broth

The ability of *B. subtilis* OTPB1 or *T. harzianum* OTPB3 to produce IAA and GA_3 in culture broth was determined. The *T. harzianum* OTPB3 significantly ($P < 0.01$) produced higher levels of both IAA (90.79%) and GA_3 (85.32%) compared to control while *B. subtilis* OTPB1 produced growth hormones by 86.42% and 82.02%, respectively (Table 3).

Table 6
Effect of culture suspensions of *B. subtilis* OTPB1 or spore suspensions of *T. harzianum* OTPB3 on infection levels of *A. solani* and *P. infestans* on leaves of tomato seedlings[A]

Treatments[B]	A.solani[C] (cm^2)	P.infestans[D] (cm^2)
T. harzianum OTPB3	0.6 ± 0.1 (77.7)a	0.5 ± 0.1 (79.8)a
B. subtilis OTPB1	1.3 ± 0.1 (50.8)b	0.8 ± 0.1 (67.3)b
Control	2.6 ± 0.3c	2.5 ± 0.2c
CD 1%	0.3	0.2

[A] Mean of three replications ± standard deviation. Each replication consists of 25 plants. Values in parentheses indicate percentage inhibition of pathogen over control. For each row values followed by a different lower case letter are significantly different at $p < 0.01$, according to Fishers LSD test.
[B] Thirty day old tomato seedlings treated with *B. subtilis* (OTPB1) and *T. harzianum* OTPB3, placed in plastic chambers (45 cm height × 40 cm length × 15 cm width) containing water to maintain humidity was used for bioassay against *A. solani* (OTA22) and *P.infestans* (PIT30).
[C] For inoculation of *A. solani*, fully colonized 5-mm-diameter V8 juice agar disk, mycelium side down, was placed on the adaxial side of the third leaf and the plants were then incubated at 25 ± 1 °C under 16 h light and 8 h dark cycle. The control plants received sterilized, uncolonized block of agar.
[D] Third intact leaf from bottom of the tomato seedlings was inoculated with 10 μl droplets of a zoospore suspension containing 2.5×10^5 zoospores/ml of *P. infestans* at the centre of the leaf. Leaf with 10 μl droplets of sterile distilled water served as control and incubated at 18 ± 1 °C under 16 h cool white fluorescent light and 8 h dark.

3.6. Growth hormones in root

The results revealed that inoculating seeds with *T. harzianum* (OTPB3) or *B. subtilis* (OTPB1) significantly increased ($P < 0.01$) the levels of IAA and GA_3 in roots of tomato seedlings compared to control (Table 4). The levels of IAA and GA_3 were significantly higher by 54.34% and 67.59%, respectively in *T. harzianum* (OTPB3) treated seedlings compared to untreated control, while *B. subtilis* (OTPB1) caused an increase of IAA by 29.12% and GA_3 by 45.82% in treated seedlings.

3.7. Induction of defense enzymes

Polyphenol oxidase, peroxidase, and superoxide dismutase activities were significantly ($P < 0.01$) higher by 67.77%, 59.93% and 46.69%, respectively in tomato seedlings treated with *T. harzianum* OTPB3 and by 65.02%, 43.72% and 42.15% in *B. subtilis* OTPB1 treated seedlings (Table 5) as compared to untreated control. Comparison between the two bio-control agents revealed that plants treated with *T. harzianum* OTPB3 exhibited higher activities of defense enzymes than *B. subtilis* OTPB1.

3.8. Effect of OTPB3 and OTPB1 on early and late blight development

The lesion size caused by *A. solani* and *P. infestans* were significantly reduced ($P < 0.01$) by 77.69% and 79.83%, respectively in OTPB3 treated seedlings and by 50.76% and 67.33%, respectively in OTPB1 treated seedlings compared to untreated control (Table 6).

4. Discussion

In the current study, application of *B. subtilis* OTPB1 or *T. harzianum* OTPB3 to coco peat, resulted in remarkable increase in seedling growth of tomato. Several strains of *B. subtilis* and *T. harzianum* have the ability to promote crop growth and yield, through increased uptake of nutrients stimulated by growth promoting factors such as IAA and GA_3 and decreased level of ethylene owing to colonization of root (Idris et al., 2007; Gravel et al., 2007; Harman, 2011; Shoresh et al., 2010; Kloepper et al., 2004; Chen et al., 2007). Our study also demonstrated that both *B. subtilis* OTPB1 and *T. harzianum* OTPB3 produced higher quantities of IAA and GA_3 in culture broth and also induced their production within the treated tomato plants. The increase in IAA and GA_3 levels is one of the direct mechanisms by which biocontrol agents promote shoot and root growth and leaf

area in tomato plants. The role of IAA in lateral and adventitious roots initiation and emergence, as well as in shoot development by influencing cell division, expansion and differentiation (Hedden and Thomas, 2006) have been reported. The role of GA_3 in the promotion of elongation in axial organs in combination with auxins, and induction of mitotic division in leaf buds and leaves are well known (Srivastava, 2002). These hormones are believed to further stimulate uptake of more nutrients in the soil (Vessey, 2003), transduce signals among plant organs and integrate them to produce adequate defense responses to biotic or abiotic stresses (Ghanashyam and Jain, 2009). *Trichoderma atroviride* has been reported to produce IAA in tomato (Gravel et al., 2007) and the effect of IAA was responsible for increased growth of hydroponic tomatoes (Gravel et al., 2007). *B. subtilis,* known biocontrol agent with growth promoting ability, has been shown to produce IAA and GA_3 (Chen et al., 2007). They also colonize the plant roots, provide protection against certain soil borne fungal pathogens as well as stimulate growth and crop yield by hormonal stimulation through induction of host resistance by elicitation (Idris et al., 2007).

The defense related enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and superoxide dismutase (SOD) were significantly higher in OTPB1 and OTPB3 inoculated tomato seedlings, as compared to uninoculated control, which may be one of the factors accounting for significant reduction in lesion size caused by *A. solani* and *P. infestans*. Several studies have demonstrated enhancement of PO and PPO activities linked disease suppression in *T. harzianum* (Jayalakshmi et al., 2009; Houssien et al., 2010) or *B. subtilis* (Nakkeeran et al., 2006; Latha et al., 2009; Thilagavathi et al., 2007) treated plants. The enhanced PO, PPO and SOD activities might increase oxidative stress due to increased production of H_2O_2 (Vance et al., 1980). H_2O_2 and other free radicals are toxic to diverse microbial pathogens (Wu et al., 1995; Pena and Kuc, 1992; Tenhaken et al., 1995; Thipyapong et al., 1995; Stout et al., 1999). The oxidative potential of H_2O_2 also contributes to the formation of lignin during plant-pathogen interactions through PO-mediated cross-linking of proline-rich structural proteins and phytoalexin biosynthesis during oxidative burst and conversion of *O*-dihydroxyphenols to toxic *o*-quinones through PPO (Mayer and Harel, 1997).

The present study has clearly demonstrated that *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) can be employed for basal application in pot trays to raise quality tomato seedlings to induce systemic resistance against blights caused by *A. solani* and *P. infestans* respectively. *T. harzianum* and *B. subtilis* were reported to induce growth promotion and systemic resistance to many soil and seed borne foliar diseases of various vegetable crops including tomato (Papavizas, 1985; Linda, 2000; Sid Ahmad et al., 2000; Kloepper et al., 2004). In India, farmers procure tomato seedlings, commonly raised in pot trays using coco peat as the growing medium, from commercial nurseries and there are a lot of chances of spread of the leaf blight pathogens through transplants. Indeed, *A. solani* and *P. infestans* are seed and/or soil borne (Khulbe and Sati, 1987; Wangsomboondee and Ristaino, 2002) and use of pathogen free tomato transplants will help tomato growers to prevent crop losses caused by early as well as late blights. This study therefore assumes significance in production of disease free quality tomato transplants. Based on this study and other data, Seed pro, a seed coating formulation, has been developed and its utility in enhancing seed germination, seedling growth and vigour with resistance to seed borne fungal pathogens in vegetable crops is in progress.

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