

Full Length Research Paper

Induction of defense-related proteins and growth promotion in tomato by mixture of *Trichoderma harzianum* OTPB3 and *Bacillus subtilis* OTPB1 and *Pseudomonas putida* OPf1 against *Phytophthora infestans*

S. P. Mohan Kumar¹, P. Chowdappa^{1*}, V. Krishna² and H. Sandhya¹

¹Division of Plant Pathology, Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore-560 089, India.

²Department of Post-Graduate Studies and Research in Biotechnology, Kuvempu University, Jnanasahyadri, Shankaraghatta 577 451 India.

Received 22 September, 2014; Accepted 22 December, 2014

Late blight incited by *Phytophthora infestans* is a destructive disease of tomato worldwide. The plant growth-promoting antagonists, which elicit induced systemic resistance (ISR) and enhance plant growth, are being used as safe alternatives to synthetic fungicides for the management of plant diseases. In this study, a combination of *Trichoderma harzianum* OTPB3 and *Bacillus subtilis* OTPB1 and *Pseudomonas putida* OPf1 alone were evaluated for induction of systemic resistance in tomato against *P. infestans* in comparison with fungicides and growth promotion. Seed treatment with fresh suspensions of a combination of *T. harzianum* OTPB3 and *B. subtilis* OTPB1 caused significant increase in growth parameters compared to *P. putida* OPf1, mancozeb and untreated control due to higher production of indole-3-acetic acid (IAA) and gibberellic acid (GA₃). Reduction in the incidence of late blight was positively linked to increase of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and β-1,3-glucanase, the defense-related enzymes in tomato seedlings treated with microbial consortium of OTPB3 and OTPB1 followed by foliar spray of *P. putida* OPf1. The effects were on par with fenamidone and mancozeb treatments. The results reveal that seed treatment with microbial consortium containing *T. harzianum* OTPB3 and *B. subtilis* OTPB1 and foliar spray of *P. putida* OPf1 have practical significance in the management of late blight disease and also plant growth enhancement in tomato.

Key words: *Trichoderma harzianum*, *Bacillus subtilis*, *Pseudomonas putida*, *Phytophthora infestans*, Growth promotion, late blight, Growth hormones, induction of systemic resistance.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.), an important protective vegetable crop, is grown in 865,000 hectares in India (<http://nhb.gov.in/area-pro/database-2011.pdf>).

Late blight, incited by *Phytophthora infestans* (Mont.) de Bary, is a devastating disease of tomato (*L. esculentum* Mill.), which often cause crop losses up to 100% (Fry et

al., 1993; Nowicki et al., 2012). *P. infestans* also causes root, foot, stem and fruit rot in addition to foliar blight in tomato (Lievens et al., 2004). The most commonly practiced methods to manage late blight are cultural, fungicide sprays, and use of resistant cultivars (Nowicki et al., 2012). Management of late blight using cultural practices alone is highly challenging particularly in areas, where tomato is grown year-round. Since commercial cultivars do not have adequate tolerance to late blight, chemical control involving fungicides scheduled at 5–7 days interval, form the basis for late blight management programs (Fry et al., 1993; Tumwine et al., 2002). Although fungicides have been successfully employed in managing late blight, their residues and environmental hazards leading to human health risks are major concerns. Development of resistance to fungicides by *P. infestans* further limits their use for disease management (Chowdappa et al., 2013a).

In recent years, biological control gained importance as an alternative to chemicals for plant disease management (Murphy et al., 2003; Woo et al., 2006; Harman, 2011). Biocontrol agents control the pathogens by several mechanisms which include direct antagonism, antibiosis, mycoparasitism and siderophore production (Compant et al., 2005; Fridlender et al., 1993; Parke et al., 1991; Daayf et al., 1997). Besides, induced systemic resistance (ISR) in plants has been demonstrated as one of the modes by which biocontrol agents limit the effects of fungal infections (Schneider and Ullrich, 1994; Ramamoorthy et al., 2002; Saravanakumar et al., 2007; Latha et al., 2009; Chitrashree et al., 2011). Microbial consortia for plant growth enhancement and induction of systemic resistance (Janisiewicz, 1988; Choure et al., 2012) were successfully used. Janisiewicz (1988) reported antagonistic mixtures that exhibited biocontrol of post-harvest diseases in apple. Combination of three strains viz. *Pseudomonas fluorescens* LPK2, *Sinorhizobium fredii* KCC5 and *Azotobacter chroococcum* AZK2, suppressed the wilt incidence in *Cajanus cajan* (Choure et al., 2012) and enhanced plant growth due to synergism. Bio-consortium containing effective *Bacillus bassiana* and *P. fluorescens* strains controlled collar rot disease in groundnut both under polyhouse and field (Senthilraja et al., 2010).

Induction of defense responses by *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp. is largely related to increase of β -1,3-glucanase, phenylalanine ammonia-lyase, 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). ISR incited by PGPR has been reported in many plants like *Arabidopsis* spp., bean, carnation, cucumber, radish, tobacco, and tomato (Van

Loon et al., 1998). These biocontrol organisms control the diseases besides plant growth promotion through production of growth hormones like IAA and GA₃ (Chowdappa et al., 2013b). Systemic acquired resistance (SAR) against late blight was reported earlier in tomato by inoculating either pathogen (Christ and Mosinger 1989; Enkerli et al., 1993; Heller and Gessler, 1986) or by applying chemicals (Cohen, 1994) proceeding to confront the pathogen. ISR induced by PGPR has also been demonstrated in tomato against late blight incited by *Phytophthora infestans* (Yan et al., 2002). In our previous study, *Trichoderma harzianum* (OTPB3) and *Bacillus subtilis* (OTPB1) strains were identified that have the ability to induce systemic resistance against *Alternaria solani* and *P. infestans* (Chowdappa et al., 2013b) and also enhance plant growth. The aim of the present investigation was to know the additive effect of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) strains as consortium and *Pseudomonas putida* (OPf1) individually through seed treatment in comparison to mancozeb followed by foliar spray of *P. putida* (OPf1) and fenamidone + mancozeb for induction of systemic resistance in tomato against *P. infestans* and also plant growth promotion.

MATERIALS AND METHODS

Isolation and identification of biocontrol strains

Biocontrol strains *B. subtilis* OTPB1 and *T. harzianum* OTPB3 identified in our previous study (Chowdappa et al., 2013b) were used in this study. *P. putida* OPf1 was isolated from the rhizosphere soil sample from tomato crop at Ranga Samudrum, Andhra Pradesh, India using King's B Medium (King et al., 1954). Soil samples from rhizosphere were collected from healthy tomato plants grown under field conditions by uprooting plants carefully without any injury to the root system. Four plants from four different places were collected and the samples were mixed together and placed in polythene bags. Ten grams of soil was added to 90 ml of sterile distilled water and vigorously shaken for 10 min. The suspensions were serially diluted up to 10⁻⁷. Then, 0.1 ml of 10⁻¹, 10⁻³, 10⁻⁵ and 10⁻⁷ diluted samples was spread on King's medium B (King et al., 1954). Three replicate plates were incubated at 27±1°C for 48 h. After 48 h of incubation, all the isolates were checked for fluorescence under UV light at 365 nm (Sharifi-Tehrani et al., 1998). Colonies that showed fluorescence were selected and further purified on King's medium B agar medium. Pure isolates were stored at -80°C after addition of 30% glycerol (v/v).

DNA was isolated from 36 h old cultures of *P. putida* OPf1, grown in nutrient broth at 26±1°C, using bacterial DNA isolation kit (Zymo Research Bacterial DNA Mini Prep., USA). PCR amplification of 16S rDNA was performed using 27F (5'-AGAGTTTGTATCCTGGCTCAG-3') (Weisberg et al., 1991) and 1492R-5'-GGTTACCTTACGACTT-3' (Reysenbach et al., 1992). PCR was carried out in 50 µl reaction volumes. Each reaction consisted approximately of 1 µl of template DNA, 5 µl 10 x PCR buffer, 40.75 µl sterile distilled water, 1 µl 2.0 mM dNTPs, 1 µl each

*Corresponding author. E-mail: pallem22@gmail.com; pallem@iihr.ernet.in. Tel: +91-80-28466421. Fax: +91-80-28466291.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

of 50 pM primers 27F and 1492R and 0.25 μ l Taq polymerase (5 U/ μ l) (Merck Bio Sciences, India). Thermocycling conditions consisted of initial one denaturation step at 94°C for 5 min followed by 32 amplification cycles at 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 1x Tris Borate-EDTA buffer and stained with ethidium bromide (5 μ g/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 *P. putida* available in NCBI.

The phylogenetic analysis of *P. putida* OPf1 was inferred using the Maximum Parsimony method. Tree 1 out of 3 most parsimonious trees (length = 74) is shown. The consistency index was 0.612245, the retention index was 0.707692, and the composite index was 0.523498 (0.433281) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown above the branches (Felsenstein, 1985). The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The tree was drawn to scale, with branch lengths computed following the average pathway method (Nei and Kumar, 2000) and expressed in the units of number of changes over the whole sequence. The analysis involved 27 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 406 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

B. subtilis OTPB1 and *T. harzianum* OTPB3 were deposited at National Bureau of Agriculturally Important Microorganisms, Mau, India bearing accession numbers NAIMCC-B-01339 and NAIMCC-F-03065, respectively and *P. putida* OPf1 was deposited at Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India, as accession number MTCC 5824.

Agar plate-based pathogen inhibition assays

Antagonistic effect of *B. subtilis* OTPB1 or *T. harzianum* OTPB3 was evaluated against *P. infestans* PIT 30 by adopting dual culture method (Webber and Hedger, 1986). For inhibition assays by *P. putida* OTPf1, a 5 mm-diameter agar plug of a 7-day-old culture of *P. infestans* PIT 30 was transferred to the center of a plate Rye agar A and incubated at 19 \pm 1°C in darkness. Then, 5 μ l from an exponentially growing bacterial culture in nutrient broth at OD600 of 0.1 was spotted 1 cm from the edge of the rye agar plate on one side of the pathogen plug. Controls consisted of a 5 mm-diameter agar plug of without *P. putida* OTPf1.

Preparation of bacterial cell suspension

Bacterial inoculum of *B. subtilis* OTPB1 and *P. putida* OPf1 were prepared by harvesting cells from nutrient broth cultures grown at 28 \pm 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 a Biomate 3 spectrophotometer (Thermo spectronic, USA) to 10⁸ cfu/ml (Thompson, 1996; Yan et al., 2002) as confirmed by plating on nutrient agar.

Preparation of spore suspension of *T. harzianum* OTPB3

Spore suspensions of *T. harzianum* OTPB3 were prepared by

scraping them 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 \pm 1°C for 7 days. Spores were suspended in sterile distilled water and the number of colony forming units (cfu) that developed was assayed on a *Trichoderma* selective medium (Elad et al., 1981) and adjusted the values to 10⁸ CFU/ml.

Preparation of zoospore suspension of *P. infestans* PIT 30

P. infestans PIT 30 (GenBank accession JF834691) was used (Chowdappa et al., 2013b) in the present study. Zoospore suspension was prepared by growing *P. infestans* PIT 30 on Rye agar B medium at 18°C under light (16 h cool white fluorescent light and 8 h dark) for 14 days. Sporangial suspension 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 2h to induce zoospore release. Zoospores were separated from sporangia by filtration through a 12- μ m mesh filter and diluted to a concentration of 3x10⁵ zoospores/ml.

Test chemicals

Mancozeb was procured from Indofil Chemicals Pvt. Ltd., India and the pre-packed mixture of fenamidone and mancozeb was obtained from Bayer Pvt. Ltd., India.

Compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1

In vitro bioassay test was done on potato dextrose agar (Himedia, Mumbai, India) to determine the compatibility of the *T. harzianum* OTPB3 and *B. subtilis* OTPB1. A Petri dish containing PDA medium was spot inoculated with a 48 h-old cell suspension of *B. subtilis* OTPB1 at four different corners on the edge of agar medium. A mycelial plug (4mm diameter, cut from the actively growing edge of a 4 day old mycelial mat on PDA) of *T. harzianum* OTPB3 was placed in centre of the plate and incubated at 25 \pm 1°C for 5 days in the dark. Each bioassay was replicated and repeated thrice. The compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1 was also studied by mixing equal ratio (1:1 ml) of cell suspension of *B. subtilis* OTPB1 (10⁸cfu ml⁻¹) and conidial suspension of *T. harzianum* OTPB3 (10⁸ spores ml⁻¹). The mixture was inoculated into potato dextrose broth and incubated at 25 \pm 1°C for 7 days and one loop of culture broth was streaked on potato dextrose agar and incubated at 25 \pm 1°C for 3 days in the dark.

Seed treatments

Tomato Cv. Arka vikas seeds were surface sterilized with 1% sodium hypochlorite for 2 min followed by three rinses with sterile distilled water. Ten grams of sterilized tomato seeds were incubated in 50 ml spore suspension (10⁸ spores/ml) of *T. harzianum* OTPB3 or cell suspension (10⁸cfu ml⁻¹) of *B. subtilis* OTPB1, amended with 0.2% (w/v) sterile carboxymethyl cellulose (CMC) sticker suspensions at 25°C in a rotary shaker at 80 rpm for 2 h for allowing attachment of bacterial cells or spore suspension or test chemicals to the seed coat. The treated seeds were placed in sterile 90 mm Petri dishes and air-dried on a laminar flow bench for 12 h. For combined inoculation of *Trichoderma* and *Bacillus* isolates, seeds were soaked in a mixture of cell suspension of *B. subtilis* OTPB1 (10⁸cfu ml⁻¹) and conidial suspension of *T. harzianum* OTPB3 (10⁸ spores ml⁻¹) in ratio of 1:1. Suspension of mancozeb (0.2%) was used. The seeds treated with sterile distilled

water amended with CMC and seeds soaked in distilled water served as controls. Inoculant densities on treated tomato seeds were determined using a dilution plating technique. Five tomato seeds treated with OTPB3, OTPB1 and consortium of OTPB3 and OTPB1 were suspended in 5 ml of 10 mM sterile phosphate buffer (pH 7.0) and sonicated in an ultrasonic bath to release adhering bacteria and *Trichoderma* and then serial dilutions (1/10) were plated on Kings B medium and *Trichoderma* selective medium. Petri dishes were incubated for 5 days at 28°C for bacteria and 7 days at 25°C for *Trichoderma*. The number of cfu per seed was determined at inoculation time (0 h), and 24 h and 48 h from inoculation time (Correa et al., 2009).

Effect of seed treatment on growth promotion under greenhouse conditions

Seeds treated with fresh suspension of microbial consortium and test chemicals along with untreated controls sown separately in pot-trays filled with sterilized coco peat. Seedlings were allowed to grow for 30 days at 25 ± 2°C under natural light. After 30 days, seedling growth parameters such as root length and shoot lengths, root and shoot weights and leaf area were measured for 1,536 seedlings. Each treatment consisted of four replicates and each replication consisting of 96 plants, thereby making a total of 384 plants per treatment and the experiment was repeated thrice. The germination percentage was calculated on the 14th day after sowing as most of the seeds germinate within this period. Seeds were considered as germinated when their two cotyledonary leaves were visible above the coco peat. About 1,536 seeds (3 independent experiments with four replicates) were scored for determining germination percentage. Seedling vigour index was calculated using the following formula as described by Baki and Anderson (1973) that is seedling vigour index = seedling length (cm) × germination percentage. The data of all 1,536 seedlings were pooled and analyzed after no block effects were noted.

Determination of growth hormones in tomato

IAA and GA₃ levels were determined in the roots of tomato seedlings treated with biocontrol agents, mancozeb 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. The supernatant was evaporated *in vacuo* at 40°C, residue dissolved in water and adjusted to pH 8.0. The alkaline extract was partitioned twice with ethyl acetate and discarded. pH of the 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, after drying over anhydrous Na₂SO₄, 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 GA₃ and IAA analyses as described below.

High performance liquid chromatography (HPLC) conditions

IAA and GA₃ were assessed by HPLC (Model-Prominence, Make-Schimidzu, Japan) as described by Kelen et al. (2004) with a few modifications. A C₁₈ reverse phase column (Synergi, 250 x 4.6 mm, 4 µm, Phenomenex, USA) and photodiode array (PDA) detector (Model SPD-M20A, Schimidzu, 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 GA₃ and IAA. The quantification of these

phytohormones was carried out at 205 and 220 nm against external standards. The experiment was repeated 12 times with five plants each time.

Induction of systemic resistance

Pot trays containing 30 days-old tomato seedlings treated with different seed treatments were placed in growth chambers (Research and Test Equipment Co., Bangalore, India). Then, each pot tray containing 96 seedlings were sprayed separately with cell suspensions of *P. putida* OPf1 (10⁸ cfu ml⁻¹), mancozeb (2 g/l) and pre-packed mixture of fenamidone + mancozeb (3 l/l) followed by zoospore suspension containing 3x10⁵ zoospores /ml of *P. infestans* PIT 30 (Chowdappa et al., 2013b). The treated plants were then kept in plant growth chambers and incubated at 18 ± 1°C with 100% relative humidity under 16 h cool white fluorescent light and 8 h dark (Chowdappa et al., 2013a). The disease incidence was recorded six days after inoculation and rated by estimating the affected percentage leaf area (James, 1971) of all leaves. Percentage of disease severity incidence was calculated using the formula (Amin et al., 2013).

$$\text{Percentage Severity Index} = \frac{\text{Sum of Individual numerical rating}}{\text{Total Number of assessed} \times \text{Maximum score in scale}} \times 100\%$$

The experiments were repeated thrice. Each experiment consists of 3 pot-trays with 96 plants/tray, totaling 288 plants. Total number of plants used for experiments are 864 seedlings. The data of all the 864 seedlings were pooled and analyzed after no block effects were recorded. The samples for enzyme assay were collected separately during three repetitions.

Sample collection and assay of defense-related proteins

Thirty days old plants were carefully uprooted without causing any damage to root and leaf tissues at intervals of 0, 1, 3, 5, 7, 9 and 11 days after challenge inoculation (Latha et al., 2009). The seedlings from each replication were separately washed in running water, blot dried and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. One gram of sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used as a crude enzyme extract for assaying peroxidase (PO; EC 1.11.1) (Hammerschmidt et al., 1982), polyphenol oxidase (PPO; EC 1.12.18.1) (Mayer et al., 1965) and phenylalanine ammonia lyase (PAL; EC 4.3.1.5) (Dickerson et al., 1984). Enzyme extracted in 0.1 M sodium citrate buffer (pH 5.0) was used for the estimation of β-1,3-glucanase (Pan et al., 1991). 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.

Assay of peroxidase (EC 1.11.1)

The assay was carried out as described by Hammerschmidt et al. (1982). The reaction mixture consisted of 1.5 ml of 0.05 pyrogallol, 0.5 ml enzyme extract and 0.5 ml of H₂O₂ and incubated at 28±1°C. The changes in absorbance were measured at 420 nm at 30 s interval for 3 min. The enzyme activity was expressed as changes in absorbance of the reaction mixture min⁻¹g⁻¹ on fresh weight source.

Assay of polyphenol oxidase (EC 1.12.18.1)

Enzyme assay was performed as described by (Mayer et al., 1965).

200 μ l of enzyme extract was added with 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). Reaction was initiated by adding 200 μ l of 0.01 catechol. Changes in absorbance of the reaction mixture were expressed in $\text{min}^{-1}\text{g}^{-1}$ on fresh weight source.

Assay of phenylalanine ammonia lyase (EC 4.3.1.5)

Enzyme assay was performed as described by Dickerson et al. (1984). Reaction mixture containing 100 μ l of enzyme with 500 μ l of 50 mM Tris HCL (pH 8.8) and 600 μ l of 1 mM L- phenylalanine were incubated for 60 min at 25°C. The reaction was arrested by adding 2 N HCl. Meanwhile 1.5 ml of toluene was added, mixed in vortex for 30 s, centrifuged at 10,000 rpm at 4°C for 5 min. Toluene portion with trans-cinnamic acid was separated and toluene phase was read at 290 nm against toluene as blank. A standard curve was plotted using cinnamic acid solution in toluene at described concentrations.

Assay of β -1, 3-glucanase (EC 3.2.1.39)

Assay was carried out as using laminarin dinitrosalicylic acid method as described by Pan et al. (1991). The reaction mixture consisted of 62.5 μ l of 4% laminarin and 62.5 μ l of enzyme extract. The assay was carried out at 40°C for 10 min. The reaction was terminated by adding 375 μ l of dinitrosalicylic acid and heating for 5 min in hot water bath, mixed well and measured absorbance at 500nm. The activity was expressed as μ g of glucose released units/mg of protein.

Protein estimation

Protein contents of the extract for all enzymes were estimated following the method of Bradford (1976) using bovine serum albumin (BSA) (Sigma, USA) as standard.

Native polyacrylamide gel electrophoresis analysis

The isoform profiles of PPO were separated by discontinuous native polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). The protein extract was prepared by homogenizing 1 g of leaf sample in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 18,000 rpm for 20 min at 4°C. After the protein content was determined (Bradford, 1976), the samples (50 μ g protein) were loaded onto 8% polyacrylamide gels (Sigma, USA). After electrophoresis, PPO isoform profiles were assessed by equilibrating gels for 30 min in 0.1% p-phenylene diamine, followed by addition of 10 mM catechol in the same buffer (Jayaraman et al., 1987).

Statistical analysis

All data were statistically analyzed using one way 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 least significant difference (LSD) at the 1% level ($p < 0.01$). Percentage data were arcsine-transformed before analysis according to $y = \arcsin [\sqrt{x/100}]$.

RESULTS

Identification of *Pseudomonas putida* OPf1

PCR amplification of the 16S rDNA gene amplified from the genomic DNA of *P. putida* OPf1 yielded fragment of 1464 bp. Blast search of the *P. putida* OPf1 16S rDNA gene sequence revealed that it had 98% similarity to the 16S rDNA gene sequences of *P. putida* strains in NCBI (Figure 1). A phylogenetic tree generated using 16S rDNA gene sequences showed that *P. putida* OPf1 was closely related to *P. putida* (Figure 1). The OPf1 was identified as *P. putida*, based on the sequence analyses of 16S rDNA gene. The 16S rDNA sequence of OPf1 was deposited in NCBI (www.ncbi.nlm.nih.gov/) with accession no. KC964109.

In vitro evaluation of antagonists

The *P. putida* OPf1 significantly reduced mycelial growth of *P. infestans* by 72.9% when evaluated under *in vitro* conditions (Table 1).

Compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1

When one loop of culture broth streaked on potato dextrose agar, both *B. subtilis* OTPB1 and *T. harzianum* exhibited growth on PDA without any antagonistic activity after 72 h of incubation (Figure 2). They also did not exhibit inhibitory effects on each other when spot inoculated on PDA. The number of colony forming units (cfu) recovered from treated seed at different time intervals after inoculation (Table 2) showed that OTPB3, OTPB1 and microbial consortium were effectively colonized tomato seeds. No differences were observed in colony forming units, irrespective of treatment and remained unaffected up to 48 h of post inoculation (Table 2). Thus, the isolates OTPB3 and OTPB1 were compatible and can be utilized for seed coating formulation (Table 2).

Growth parameters

Tomato seeds treated with a mixture of *B. subtilis* OTPB1 and *T. harzianum* (OTPB3) or singly with OTPB1, OTPB3 and *P. putida* OPf1 exhibited increase ($p < 0.01$) in seedling growth parameters (Table 3) significantly compared to mancozeb (0.2%) and untreated control. The consortium enhanced root and shoot lengths, leaf area, fresh weight of shoots and roots by 56.3, 40.9, 34.0, 50.2 and 56.9% respectively as compared to the control seedlings (Table 3). The data also indicated that the microbial consortium stimulated better growth than

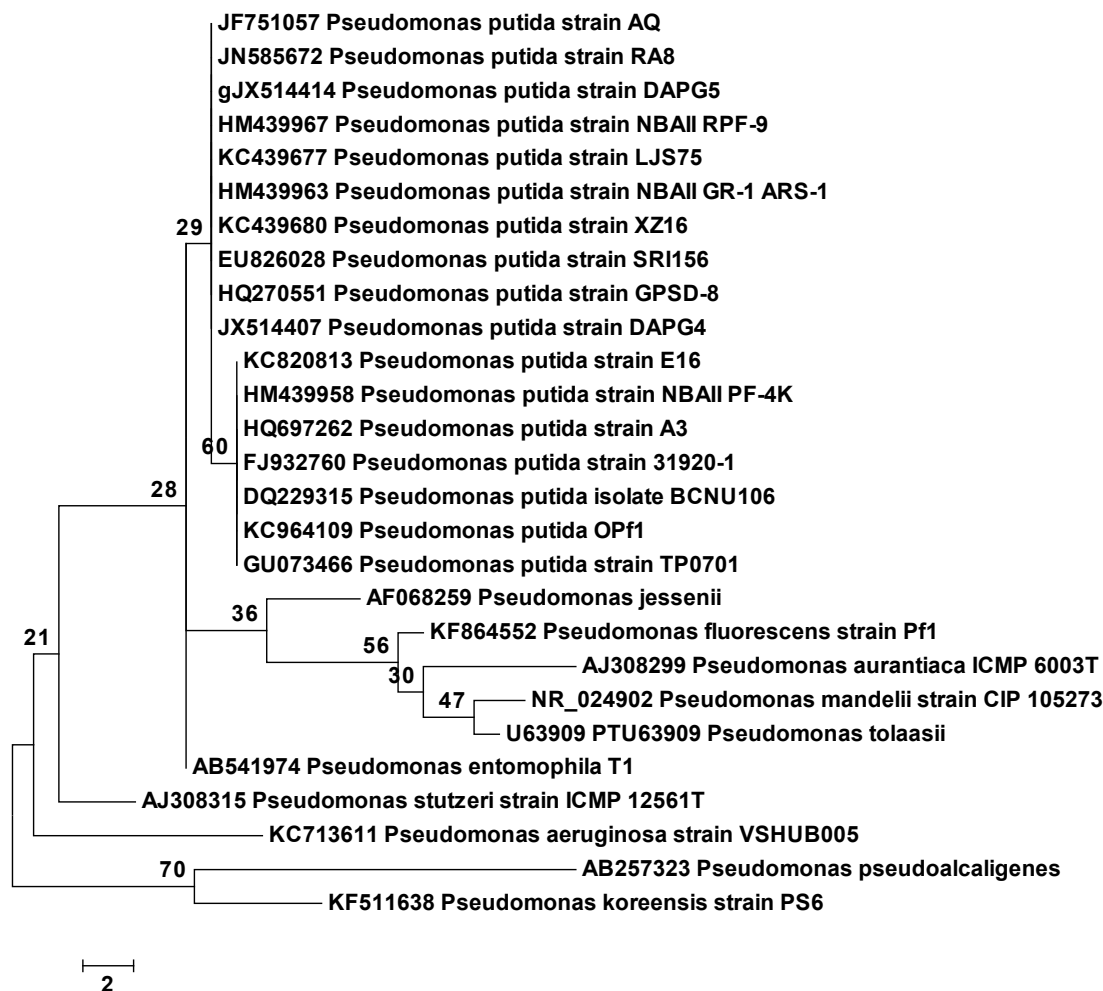


Figure 1. Phylogenetic tree of the *Pseudomonas putida* OPf1 based on the 16s rDNA gene sequences.

Table 1. *In vitro* inhibition of and *P. infestans* (OTA 30) by *P. putida* (OPf1)^A.

Isolate	Pathogen	Pathogen growth (mm)	
<i>P. putida</i> (OPf1)	<i>P. infestans</i> (PIT30)	21.0 ± 1.0	(72.9)
Control	<i>P. infestans</i> (PIT30)	77.7 ± 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 $y = \arcsin [\text{sqr.} (1/100)]$. Data are the means and standard deviation of nine independent experiments. Each experiment contained three replicates. Each replicate contained six Petri plates.^A *Phytophthora infestans* inhibition assay on rye A agar $19 \pm 1^\circ\text{C}$ 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).

other treatments including treatments with OTPB1, OTPB3 and *P. putida* OPf1 separately. Further studies were restricted to consortium only as they stimulated

higher growth and the data on stand-alone treatments of OTPB1, OTPB3 was published earlier (Chowdappa et al., 2013b).

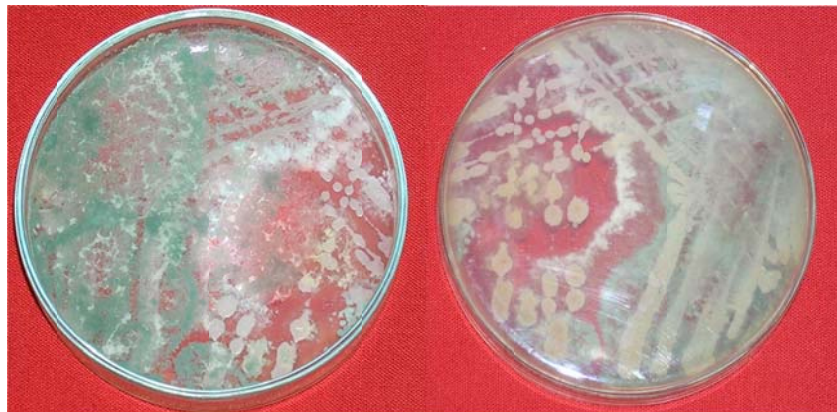


Figure 2. Compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1. Both *B. subtilis* OTPB1 and *T. harzianum* exhibited growth on PDA without any antagonistic activity after 72 h of incubation. One loop of culture broth inoculated with *T. harzianum* OTPB3 and *B. subtilis* OTPB1 was streaked on potato dextrose agar, both *T. harzianum* OTPB3 and *B. subtilis* OTPB1, exhibited growth on PDA without any antagonistic activity.

Table 2. Viable inoculum densities per tomato seed treated with biocontrol agents OTPB1 and OTPB3^A.

Treatments ^B	0 h	24 h	48 h
OTPB3	5.8x10 ⁴ a	5.6x10 ⁴ b	5.8x10 ⁴ c
OTPB1	5.3x10 ⁴ a	5.3x10 ⁴ b	5.6x10 ⁴ c
OTPB3+OTPB1 (OTPB1)	5.6x10 ⁴ a	5.3x10 ⁴ b	5.3x10 ⁴ c
OTPB3+OTPB1 (OTPB3)	5.6x10 ⁴ a	5.7x10 ⁴ b	5.2x10 ⁴ c
CD1%	0.9	1.2	1.3

^AValues are mean of five independent experiments. Each experiment consists of five seeds. For each row values are followed by a different lower case letter indicates significantly different at $p < 0.01$ for each of pair of treatment according to Fishers LSD test. ^BFive tomato seeds var. Arka vikas, treated with OTPB3, OTPB1 and Consortia with OTPB3 and OTPB1 were suspended in 5ml of 10mM sterile phosphate buffer (pH 7.0) and sonicated in an ultrasonic bath to release adhering bacteria and *Trichoderma* and then serial dilutions (1/10) were plated on Kings B medium for bacteria, *Trichoderma* selective media for *Trichoderma* and Potato dextrose agar for microbial consortia. Petri dishes were incubated for 5 days at 28°C for bacteria and 7days for *Trichoderma*. The number of cfu per seed was determined at inoculation time (0 h), 24 h and 48 h from inoculation time (Correa et al., 2009).

Growth hormones in tomato

The endogenous levels of IAA and GA₃ in roots of tomato seedlings treated with microbial consortium of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) were significantly higher ($P < 0.01$) compared to treatment with *P. putida* OPf1 and mancozeb and untreated control (Table 4). The IAA and GA₃ levels were higher by 71.1 and 78.8%, respectively in seedlings treated with microbial consortium as compared to untreated control, while *P. putida* (OPf1) treated seedlings showed an increase of IAA by 44.7% and GA₃ by 60.7%.

Late blight incidence under controlled conditions in growth chamber

Seed treatment with OTPB3 and OTPB1 combination, OPf1 strain and mancozeb as chemical check coupled with foliar sprays of OPf1 and pre-packed mixture of fenamidone - mancozeb were evaluated for their efficiency against *P. infestans* under pot culture conditions in growth chamber (Table 5). Seed treatment with OTPB3+OTPB1 followed by foliar spray of OPf1 significantly reduced late blight incidence by 73.1% compared to untreated controls. The results showed that disease reduction with OTPB3+OTPB1+ OPf1 mixture was on par with the fungicide check (mancozeb + fenamidone – mancozeb), which also recorded 72.8% reduction in late blight incidence. The combinations of OPf1+ fenamidone – mancozeb, OTPB3+OTPB1+ fenamidone – mancozeb, OTPB3+OTPB1+ mancozeb also caused reduction in late blight incidence similar to OTPB3+OTPB1+ OPf1 combination. Seed treatment alone with OTPB3+OTPB1 combination showed lower incidence of late blight (38.4%) compared to OPf1 (61.2%) and mancozeb (51.4%).

Response of defense-related proteins

PO, PPO, PAL and β -1,3-glucanase activities were measured in leaves from *P. infestans* inoculated and OTPB3 + OTPB1 + OPf1, OTPB3 + OTPB1 + mancozeb, OTPB3 + OTPB1 + fenamidone – mancozeb, OPf1+ fenamidone – mancozeb and OTPB3 + OTPB1, mancozeb, and OPf1 pre-treated tomato plants. These treatments differed in their ability to stimulate PO, PPO, PAL and β -1,3- glucanase activities in tomato plants inoculated with *P. infestans*. The data showed that tomato plants treated with OTPB3 + OTPB1 + OPf1 mixture exhibited higher

Table 3. Effect of seed treatment of fresh suspensions on growth of tomato seedlings^A.

Treatment ^B	Root Length(cm)	Shoot length (cm)	Seedling vigour index ^C	Root weight (g)	Shoot weight (g)	Leaf area(cm ²)
OTPB3	14.4±2.2(41.1) ^b	20.5±2.4(42.0) ^b	3434.0±348.1(45.7) ^b	1.3±0.3(52.9) ^b	2.0±0.4(63.2) ^{ab}	7.1±1.3(46.6) ^b
OTPB1	11.7±2.7(27.8) ^c	22.3±3.1(35.9) ^c	2935.9±271.3(36.4) ^c	1.0±0.1(26.9) ^b	1.8±0.3(60.4) ^b	6.3±1.8(40.3) ^c
OTPB3+OTPB1	16.5±2.3(56.3) ^a	23.5±2.2(40.9) ^a	3708.9±178.2(53.5) ^a	0.3±0.05(56.9) ^a	2.4±0.2(50.2) ^a	8.8±1.1(34.0) ^a
OPf1	11.0±1.4(34.5) ^c	18.4±1.8(37.9) ^c	2877.7±118.5(40.1) ^c	0.2±0.03(38.2) ^c	1.8±0.3(46.2) ^c	6.2±1.2(19.2) ^c
Mancozeb (0.2%)	7.3±1.1(1.5) ^d	16.6±1.4(16.4) ^d	1952.7±114.3(11.7) ^d	0.1±0.05(8.5) ^d	1.6±0.2(23.2) ^d	6.2±1.4(5.4) ^d
Control CMC	7.8±1.3d	14.0±1.6 ^d	1785.8±145.2 ^d	0.1±0.05 ^d	1.3±0.1 ^d	6.0±1.1 ^d
Control	7.2±1.2 ^d	13.8±1.8 ^d	1723.3±138.6 ^d	0.1±0.025 ^d	1.2±0.2 ^d	5.8±1.4 ^d
CD 1%	1.3	0.9	127.1	0.04	0.3	0.5

^A Values are mean of 3 independent experiments ± standard deviation. Each experiment consists of 4 pot trays with 96 plants/tray, totaling 384 plants. Total number of plants used for experiments are 1536 seedlings. Seedling growth parameters like root length, shoot length, root fresh weight, shoot fresh weight and leaf area were determined for 1536 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. ^BBacterial isolate *B. subtilis* OTPB1 (10⁸) and one isolate *Trichoderma harzianum* OTPB3 suspension (10⁸), *P. putida* OPf1 suspension (10⁸) and consortium of OTPB1 (10⁸) and OTPB3 (10⁸) and 0.25% suspension of mancozeb were used as fresh suspension for seed treatment and each treated tomato seed var. Arka vikas was placed in each cavity of pot trays containing sterilized cocpeat. Seed receiving only sterile distilled water and CMC for seed treatment served as untreated control and growth parameters were recorded after 30 days of sowing. ^CSeedling vigour index = seedling length (cm) x germination percentage. Vigor indices were calculated after 4 weeks.

Table 4. Ability of Biocontrol agents to induce growth hormones in tomato roots^A.

Treatments ^B	IAA (nmol/g) ^B	GA3 (nmol/g) ^B
OTPB1+OTPB3	35.8±0.8(71.1)a	10.4±0.4(78.8)a
OPf1	18.7±0.9(44.7)b	5.6±0.2(60.7)b
Mancozeb	11.01±0.8(6.08)c	2.2±0.2c
Control	10.34±0.2ce	2.2±0.3c
CD1%	5.7	0.5

^AValues 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. ^BIndole-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.

defense enzyme activities against *P. infestans* compared to other treatments (Figure 3). The enzyme activities were increased after 3 days and reached to a maximum after 5 days of pathogen inoculation and decreased, thereafter. However, the enzyme activities in tomato plants treated with a combination of OTPB3+OTPB1+ OPf1 remained high, up to 11 days after inoculation as compared to all other treatments. In contrast, the increased activities of enzymes were observed only up to the seventh day of *P. infestans* inoculation in other treatments and, thereafter, a drastic decline was recorded. Control plants or inoculation with pathogen alone did not exhibit any noticeable changes in the activities of the enzyme (Figure 3).

Native polyacrylamide gel electrophoresis analysis of PPO

An analysis of PPO extract from tomato plants treated

with OTPB3+OTPB1+ OPf1 combination and inoculated with *P. infestans* by native PAGE exhibited three isoforms PPO1, PPO2 and PPO3, whereas in other treatments only two isoforms PPO1 and PPO2 were observed with very low intensity and were absent in untreated plants (Figure 4). Further, the expression of isoform banding patterns in plants treated with OTPB3+OTPB1+ OPf1 was more intense compared to other treatments and untreated control.

DISCUSSION

The results from agar plate and seed assays indicated that the isolates of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) were compatible. Previous studies showed that biocontrol agents should be compatible when combined in order to obtain desired and consistent plant growth promotion and disease suppression (Janisiewicz and Bors 1995; Raaijmakers et al., 1995; Janisiewicz 1996; Li and Alexander, 1988). Many earlier reports also illustrated

Table 5. Effect of seed treatments with fresh suspensions of OTPB3+OTPB1, OPf1 and fungicides and foliar sprays of fungicides on late blight incidence of tomato under controlled conditions in growth chamber ^A.

Treatments ^B	Late blight incidence (%) ^C
Pathogen spray alone	76.6±2.3 ^e
OTPB3+OTPB1*	38.4±2.4 (49.9) ^c
Mancozeb*	51.4±3.2 (32.9) ^b
OPf1*	61.2±4.1(20.1) ^d
OTPB3+OTPB1+OPf1**	20.6±2.4(73.1) ^a
OTPB3+OTPB1+ Fenamidone + Mancozeb**	20.0±2.4(73.9) ^a
Mancozeb + Fenamidone + Mancozeb**	20.6±2.2(72.8) ^a
Mancozeb + OPf1**	25.4±2.1(66.8) ^a
OPf1+ Fenamidone + Mancozeb**	20.5±3.1(68.0) ^a
OPf1+OPf1**	48.2±3.5(37.1) ^c
Control CMC	71.4±3.4(6.8) ^e
CD 5%`	14.6

*Seed treatment; **Seed treatment with foliar spray. ^AValues are mean of 3 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 864 seedlings. Percentage of disease severity index was estimated after initiation of symptom, *i.e.*, 72 hrs of pathogen spray. 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 late blight incidence of control plant and T is the late blight incidence of treated. Percentage data were arcsin-transformed before analysis according to $y = \arcsin [\text{sqr. } (_/100)]$. For each row values followed by a different lower case letter are significantly different at $p < 0.05$, according to Fishers LSD test.

^BPot trays containing tomato seedlings of 30 days old treated with different seed treatments were placed in growth chambers were sprayed with different foliar treatments which includes *P. putida* OPf1, Mancozeb and Famaxodine + Mancozeb followed with spray of *P.infestans* PIT 30 spore suspension and plants were incubated in 100% relative humidity (RH) and maintained at 25 °C at day and 20°C at night, with a 12-h photoperiod (Yan et al., 2002). Six days after inoculation with the pathogen, disease was rated by estimating the affected percentage leaf area (James, 1971) of all leaves and percentage of disease severity incidence was calculated using the formula (Amin et al., 2013).

$$^{\circ}\text{Percentage Severity Index} = \frac{\text{Sum of Individual numerical rating}}{\text{Total Number of assessed} \times \text{Maximum score in scale}} \times 100$$

that disease suppression can be increased by utilizing combinations of biological control agents and plant growth promoting rhizobacteria (PGPR) and their combined effects are pronounced in improving crop yields and enhancing nutrient uptake by plants (Alagawadi and Gaur, 1988; Alagawadi and Gaur, 1992, Jisha and Alagawadi, 1996; Guetsky et al., 2002; (van Peer et al., 1991; Duffy et al., 1996; de Boer et al., 1999; Nandakumar et al., 2001; Domenech et al., 2006; Saravanakumar et al., 2007; Thilagavathi et al., 2007; Ganeshmoorthi et al., 2008;

Latha et al., 2009) over single organism inoculations. Meanwhile Yobo et al. (2009) demonstrated that *Trichoderma* and *Bacillus* combinations were better than the *Trichoderma* isolated and *Bacillus* isolates used alone. They reported that there was potential in using mixtures of *Trichoderma* and *Bacillus* for improving plant growth and disease control. Earlier studies also demonstrated that the mixtures of *T. harzianum* and *B. subtilis* may not affect each other *in vivo* due to spatial separation on the roots or production of antimicrobial

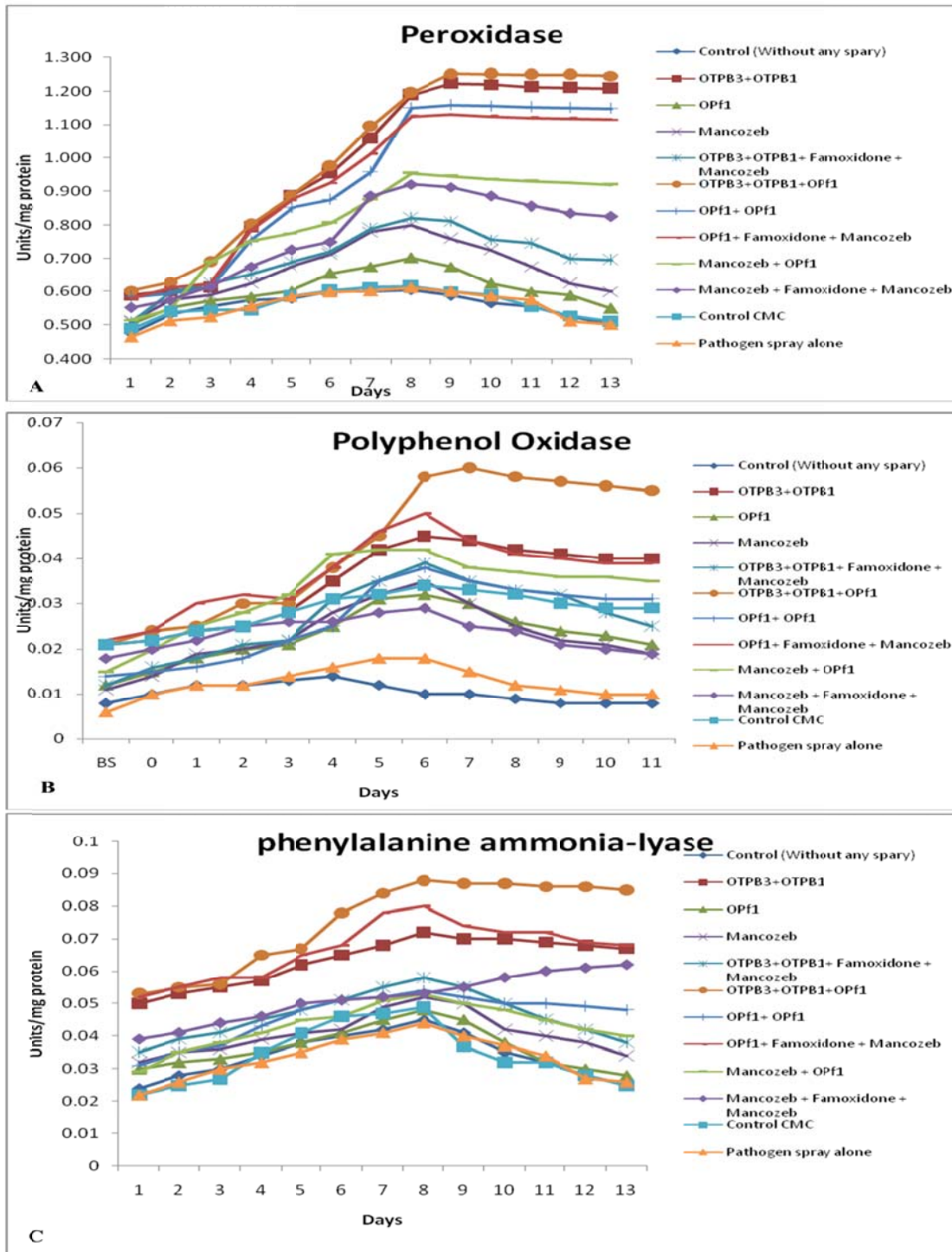


Figure 3. Induction of peroxidase (A), polyphenol oxidase (B), phenylalanine ammonia-lyase (C) and β -1,3-glucanase (D) activities in tomato plants treated with bio-control agents and fungicides extract against *P. infestans*. Thirty day old plants root and leaf tissues were collected at different day intervals viz., 0, 1, 3, 5, 7, 9 and 11 days after challenge inoculation (Latha, 2009). Four fresh seedlings were selected from each replication and they were washed in running water, blot dried and homogenized. One gram of sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used as a crude enzyme extract for assaying peroxidase (PO; EC 1.11.1) (Hammerschmidt et al., 1982), polyphenol oxidase (PPO; EC 1.12.18.1) (Mayer et al., 1965) and phenylalanine ammonia lyase (PAL; EC 4.3.1.5) (Dickerson et al., 1984). Enzyme extracted in 0.1 M sodium citrate buffer (pH 5.0) was used for the estimation of β -1,3-glucanase (Pan et al., 1991). Each of the enzyme assays were repeated three times. BS, before pathogen spray.

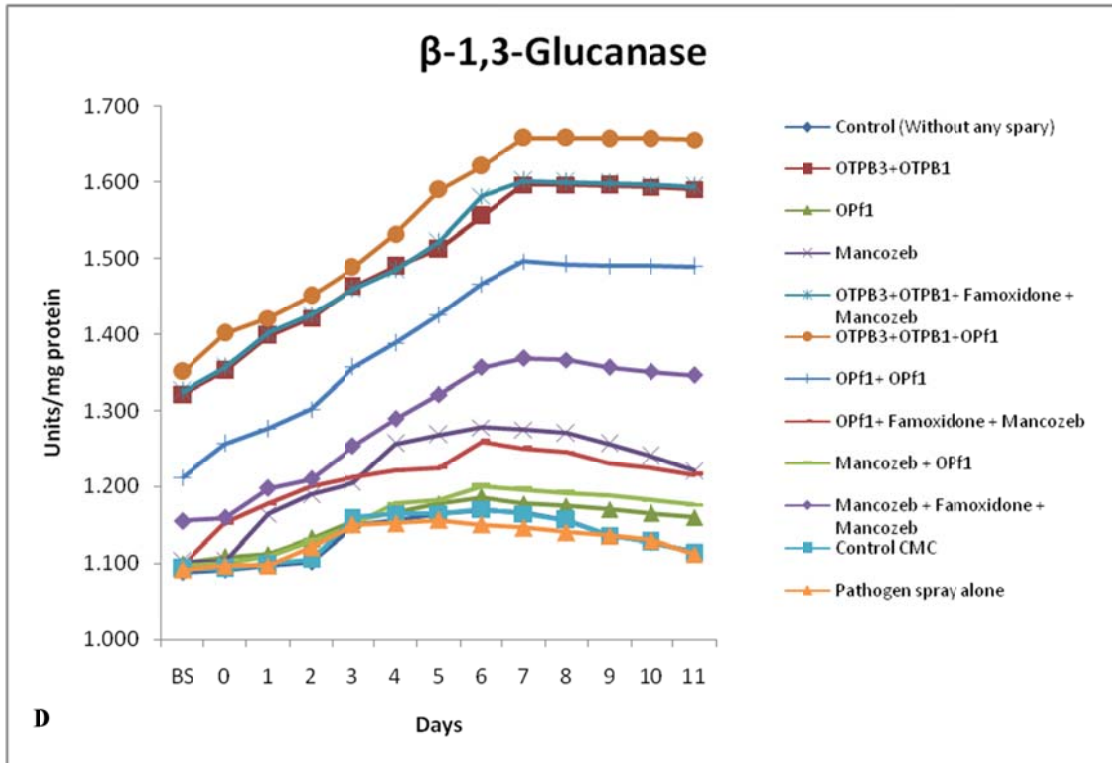


Figure 3. Cntd.

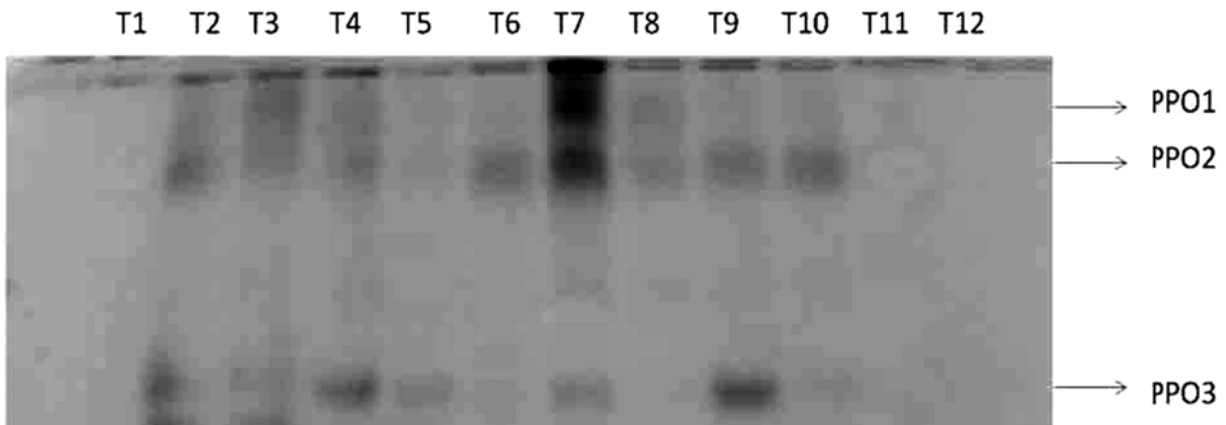


Figure 4. Native polyacrylamide gel electrophoresis analysis of polyphenol oxidase in tomato plants treated with biocontrol agents and fungicides against *P. infestans*. Lane, T1: Control (without any spray); T2, OTPB3+OTPB1; T3, Mancozeb; T4, OPf1; T5, Mancozeb + Fenamidone + Mancozeb; T6, Mancozeb + OPf1; T7, OTPB3+OTPB1+OPf1; T8, OTPB3+OTPB1+ Fenamidone + Mancozeb; T9, OPf1+ Fenamidone + Mancozeb; T10, OPf1+OPf1; T11, Control CMC; T12, Pathogen alone spray.

compounds *in vitro* in the stationary phase (Fukui et al., 1994; Duffy et al., 1996). Compatible combinations of biocontrol agents might be useful to deal with multiple diseases or multiple infection sites of a disease or wide range of environmental conditions (Fukui et al., 1994) as

single isolate may not work in different situations or against different pathogens. Most cases of naturally occurring biological control results from mixtures of antagonists rather than from high population of a single antagonist (Bin et al., 1991). Accordingly, application of a

mixture of pioneered biocontrol agents would further closely imitate the natural condition and might broaden the spectrum of biocontrol activity, improve the efficiency and consistency of biological control (Mishra et al., 2011). Direct interactions taking place among members of dissimilar microbial types often result in the promotion of key processes benefiting plant growth and health. Syntrophic relationships between different organisms have been demonstrated in several microbial ecosystems. Hence combinations of microorganisms that interact synergistically are currently being devised, which yield better and quick results (Bashan, 1998). Hence microbial consortium was suggested for plant growth promotion and disease suppression (Seneviratne, 2003). However, information pertaining to combined inoculations of *Trichoderma* and *Bacillus* species on plant growth and especially on disease control appears to be very sparse, even though both *Bacillus* and *Trichoderma* species are well known for their biological control and plant growth promoting properties (Yobo et al., 2009).

Tomato seeds coated with fresh suspensions of microbial mixture containing *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) resulted in significant increase in growth parameters in comparison with *P. putida* OPf1 and mancozeb treatments and untreated control. Many strains of *Trichoderma* spp., *Bacillus* spp. and *Pseudomonas* spp. were reported as potential plant growth promoters and disease resistance inducers in a range of crops (Schneider and Ullrich, 1994; Raupach and Kloepper, 1998; Nandakumar et al., 2001; Ramamoorthy et al., 2002; Harman et al., 2004; Kleifeld and Chet, 1992; MacKenzie et al., 1995; Windham et al., 1986; Yedia et al., 1999; Chithrashree et al., 2011; Chowdappa et al., 2013b). Choure et al. (2012) demonstrated that use of microbial consortia promoted early growth in *Cajanus cajan*, compared to individual strains of *S. fredii* KCC5, *P. fluorescens* LPK2 and *Azotobacter chroococcum* AZK2. Senthilraja et al. (2010) also reported that *B. bassiana* and *P. fluorescens* formulation has effectively decreased the collar rot and increased yield in groundnut production.

The significant increase in growth parameters of tomato was possible due to higher production of IAA and GA₃ in roots of tomato seedlings raised from seeds coated with *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) consortium. The enhancement of IAA and GA₃ levels is one of the mechanisms by which biocontrol organisms can enhance shoot and root growth and leaf area in tomato plants. IAA plays a vital role in initiation and elongation of lateral and adventitious roots and also influence shoot development (Hedden and Thomas, 2006). GA₃ in combination with auxins promotes axial part elongation (Srivastava, 2002). IAA stimulates cell elongation or cell division by reducing the effect of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and promotes root growth. ACC is a known inhibitor of root growth and several bacteria produce ACC-deaminase (Jacobson et al., 1994). Many studies demonstrated that certain bacteria and fungi promote

plant growth directly through increased nutrition uptake excited by growth regulators (Idris et al., 2007; Gravel et al., 2007; Harman, 2011; Shores et al., 2010; Kloepper et al., 2004; Chen et al., 2007; Chowdappa et al., 2013b). They also colonize plant roots, suppress many soil borne fungal pathogens and also stimulate growth and crop yield (Idris et al., 2007).

Accumulation of enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and β -1, 3-glucanase were significantly higher in tomato seedlings treated with OTPB3+OTPB1 consortium followed by *P. putida* OPf1 foliar spray after challenge inoculation with *P. infestans* as compared to other treatments including fungicidal check, mancozeb, fenamidone – mancozeb, and untreated control and is presumably responsible for the reduction of late blight disease index in plants challenged with *P. infestans*. Enhanced activities of the enzymes related to defense in the PGP microbes treated tomato plants may play a role in suppression of pathogen interference in the host eventually preventing development of disease. Several studies have demonstrated that enhancement of PO, PPO, PAL and β -1, 3-glucanase activities were responsible for fungal disease suppression in plants treated with *T. harzianum* (Jayalakshmi et al., 2009; Houssien et al., 2010) or *B. subtilis* (Nakkeeran et al., 2006; Thilagavathi et al., 2007; Latha et al., 2009; Chithrashree et al., 2011) or *Pseudomonas* spp (Latha et al., 2009, Sundaramoorthy et al., 2012).

In the present study, enzyme activities were initiated 72 h after pathogen inoculation and were maximum on 5th day in all treatments. Plants treated with OTPB3+OTPB1 consortia followed by OPf1 foliar spray exhibited maximum activities of the defense enzymes during the initial stage of pathogen attack and persisted up to 11th day after pathogen inoculation, which may be the cause of reduction in late blight disease incidence. Similar kind of responses were reported in many host-pathogen interactions (Dalisay and Kuc, 1995; Chen et al., 2007; Ramamoorthy et al., 2002; Rajendran and Samiyappan, 2008). Increased accumulation of both PO and PAL plays an important role in biosynthesis of secondary metabolites and phytoalexins and attributed their role in disease resistance (Daayf et al., 1997; Ryals et al., 1996; Kosuge, 1969). Increased activity of PO and PAL was reported in tomato treated with *P. fluorescens* infected by *Fusarium oxysporum* (Ramamoorthy et al., 2002), PO, PPO and PAL activity in rice, treated with *B. pumilus* SE34 and *B. subtilis* GBO3 after challenge inoculation with *Xanthomonas oryzae* pv. *oryzae* (Chithrashree et al., 2011). β -1-3-glucanase have the ability to hydrolyze β -1-3-glucan, a major component of cell wall of Stramenopile fungus like, *P. infestans* leading to direct the inhibition of growth of pathogen (Karthikeyan et al., 2005). Umamaheswari et al. (2009) reported that watermelon plants pre-treated with bio-agents showed enhanced PAL, PO, PPO, β -1-3-glucanase activities upon challenge inoculation with *Alternaria alternata*.

The present study is clearly demonstrated better ability of the 'synthetic microbial consortium' of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) to promote plant growth and induce systemic resistance against *P. infestans* in tomato than those of seed treatments with mancozeb and stand-alone treatments of OTPB3 and OTPB1. Thus, development of seed coating formulation with the microbial consortium of OTPB3 and OTPB1 is crucial to raise healthy tomato seedlings as *P. infestans* is a soil/seed borne pathogen (Wangsomboondee and Ristaino, 2002). In addition to seed and soil borne inoculums, airborne inoculum is also vital to late blight outbreaks under congenial tropical and subtropical conditions. In practice, protective foliar fungicidal applications at weekly intervals are used to effectively control the late blight disease. Thus, seeds treated with consortium of OTPB3 and OTPB1 followed by OPf1 foliar spray showed persistence of higher activities of the defense enzymes up to 11th day after pathogen inoculation leading to reduction in late blight disease incidence. This synthetic microbial consortium has the ability to protect plants from soil/seed/air borne inoculums. As most of the vegetable growers in India purchase tomato seedlings from commercial vegetable nurseries grown in pot trays using coco peat, movement of the *P. infestans* through seedlings is very high and this can be contained through seed treatments. Systemic resistance can be extended in field by foliar spray of *P. putida* OPf1 comparable with results of fungicide check fenamidone-mancozeb.

Therefore, in comparison with our previous work, where basal application of isolates of *T. harzianum* OTPB3 or *B. subtilis* OPTB1 individually promoted growth and induced systemic resistance against early and late blight of tomato, and in present paper, the effects of growth promotion and induction of systemic resistance are more in the tomato seedlings when seeds treated with consortium of OTPB3 and OTPB1 followed by OPf1 spray..

We, therefore, suggest that a combination of OTPB3 and OTPB1 can be effectively used for development of seed coating formulations to produce disease free and quality tomato seedlings and *P. putida* OPf1 as foliar spray for effective management of late blight disease.

However, this technology 'synthetic microbial consortia' needs to be validated further under field conditions at multi-locations before any recommendations are made.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENT

The financial support received from Indian Council of Agricultural Research, New Delhi in form of ALCOCERA, an outreach programme on *Alternaria*, *Colletotrichum* and

Cercospora diseases of field and horticultural crops is highly acknowledged.

REFERENCES

- Ahmed AS, Sanchez CP, Candela ME (2000). Evaluation of induction of systemic resistance in pepper plants (*Capsicum annuum*) to *Phytophthora capsici* using *Trichoderma harzianum* and its relation with capsidiol accumulation. Eur. J. Plant Pathol. 106:817-824.
- Amin M, Mulugeta N, Selvaraj T (2013). Field Evaluation of New Fungicide, Victory 72 WP for Management of Potato and Tomato Late Blight (*Phytophthora infestans* (Mont) de Bary) in West Shewa Highland, Oromia, Ethiopia. J. Plant Pathol. Microbiol. 4:192.
- Babitha MP, Bhat SG, Prakash HS, Shetty HS (2002). Differential induction of superoxide dismutase in downy mildew-resistant and susceptible genotypes of pearl millet. Plant Pathol. 51:480-486.
- Baki AAA, Anderson JD (1973). Vigour determination in soybean seed by multiple criteria. Crop Sci. 31:630-833.
- Bradford MM (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72:248-254.
- Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, Morgenstern B, Voss B, Hess WR, Reva O, Junge H, Voigt B, Jungblut PR, Vater J, Süßmuth, R, Liesegang H, Strittmatter A, Gottschalk G, Borriss R. (2007). Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat. Biotechnol. 25(9):1007-14.
- Chithrashree C, Udayashankar AC, Chandra Nayaka S, Reddy MS, Srinivas C (2011). Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. Biol. Control 59:114-122.
- Choure K, Dubey RC, Maheshwari DK (2012). Development of Plant Growth Promoting Microbial Consortium Based on Interaction Studies to Reduce Wilt Incidence in *Cajanus cajan* L var. Manak. Middle-East J. Sci. Res. 12:1459-1470.
- Chowdappa P, Kumar NBJ, Madhura S, Kumar MSP, Myers KL, Fry WE, Squires JN, Cooke DEL (2013a). Emergence of 13_A2 blue lineage of *Phytophthora infestans* was responsible for severe outbreaks of late blight on tomato in South-West India. J. Phytopathol. 161:49-58.
- Chowdappa P, Mohan Kumar SP, Jyothi Lakshmi M, Upreti KK (2013b). Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3. Biol. Control 65:109-117.
- Christ U, Mössinger E (1989). Pathogenesis-related proteins of tomato: I. Induction by *Phytophthora infestans* and other biotic and abiotic inducers and correlations with resistance. Physiol. Mol. Plant Pathol. 35:53-65.
- Cohen Y (1994). Local and systemic control of *Phytophthora infestans* in tomato plants by DL-3-amino-n-butanoic acids. Phytopathology 84: 55-59.
- Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005). Use of plant growth promoting bacteria for bio-control of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol. 71:4951-4959.
- Correa OS, Montecchia MS, Berti MF, Ferraria MCF, Pucheu NL, Kerber NL, Garcia AF (2009). *Bacillus amyloliquefaciens* BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities. Appl. Soil Ecol. 41:185-194.
- Daayf F, Bel-Rhild R, Belanger RR (1997). Methyl ester of p-coumaric acid: a phytoalexin-like compound from long English cucumber leaves. J. Chem. Ecol. 23:1517-1526.
- Dalisay R, Ku'c J (1995). Persistence of induced resistance and enhanced peroxidase and chitinase activities in cucumber plants. Physiol. Mol. Plant P 47:315-327.
- de Boer M, Van der Sluis I, Van Loon LC, Bakker PAHM (1999). Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of *Fusarium* wilt of radish. Eur. J. Plant Pathol. 105:201-210.

- Dickerson DP, Pascholati SF, Hagerman AE, Butler LG, Nicholson RL (1984). Phenylalanine ammonia-lyase and hydroxy cinnamate CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. *Physiol. Plant Pathol.* 25:111-123.
- Domenech J, Reddy MS, Kloepper JW, Ramos B, Gutierrez-Manero J (2006). Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. *Biocontrol* 51:245-258.
- Duffy BK, Simon A, Weller DM (1996). Combination of *Trichoderma koningii* with fluorescent pseudomonads for control of take-all on wheat. *Phytopathology* 86:188-194.
- Elad Y (2000). Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot.* 19:709-714.
- Elad Y, Chet I, Henis Y (1981) A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica* 9: 59-67.
- Enkerli J, Gist U, Mösinger E (1993). Systemic acquired resistance to *Phytophthora infestans* in tomato and the role of pathogenesis related proteins. *Physiol. Mol. Plant Pathol.* 43:161-171.
- Felsenstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Fridlender M, Inbar J, Chet I (1993). Biological control of soilborne plant pathogens by a β -1,3 glucanase-producing *Pseudomonas cepacia*. *Soil Biol. Biochem.* 25:1121-1221.
- Fry WE, Goodwin SB, Dyer AT, Matuszak JM, Drenth A, Tooley PW, Sujkowski LS, Koh YJ, Cohen BA, Spielman LJ, Deahl KL, Inglis DA, Sandlan KP (1993). Historical and recent migrations of *Phytophthora infestans*: Chronology, pathways, and implications. *Plant Dis.* 77:53-661.
- Fukui R, Poinar EI, Bauer PH, Schroth MN, Hendson M, Wang WL, Hancock JG (1994). Spatial colonization patterns and interaction of bacteria on inoculated sugar beet seed. *Phytopathology* 84:1338-1345.
- Ganeshmoorthi P, Anand T, Prakasan V, Bharani, M, Ragupathi N., Samiyappan R (2008). Plant growth promoting rhizobacterial (PGPR) bioconsortia mediates induction of defense-related proteins against infection of root rot pathogen in mulberry plants. *J. Plant Interact.* 3:233-244.
- Gravel V, Antoun H, Tweddell RJ (2007) Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). *Soil Biol. Biochem.* 39:1968-1977.
- Guetsky R, Shtienberg D, Elad Y, Fischer E, Dinoor A (2002). Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* 92: 976-985.
- Hammerschmidt R, Nuckels EM, Kuc J (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol. Plant Pathol.* 20:73-82.
- Harman GE (2011). Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. *New Phytol.* 189:647-649.
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. (2004). *Trichoderma* species- opportunistic, avirulent plant symbionts, A reviews. *Nat. Rev. Microbiol.* 2:43-56.
- Hedden P, Thomas SG. (eds) (2006). Plant hormone signaling. Annual plant reviews. Blackwell, Oxford, UK, p. 348.
- Heller WE, Gessler C (1986). Induced systemic resistance in tomato plants against *Phytophthora infestans*. *J. Phytopathol.* 116: 323-328.
- Houssien AA, Ahmed SM, Ismail AA (2010). Activation of tomato plant defense response against *Fusarium* wilt disease using *Trichoderma harzianum* and salicylic acid under greenhouse conditions. *Res. J. Agric. Biol. Sci.* 6:328-338.
- Idris HA, Labuschagne N, Korsten L. (2007). Screening rhizobacteria for biological control of *Fusarium* root and crown rot of sorghum in Ethiopia. *Biol. Control* 40:97-106.
- Jacobson CB, Pasternak JJ, Glick BR (1994). Partial purification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Can. J. Microbiol.* 40:1019-1025.
- James C (1971). A manual of assessment keys for plant diseases. Canada Department of Agriculture Publication No. 1458.
- Janisiewicz WJ (1988). Biocontrol of post-harvest disease of apple with antagonist mixtures. *Phytopathology* 78:194-198.
- Janisiewicz WJ (1996). Ecological diversity, niche overlap and coexistence of antagonists used in developing mixtures for biocontrol of postharvest diseases of apples. *Phytopathology* 86:473-479.
- Janisiewicz WJ, Bors B (1995). Development of a microbial community of bacterial and yeast antagonists to control wound-invading post-harvest pathogens of fruits. *Appl. Environ. Microbiol.* 61: 3261-3267.
- Jayalakshmi SK, Raju S, Usha-Rani S, Benagi VI, Sreeramulu K. (2009). *Trichoderma harzianum* L1 as a potential source for lytic enzymes and elicitor of defense responses in chickpea (*Cicer arietinum* L.) against wilt disease caused by *Fusarium oxysporum* f. sp. *ciceri*. *AJCS* 3:44-52.
- Jayaraman KS, Ramunja MN, Vijayaraghavan PK, Vaidhyanathan CS (1987). Studies on the purification of banana polyphenol oxidase. *Food Chem.* 24:203-217.
- Karthikeyan M, Jayakumar V, Radhika K, Bhaskaran R, Velazhahan R, Alice D (2005). Induction of resistance in host against the infection of leaf blight pathogen (*Alternaria palandui*) in onion (*Allium cepa* var *aggregatum*). *Indian J. Biochem. Biol.* 42: 371-377.
- Kelen M, Demiralay EC, Sen S, Ozkan G (2004). Separation of abscisic acid, indole acetic acid and gibberellic acid in 99R (*Vitis berlandieri* x *Vitis rupestris*) and rose oil (*Rosa damascene* Mill) by reverse phase liquid chromatography. *Turk. J. Chem.* 28:603-610.
- King EO, Ward MK, Raney DE (1954). Two simple media for the demonstration of pyocyanin and fluoresin. *J. Lab. Clin. Med.* 44:301-307.
- Kleefeld O, Chet I (1992). *Trichoderma harzianum*-interaction with Plants and Effects on Growth Response. *Plant Soil* 144:267-272.
- Kloepper JW, Ryu CM, Zhang S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* 94:1259-1266.
- Kosuge T (1969) The role of phenolics in host response to infection. *Annu. Rev. Phytopathol.* 7:195-222.
- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Latha P, Anand T, Ragupathi N, Prakasam V, Samiyappan R (2009). Antimicrobial activity of plant extracts and induction of systemic resistance in tomato plants by mixtures of PGPR strains and Zimmu leaf extract against *Alternaria solani*. *Biol. Control* 50:85-93.
- Li DM, Alexander M (1988). Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. *Plant Soil* 108: 211-219
- Lievens B, Hanssen IRM, Vanachter ACRC, Cammue BPA, Thomma BPHJ (2004). Root and foot rot on tomato caused by *Phytophthora infestans* detected in Belgium. *Plant Dis.* 88:86.
- MacKenzie AJ, Starman TW, Windham MT (1995). Enhanced Root and Shoot Growth of Chrysanthemum Cutting Propagated with the Fungus *Trichoderma harzianum*. *Hortic Sci.* 496-498.
- Mayer AM, Harel E, Shaul RB (1965). Assay of catechol oxidase, a critical comparison methods. *Phytochemistry* 5:783-789.
- Murphy JF, Reddy MS, Ryu CM, Kloepper JW, Li R (2003). Rhizobacteria-mediated growth promotion of tomato leads to protection against cucumber mosaic virus. *Phytopathology* 93:1301-1307.
- Nakkeeran S, Kavitha K, Chandrasekar G, Renukadevi P, Fernando WGD (2006). Induction of plant defense compounds by *Pseudomonas chlororaphis* PA23 and *Bacillus subtilis* BSCBE4 in controlling damping-off of hot pepper caused by *Pythium aphanidermatum*. *Biocontrol Sci. Technol.* 16: 403-416.
- Nandakumar R, Babu S, Viswanathan R, Sheela J, Raguchander T, Samiyappan R (2001). A new bio-formulation containing plant growth promoting rhizobacterial mixture for the management of sheath blight and enhanced grain yield in rice. *Biocontrol* 46 : 493-510.
- Nei M, Kumar S (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.
- Nowicki M, Foolad MR, Nowakowska M, Kozik EU (2012). Potato and tomato late blight caused by *Phytophthora infestans*: An overview of pathology and resistance breeding. *Plant Dis.* 96:4-17.
- Pan SQ, Ye XS, Kuc J (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.
- Parke JL, Rand R, Joy A, King EB (1991). Biological control of *Pythium*-damping off and *Aphanomyces* root rot of peas by application of *Pseudomonas cepacia* or *Pseudomonas fluorescens* to seed. *Plant Dis.* 75: 987-992.
- Raaijmakers JM, van der Sluis I, Koster M, Bakker, PAHM, Weisbeek PJ, Schippers B (1995). Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp. *Can. J. Microbiol.* 41:126-135.
- Rajendran L, Samiyappan R (2008) Endophytic *Bacillus* Species Confer Increased Resistance in Cotton Against Damping off Disease Caused by *Rhizoctonia solani*. *J. Plant Pathol.* 7:1-12.
- Ramamoorthy V, Raguchander T, Samiyappan R (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.
- Raupach GS, Kloepper JW (1998). Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 88:1158-1164.
- Reysenbach AL, Giver LJ, Wickham GS, Pace NR (1992). Differential amplification of rDNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58: 3417-3418.
- Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996). Systemic acquired resistance. *Plant Cell* 8:1809-1819.
- Saravanakumar D, Vijayakumar C, Kumar N, Samiyappan R (2007) PGPR induced defense responses in the tea plant against blister blight disease. *Crop Prot.* 26:556-565.
- Schneider S, Ullrich WR (1994). Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. *Physiol. Mol. Plant Pathol.* 45:291-304.
- Senthilraja G, Ananda T, Durairajb C, Kennedyb JS, Sureshb S, Raguchandera T, Samiyappana R (2010). A new microbial consortia containing entomopathogenic fungus, *Beauveria bassiana* and plant growth promoting rhizobacteria, *Pseudomonas fluorescens* for simultaneous management of leafminers and collar rot disease in groundnut. *Biocontrol Sci. Technol.* 20: 449-464.
- Sharifi-Tehrani A, Zala M, Natsch A, Moenne-Loccoz Y, Defago G (1998). Biocontrol of soil-borne fungal plant diseases by 2,4-diacetylphloroglucinol producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA. *Eur. J. Plant Pathol.* 104:631-643.
- Shoresh M, Mastouri F, Harman GE (2010). Induced systemic resistance and plant responses to fungal bio-control agents. *Annu. Rev. Phytopathol.* 48:21-43.
- Srivastava LM (2002) Plant growth and development-hormones and environment. Elsevier, San Diego, pp. 307-314.
- Sundaramoorthy S, Raguchander T, Ragupathi N, Samiyappan R (2012). Combinatorial effect of endophytic and plant growth promoting rhizobacteria against wilt disease of *Capsicum annum* L. caused by *Fusarium solani*. *Biol. Control* 60:59-67.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* 28:2731-2739.
- Thilagavathi R, Saravanakumar D, Ragupathi N, Samiyappan R (2007). A combination of bio-control agents improves the management of dry root rot (*Macrophomina phaseolina*) in green gram. *Phytopathol. Mediterr.* 46: 157-167.
- Thompson DC (1996) Evaluation of bacterial antagonist for reduction of summer patch symptoms in Kentucky blue grass. *Plant Dis.* 80: 856-862.
- Tumwine J, Frinking HD, Jeger MJ (2002). Integrating cultural control methods for tomato late blight (*Phytophthora infestans*) in Uganda. *Ann. Appl. Biol.* 141:225-236.
- Umamaheswari C, Sankaralingam A, Nallathambi P (2009). Induced systemic resistance in watermelon by biocontrol agents against *Alternaria alternata*. *Arch. Phytopathol. Plant Prot.* 42:1187-1195.
- Van Loon LC, Bakker PAHM, Pieterse CMJ (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36:453-483.
- Wangsomboondee T, Ristaino JB (2002). Optimization of sample size and DNA extraction methods to improve PCR detection of different propagules of *Phytophthora infestans*. *Plant Dis.* 86:247-253.
- Webber JF, Hedger JN (1986). Comparison of interactions between *Ceratocystis ulmi* and Elm bark saprobes *in vitro* and *in vivo*. *T Br. Mycol. Soc.* 86:93-101.
- Weisberg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173: 697-703.
- Windham MT, Elad Y, Baker R (1986). A Mechanism for Increased Plant Growth Induced by *Trichoderma* spp. *Phytopathology* 76: 18-521.
- Woo SL, Scala F, Ruocco M, Lorito M (2006). The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathology* 96:181-185.
- Yan Z, Reddy MS, Ryu CM, McInroy JA, Wilson M, Kloepper JW (2002). Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. *Phytopathology* 92:1329-1333.
- Yang HH, Yang SL, Peng KC, Lo CT, Liu SY (2009). Induced proteome of *Trichoderma harzianum* by *Botrytis cinerea*. *Mycol. Res.* 113: 924-932.
- Yedidia I, Benhamou N, Chet I (1999) Induction of defense in cucumber plants (*Cucumis sativus* L.) by the bio-control agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.* 65:1061-1070.
- Yobo KS, Laing MD, Hunter CH (2009). Effects of single and dual applications of selected *Trichoderma* and *Bacillus* isolates on performance of dry bean seedlings grown in composted pine bark growth medium under shade house conditions. *J. Plant Nutr.* 32: 1271-1289.