Research articles


**Book chapters**


**Technical bulletins**

PRODUCT

Seedpro has been evaluated in tomato and across the crop and commercialized to M/s Multiplex Biotech. Pvt. Ltd, Bangalore, M/s. Agri Life Pvt. Ltd., Hyderabad and M/s. Poabs Biotech, Kerala.

AWARDS

1. Sharada Lele award for best oral presentation on “Growth stimulation and induction of systemic resistance in tomato against early and late blight by Bacillus subtilis OTPB1 or Trichoderma harzianum OTPB3” by Indian Phytopathological Society, New Delhi during National symposium on “Blending Conventional and Modern Plant Pathology for Sustainable Agriculture”, 4-6, December, 2012, Bangalore.


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*

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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
Pseudomonas (Senthilraja et al., 2010). Disease in groundnut both under polyhouse and field synergism. Bio-consortium containing effective (Choure et al., 2012) and enhanced plant growth due to programs (Fry et al., 1993; Tumwine et al., 2002). Although interval, form the basis for late blight management do not have adequate tolerance to late blight, chemical tomato is grown year–round. Since commercial cultivars alone is highly challenging particularly in areas, where 2012). Management of late blight using cultural practices methods to manage late blight are cultural, fungicide sprays, and use of resistant cultivars (Nowicki et al., 2012). 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, antibiotic hypersensitivity, 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 Ulrich, 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 of strains which exhibited biocontrol of post-harvest diseases in apple. Combination of three strains viz. Pseudomonas fluorescens LPK2, Sinorhizobium fredii KC5 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 (Senthilraj 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 mancozeb + 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^-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'-AGAGTTTGTATCTGGCTCAG-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

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of 50 pM primers 27F and 1492R and 0.25 µl Taq polymerase (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 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 *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 ± 1°C for 5 days and then, 5 µl of 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 ± 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 10^8 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±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 10^8 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 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 3x10^5 zoospores/ml.

Test chemicals

Mancozeb was procured from Indoffil 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 ± 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 (10^6 cfu ml^-1) and conidial suspension of *T. harzianum* OTPB3 (10^6 spores ml^-1). The mixture was inoculated into potato dextrose broth and incubated at 25 ± 1°C for 7 days and one loop of culture broth was streaked on potato dextrose agar and incubated at 25 ± 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 (10^8 spores/ml) of *T. harzianum* OTPB3 or cell suspension (10^6 cfu 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 (10^6 cfu ml^-1) and conidial suspension of *T. harzianum* OTPB3 (10^6 spores ml^-1) in ratio of 1:1. Suspension of mancozeb (0.2%) was used. The seeds treated with sterile distilled
overnight at 4°C. Seedlings were macerated in 80% chilled methanol (50 ml) and control according to the method of Kelen et al. (2004) with a few modifications. 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 ethyl acetate and the ethyl ether portion, after drying over anhydrous Na₂SO₄, was filtered in vacuo through Whatman No. 1 filter paper, and the ether was removed.

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. Germination 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 acetoni trile (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 (10⁶ cfu ml⁻¹), mancozeb (2 g/l) and pre-packed mixture of fenamidone + mancozeb (3 /l) followed by zoospore suspension containing 3 x 10⁷ zoospores/ml of P. infestans PIT 30 (Chowdappa et al., 2013b). 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).

\[
\text{Percentage Severity Index} = \left(\frac{\text{Sum of Individual numerical rating}}{\text{Total Number of assessed}}\right) \times 100\%
\]

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, blotted 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 1.11.11) (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 1.11.1.1)

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⁻¹ g⁻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 µl 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 µl of 0.01 catechol. Changes in absorbance of the reaction mixture were expressed in min−1 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 µl of enzyme with 500 µl of 50 mM Tris HCl (pH 8.8) and 600 µl 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 µl of 4% laminarin and 62.5 µl of enzyme extract. The assay was carried out at 40°C for 10 min. The reaction was terminated by adding 375 µl of dinitrosalicylic acid and heating for 5 min in hot water bath, mixed well and measured absorbance at 500 nm. The activity was expressed as µg 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 isofrom 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 µg 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 [(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 OPf116S 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)*.  

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pathogen</th>
<th>Pathogen growth (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. putida</em> (OPf1)</td>
<td><em>P. infestans</em> (PIT30)</td>
<td>21.0 ± 1.0 (72.9)</td>
</tr>
<tr>
<td>Control</td>
<td><em>P. infestans</em> (PIT30)</td>
<td>77.7 ± 0.5</td>
</tr>
</tbody>
</table>

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 \(y = \text{arcsin} \left[ \text{sqr.} (x/100) \right] \). Data are the means and standard deviation of nine independent experiments. Each experiment contained three replicates. Each replicate contained six Petri plates. *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).
Late blight incidence under controlled conditions in growth chamber

Seed treatment with OTPB3 and OTPB1 combination, OP1 strain and mancozeb as chemical check coupled with foliar sprays of OP1 and pre-packed mixture of fenamidone - mancozeb were evaluated for their efficiency against *F. infestans* under pot culture conditions in growth chamber (Table 5). Seed treatment with OTPB3+OTP1 followed by foliar spray of OP1 significantly reduced late blight incidence by 73.1% compared to untreated controls. The results showed that disease reduction with OTPB3+OTP1+ OP1 mixture was on par with the fungicide check (mancozeb + fenamidone – mancozeb), which also recorded 72.8% reduction in late blight incidence. The combinations of OP1+ fenamidone – mancozeb, OTPB3+OTP1+ fenamidone – mancozeb, OTPB3+OTP1+ mancozeb also caused reduction in late blight incidence similar to OTPB3+OTP1+ OP1 combination. Seed treatment alone with OTPB3+OTP1 combination showed lower incidence of late blight (38.4%) compared to OP1 (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 + OTP1 + OP1, OTPB3 + OTP1 + mancozeb, OTPB3 + OTP1 + fenamidone – mancozeb, OP1+ fenamidone – mancozeb and OTPB3 + OTP1, mancozeb, and OP1 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*.

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* (OTP1) were significantly higher (*P < 0.01*) compared to treatment with *P. putida* OP1 and mancozeb and untreated control (Table 4). The IAA and GA3 levels were higher by 71.4 and 78.8%, respectively in seedlings treated with microbial consortium as compared to untreated control, while *P. putida* (OP1) treated seedlings showed an increase of IAA by 44.7% and GA3 by 60.7%.

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

<table>
<thead>
<tr>
<th>Treatments</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTPB3</td>
<td>5.6x10³a</td>
<td>5.6x10³b</td>
<td>5.8x10³c</td>
</tr>
<tr>
<td>OTPB1</td>
<td>5.3x10³a</td>
<td>5.3x10³b</td>
<td>5.6x10³c</td>
</tr>
<tr>
<td>OTPB3+OTP1 (OTP1)</td>
<td>5.6x10³a</td>
<td>5.3x10³b</td>
<td>5.3x10³c</td>
</tr>
<tr>
<td>OTPB3+OTP1 (OTP3)</td>
<td>5.6x10³a</td>
<td>5.7x10³b</td>
<td>5.2x10³c</td>
</tr>
<tr>
<td>CD1%</td>
<td>0.9</td>
<td>1.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Values 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 pair of treatment according to Fishers LSD test.

Five tomato seeds var. Arkha vikas, treated with OTPB3, OTP1 and Consortia with OTPB3 and OTP1 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).

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.
defence enzyme activities against *P. infestans* compared to other treatments (Figure 3). The enzyme activities were increased after 3 days and reached 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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root Length (cm)</th>
<th>Shoot length (cm)</th>
<th>Seedling vigour index</th>
<th>Root weight (g)</th>
<th>Shoot weight (g)</th>
<th>Leaf area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTPB3</td>
<td>14.4±2.4 (41.1)</td>
<td>20.5±2.4 (42.0)</td>
<td>3434.0±348.1 (45.7)</td>
<td>1.3±0.3 (52.9)</td>
<td>2.0±0.4 (63.2)</td>
<td>7.1±1.3 (46.6)</td>
</tr>
<tr>
<td>OTPB1</td>
<td>11.7±2.7 (27.8)</td>
<td>22.3±3.1 (35.9)</td>
<td>2935.9±271.3 (36.4)</td>
<td>1.0±0.1 (26.9)</td>
<td>1.8±0.3 (60.4)</td>
<td>6.3±1.8 (40.3)</td>
</tr>
<tr>
<td>OTPB3+OTPB1</td>
<td>16.5±2.3 (56.3)</td>
<td>23.5±2.2 (40.9)</td>
<td>3708.9±178.2 (53.5)</td>
<td>0.3±0.05 (56.9)</td>
<td>2.4±0.2 (50.2)</td>
<td>8.8±1.1 (34.0)</td>
</tr>
<tr>
<td>OPf1</td>
<td>11.0±1.4 (34.5)</td>
<td>18.4±1.8 (37.9)</td>
<td>2877.7±118.5 (40.1)</td>
<td>0.2±0.03 (38.2)</td>
<td>1.8±0.3 (46.2)</td>
<td>6.2±1.2 (19.2)</td>
</tr>
<tr>
<td>Mancozeb (0.2%)</td>
<td>7.3±1.1 (15.1)</td>
<td>16.6±1.4 (16.4)</td>
<td>1952.7±114.3 (11.7)</td>
<td>0.1±0.05 (8.5)</td>
<td>1.6±0.2 (23.2)</td>
<td>6.2±1.4 (5.4)</td>
</tr>
<tr>
<td>Control CMC</td>
<td>10.34±0.2c</td>
<td>2.2±0.3c</td>
<td>2877.7±118.5 (40.1)</td>
<td>0.2±0.03 (38.2)</td>
<td>1.8±0.3 (46.2)</td>
<td>6.2±1.2 (19.2)</td>
</tr>
<tr>
<td>Control</td>
<td>7.2±1.2c</td>
<td>13.8±1.8d</td>
<td>1723.3±138.6 (60.1)</td>
<td>0.1±0.025d</td>
<td>1.2±0.2d</td>
<td>5.8±1.4d</td>
</tr>
<tr>
<td>CD 1%</td>
<td>1.3±0.9</td>
<td>127.1</td>
<td>2877.7±118.5 (40.1)</td>
<td>0.2±0.03 (38.2)</td>
<td>1.8±0.3 (46.2)</td>
<td>6.2±1.2 (19.2)</td>
</tr>
</tbody>
</table>

*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 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. Bacterial isolate B. subtilis OTPB1 (10⁸) and one isolate *Trichoderma harzianum* OTPB3 suspension (10⁷), *P. putida* OPf1 suspension (10⁸) and consortium of OTPB1 (10⁸) and OTPB3 (10⁷) 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 cocopeat. 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. Seedling 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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>IAA (nmol/g)</th>
<th>GA3 (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTPB3+OPf1</td>
<td>35.8±0.8 (71.1)a</td>
<td>10.4±0.4 (78.8)ja</td>
</tr>
<tr>
<td>OPf1</td>
<td>18.7±0.9 (44.7)b</td>
<td>5.6±0.2 (60.7)jb</td>
</tr>
<tr>
<td>Mancozeb</td>
<td>11.0±0.8 (60.8)jc</td>
<td>2.2±0.2c</td>
</tr>
<tr>
<td>Control</td>
<td>10.3±0.2ce</td>
<td>2.2±0.3c</td>
</tr>
<tr>
<td>CD 1%</td>
<td>5.7</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*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. 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.

**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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Late blight incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogen spray alone</td>
<td>76.6±2.3°</td>
</tr>
<tr>
<td>OTPB3+OTPB1*</td>
<td>38.4±2.4 (49.9)‡</td>
</tr>
<tr>
<td>Mancozeb*</td>
<td>51.4±3.2 (32.9)§</td>
</tr>
<tr>
<td>OPf1*</td>
<td>61.2±4.1(20.1)²</td>
</tr>
<tr>
<td>OTPB3+OTPB1+OPf1**</td>
<td>20.6±2.4(73.1)³</td>
</tr>
<tr>
<td>OTPB3+OTPB1+ Fenamidone + Mancozeb**</td>
<td>20.0±2.4(73.9)⁴</td>
</tr>
<tr>
<td>Mancozeb + Fenamidone + Mancozeb**</td>
<td>20.6±2.2(72.8)⁵</td>
</tr>
<tr>
<td>Mancozeb + OPf1**</td>
<td>25.4±2.1(66.8)⁶</td>
</tr>
<tr>
<td>OPf1+ Fenamidone + Mancozeb**</td>
<td>20.5±3.1(68.0)⁷</td>
</tr>
<tr>
<td>OPf1+OPf1**</td>
<td>48.2±3.5(37.1)⁸</td>
</tr>
<tr>
<td>Control CMC</td>
<td>71.4±3.4(6.8)⁹</td>
</tr>
<tr>
<td>CD 5%</td>
<td>14.6</td>
</tr>
</tbody>
</table>

*Seed treatment; **Seed treatment with foliar spray. Values 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 [sqr. (/100)]. For each row values followed by a different lower case letter are significantly different at p < 0.05, according to Fisher's LSD test.

Pot 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).

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

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 1.11.1.1) (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 benefitting plant growth and health. Symbiotic 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 OPfi 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 Kloeper, 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; Yedidia 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 KCCS, P. fluorescens LPK2 and Azotobacter chroococcum AZK2. Senthiraja 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; Kloeppe, 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; Chitrashree 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 (Karthekeyan 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 congenital 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**

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Development of seed coating formulation using consortium of *Bacillus subtilis* OTPB1 and *Trichoderma harzianum* OTPB3 for plant growth promotion and induction of systemic resistance in field and horticultural crops

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**ABSTRACT:** An experiment was conducted to develop seed-coating formulation using microbial consortium of *Bacillus subtilis* OTPB1 and *Trichoderma harzianum* OTPB3. Tomato (*Lycopersicon esculentum* Mill.) cv. Arka vikas was used as the model crop to study the efficacy of formulation on growth promotion and induction of systemic resistance. The microbial consortium led to a significant increase (p < 0.01) in all growth parameters of tomato seedlings compared to stand alone treatments of *B. subtilis* OTPB1 and *T. harzianum* (OTPB3) and the control. Defence related enzymes such as polyphenol oxidase, peroxidase, and superoxide dismutase activities were significantly (P<0.01) higher by 56.7, 69.7 and 55.5%, respectively in tomato seedlings treated with formulation compared to other treatments and the control. The lesion size caused by *Alternaria solani* and *Phytophthora infestans* on intact tomato leaves were significantly reduced by 78.7 and 82.1%, respectively in microbial consortium formulation treated seedlings. When microbial consortium formulation evaluated against vegetable crops (brinjal, beans, bitter gourd, bottle gourd, cabbage, chili, carrot, cauliflower, pumpkin, ridged gourd), fruit crop (papaya in plastic trays in glasshouse), tuber crops (potato), ginger and turmeric, significant increase in growth parameters under greenhouse conditions and growth and yield parameters under field conditions were recorded. This study, therefore, assumes significance in production of disease-free quality vegetable transplants and enhancement of yield in potato, ginger and turmeric crops.

**Key words:** *Trichoderma harzianum, Bacillus subtilis, growth promotion, induced systemic resistance, microbial consortium*

The extensive usage of chemical fertilizers and pesticides for increasing productivity and production led to health hazards and environmental pollution. The use of biofertilizers and biopesticides has been advocated as a safe substitute for sustaining high production with low environmental impact (Hermosa et al., 2012). Apart from mycorrhizal fungi and rhizobium bacteria, plant-growth-promoting fungi such as *Trichoderma* spp. and *Piriformospora indica* have the ability to colonize roots and stimulate plant growth and suppress plant diseases (Van Wees et al., 2008; Mastouri et al., 2010). These microorganisms also have the capacity to form endophytic associations and interrelate with other microbes in rhizosphere and influence disease protection, plant growth and yield. *Trichoderma* plays a vital and unique role as plant growth promoter compared with other plant growth-promoting microbes, where their effects are greater when plants are under biotic, abiotic, or physiological stresses (Bae et al., 2009; Harman 2000; Harman et al., 2004). *Trichoderma harzianum* Rifai is widely used in seed treatment, where they may improve plant stands and induce long-term improvements in plant quality and suppress the diseases (Harman 2000; 2011; Harman et al., 2004; Murphy et al., 2003; Woo et al., 2006).

Apart from *Trichoderma*, many species of bacteria associated with plants, known to stimulate plant growth and also manage soil and plant health (Glick 1995; Hallman, 1997; Welbaum, 2004 and Compant et al., 2005). Among plant growth-promoting bacteria, *Bacillus subtilis* plays a vital role. It produces 60 different types of secondary metabolites and most of them have antifungal effects and some compounds may also help in plant growth promotion (Compant et al., 2005). These biocontrol organisms also promote plant growth by production of plant growth hormones like IAA and GA3 coupled with increased availability of nutrients (Cattelan et al., 1999; Chen et al., 2007; Harman, 2011; Chowdappa et al., 2013). Therefore, an experiment was conducted to develop simple and economically-viable seed coating formulation using *Bacillus subtilis* OTPB1 and *Trichoderma harzianum* OTPB3 and to evaluate its efficacy for stimulation of growth and yield, and to induce systemic resistance for disease protection in different vegetables, cereals, oilseeds, pulses and tuber crops.

**MATERIALS AND METHODS**

Chemicals, microbial growth media and ingredients used for the preparation of various formulations were of laboratory grade and were purchased from Himedia Pvt. Ltd, Mumbai. The media included nutrient broth (NB), potato dextrose agar (PDA), *Trichoderma* Selective Media and King's B. The detailed methods of isolation
and identification of *B. subtilis* OTPB1 and *T. harzianum* OTPB3 were described earlier (Chowdappa et al., 2013). Based on basic studies (Chowdappa et al., 2013), a seed coating formulation was developed using vermicompost (VC) as inert carrier material. Pure VC was ground into fine powder and steam sterilized at 121°C for 20 min, and dried aseptically in glass trays for 12 h at 50°C before use.

**Preparation of inoculum of biocontrol strains**

Inoculum of *B. subtilis* OTPB1 was prepared 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 concentration was adjusted using spectrophotometer to 10^6 CFU/ml (Thompson, 1996; Yan et al., 2002). Spore suspensions of *T. harzianum* OTPB3 were prepared by scraping the spores from cultures grown on PDA 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 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.

**Development of seed coating formulation**

Seed coating formulations were prepared by adding 500ml of OTPB1 suspension or 500ml of OTPB3 suspension or microbial consortium of 250ml of OTPB1 suspension and 250ml of OTPB3 spores to 1000 g of VC with 25% of moisture, and mixed separately as three independent formulations. The products were shade-dried to reduce the moisture content (less than 20%), then packed in polypropylene bags and sealed for further use.

**Effect of formulation on tomato cv. Arka Vikas**

Tomato cv. Arka Vikas was used as the model crop to study the efficacy of formulation on growth promotion, induction of defense related proteins and disease suppression. Tomato seeds were moistened with minimal amount of water and coated uniformly with individual formulations @ 20g/kg of seed and seeds were shade-dried for 30 min. and sown in plastic pot trays containing 96 cavities with 10 cm diameter, filled with well-composted and sterilized coco peat (soiless growth media). Seeds treated with only VC served as the control. Seedlings were allowed to grow for 30 days at 25 ± 2°C under natural light.

**Assay of proteins induced by formulations**

For extraction of anti-oxidative enzymes, modified method (Argandona et al., 2001) was adopted. Freshly collected leaf samples (1g) were ground in 10 ml of 0.1 M phosphate buffer pH 7.0 in pre-chilled pestle and mortar. The homogenate was filtered and centrifuged at 10,000 rpm for 15 min. The supernatant was used as enzyme source for assay of peroxidase, polyphenol oxidase and superoxide dismutase. All the steps, in preparation of extract, were carried out at 0-4°C. Enzyme activities were expressed as unit/g fresh weight (fw).

**Peroxidase**

Peroxidase activity was done by pipetting out 2.9 ml of 0.05M phosphate buffer, pH 6.8, 0.05 ml of 0.05M freshly prepared enzyme substrate pyrogallol, 0.05ml 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 sec interval for 3 minute (Manoranjan and Dinabandhu, 1976). The enzyme activity was expressed as changes in the absorbance of reaction mixture for min^-1 g^-1.

**Polyphenol Oxidase**

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

**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 William, 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.

**Isolation, identity and inoculum preparation of Alternaria solani and Phytophthora infestans**

The early blight pathogen, *A. solani* OTA 22 and late blight agent *P. infestans* PIT 30 were used in this study. The detailed protocols for isolation, identification and inoculum preparation of *A. solani* OTA 22 and *P. infestans* PIT 30, were described earlier (Chowdappa et al., 2013).

**Inoculation of tomato plants with Alternaria solani and Phytophthora infestans**

The tomato seedlings treated with biocontrol formulations were placed in plastic chambers (45cm height x 40cm length x 15 cm width) containing water to maintain humidity. Then, intact third leaf from bottom of seedlings were kept on wire mesh in horizontal position and inoculated with 10 μl droplets of a zoosporangium
Effect of microbial consortium formulation on different crops

The formulation with microbial consortium was used to test efficacy on wide range of vegetable (brinjal, beans, bitter gourd, bottle gourd, cabbage, chili, carrot, cauliflower, pumpkin and ridged gourd), fruit (papaya in plastic trays in glasshouse), tuber crops (potato), ginger and turmeric crops under field conditions at different locations. The field trials on potato were conducted at Kasaragod, Kerala. Sprouted rhizomes were broken into pieces keeping 2-3 sprouted eye buds on each rhizome of ginger and turmeric and were treated with vermicompost as a carrier material with B. subtilis OTPB1 or T. harzianum (OTPB3) and the control rhizome of ginger and turmeric and were treated with only VC served as the control. For sowing rhizomes, land was prepared by ploughing and digging. Beds were prepared with 1 m width, 25 cm height with 40 cm spacing between the beds. Then rhizomes were planted in small pits at a spacing of 20 cm x 20 cm to 25 cm x 25 cm and at a depth of 4-5 cm with at least one viable healthy bud facing upwards @ 1500 kg rhizomes/ha. At regular intervals, normal cultural practices were carried out.

Statistical analysis

The 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 LSD (least significant difference) at 1% level (p<0.01). Percentages data were arcsin-transformed before analysis according to y = arcsin [sqrt (x/100)].

RESULTS AND DISCUSSION

A seed coating formulation was developed using vermicompost as a carrier material with B. subtilis OTPB1 and T. harzianum (OTPB3) as a stand alone formulations and microbial consortium. The methods employed to produce, formulate and deliver biocontrol agents could influence their efficacy despite they have inherent qualities of growth promotion and induction of systemic resistance (Lo et al., 1997). Delivery systems employing biocontrol agent include dust or powder, alginate pellet, and starch or extruded granule, diatomaceous earth, manure or animal dung (Raj et al., 2003; Schisler et al., 2004; Omer, 2010; Senthilraja et al., 2010; Siripornvisal and Trilux, 2011). There are several formulated products of these microbial agents are already available in the market (Leggett et al., 2011). The results showed that microbial consortium with VC as carrier led to a significant increase (p <0.01) in all growth parameters of tomato seedlings compared to stand alone treatments of B. subtilis OTPB1 or T. harzianum (OTPB3) and the control (Table 1). The consortium increased root length, shoot length, leaf area, fresh weight of shoots and roots by 35.5, 40.8, 59.3, 74.2 and 54.5%, respectively as compared to the control seedlings (Table 1).

Table 1. Effect of seed coating formulation on growth of tomato seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root length (cm)</th>
<th>Shoot length (cm)</th>
<th>Seedling vigor index</th>
<th>Root weight (g)</th>
<th>Shoot weight (g)</th>
<th>Leaf area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC with OTPB3</td>
<td>14.1±2.1</td>
<td>18.4±1.4</td>
<td>3185.0±328</td>
<td>1.4±0.4</td>
<td>2.1±0.2</td>
<td>7.9±1.3</td>
</tr>
<tr>
<td></td>
<td>(35.5)b</td>
<td>(40.8)b</td>
<td>(38.5)b</td>
<td>(59.3)b</td>
<td>(61.9)b</td>
<td>(48.1)b</td>
</tr>
<tr>
<td>VC with OTPB1</td>
<td>12.7±1.7</td>
<td>15.3±3.1</td>
<td>2744.0±251</td>
<td>0.9±0.1</td>
<td>1.7±0.2</td>
<td>7.1±1.6</td>
</tr>
<tr>
<td></td>
<td>(28.3)c</td>
<td>(28.8)c</td>
<td>(28.6)c</td>
<td>(36.7)c</td>
<td>(52.9)b</td>
<td>(42.3)c</td>
</tr>
<tr>
<td>VC with OTPB3+OTPB1</td>
<td>19.1±2.3</td>
<td>21.4±2.4</td>
<td>3969.0±309.1</td>
<td>2.0±0.3</td>
<td>3.1±0.4</td>
<td>9.0±2.1</td>
</tr>
<tr>
<td></td>
<td>(52.4)a</td>
<td>(46.1)a</td>
<td>(50.6)a</td>
<td>(70.8)a</td>
<td>(74.2)a</td>
<td>(54.5)a</td>
</tr>
<tr>
<td>VC control</td>
<td>9.1±3.7d</td>
<td>10.9±3.1d</td>
<td>1960.0±312.1d</td>
<td>0.6±0.1d</td>
<td>0.8±0.5c</td>
<td>4.1±0.5</td>
</tr>
<tr>
<td>CD1%</td>
<td>2.1</td>
<td>1.7</td>
<td>354.7</td>
<td>0.3</td>
<td>0.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Values are mean of 12 independent experiments. Each experiment consists of 3 pot trays with 96 plants/tray, totaling 294 plants. Data were analyzed using ANOVA in completely randomized design (CRD). Significance of treatments were tested using LSD. Mean values are significant at 1% level (p<0.01). Values in parentheses indicates percentage increase over control.
Many strains of *Trichoderma* spp., *Bacillus* spp. and *Pseudomonas* spp. reported as potential plant growth promoters and disease resistance inducers in a wide range of crops (Schneider and Ullrich, 1994; Raupach and Kloepper, 1998; Nandakumar et al., 2001; Ramamoorthy et al., 2002; Harman et al., 2004; Klefield and Chet, 1992; MacKenzie et al., 1995; Windham et al., 1986; Yedidia et al., 2000; Chithrashree et al., 2011; Chowdappa et al., 2013). The biocontrol agents should be compatible when combined in order to obtain desired and consistent plant growth promotion and disease suppression (Janisiewicz, 1996; Li and Alexander, 1998). Compatible combination of biocontrol agents might be useful to deal with multiple diseases or multiple infection sites of a disease or a wide range of environmental conditions (Fukui et al., 1994) as single isolate may not work in different situations. The lesion size caused by *A. solani* and *P. infestans* were significantly reduced (P<0.01) by 78.7 and 82.1% respectively in microbial consortium formulation treated seedlings compared to other treatments and the control (Table 3).

There were significant increase in growth parameters under greenhouse conditions (Table 4) and growth parameters and yield under field (Tables 5-7). The *T. harzianum*, *B. subtilis* and *P. fluorescens* are being marketed as dry formulations with talc or peat as carriers, and are also used for mixing with potting soil or with compost for amalgamation into nursery beds or field soil (Sridhar et al., 1993; Kannan and Jayaraj, 1998). Ardakani et al. (2010) developed new formulation based on *P. fluorescens* using carrier materials powdered talc, bentonite, and powdered organic compounds of peat and rice bran and successfully evaluated these formulations against damping-off of cotton seedlings.

This study, therefore, assumes significance in production of disease-free quality vegetable seedlings. In India, farmers procure vegetable 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 seed-borne pathogens through transplants. For example, certain *Alternaria* spp, *Colletotrichum* spp. and *Phytophthora* spp. are seed- and/or soil-borne (Khulbe and Sati, 1987; Wangsomboondee and Ristaino, 2002) and use of pathogen free vegetable seedlings will help vegetable growers to reduce crop losses caused by soil-borne diseases as well as foliar blights. Thus, seed coating formulation was christened as Seedpro. Commercialization of Seedpro, having capacity in enhancing seed germination, seedling growth and vigour and resistance to seed-borne fungal pathogens in vegetable crops is under progress.

### Table 2. Induction of defense enzyme activities in tomato seedlings treated biocontrol formulations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Polyphenoloxidase (U/g/min)</th>
<th>Peroxidase (U/g/min)</th>
<th>Superoxide Dismutase (U/g/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC with OTPB3+OTPB1</td>
<td>2.5±0.2 (56.7)a</td>
<td>10.9±0.3 (69.7)a</td>
<td>1.8±0.3 (55.5)a</td>
</tr>
<tr>
<td>VC with OTPB3</td>
<td>1.9±0.1 (42.9)b</td>
<td>7.3±0.1 (54.7)b</td>
<td>1.4±0.2 (42.8)b</td>
</tr>
<tr>
<td>VC with OTPB1</td>
<td>1.2±0.4 (11.3)c</td>
<td>5.6±0.3 (41.0)c</td>
<td>1.2±0.5 (33.3)b</td>
</tr>
<tr>
<td>VC control</td>
<td>1d</td>
<td>3.3d</td>
<td>0.8c</td>
</tr>
<tr>
<td>CD1%</td>
<td>0.3</td>
<td>2.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Mean of three replications ± standard deviation. Each replication consists of five plants. Values in parentheses indicate percentage increase over the control. For each row values followed by a different lower case letter are significantly different at p < 0.01, according to Fishers LSD test.

### Table 3. Effect of biocontrol formulations on infection levels of *A. solani* and *P. infestans* on leaves of tomato seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>A. solani</em> (cm²)</th>
<th><em>P. infestans</em> (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTPB3+OTPB1</td>
<td>0.6±0.2 (78.7)a</td>
<td>0.5±0.1 (82.1)a</td>
</tr>
<tr>
<td><em>T. harzianum</em> OTPB3</td>
<td>0.8±0.1 (73.5)a</td>
<td>0.6±0.2 (78.5)b</td>
</tr>
<tr>
<td><em>B. subtilis</em> OTPB1</td>
<td>1.3±0.4 (58.0)b</td>
<td>0.9±0.1 (67.8)c</td>
</tr>
<tr>
<td>Control</td>
<td>3.1c</td>
<td>2.8d</td>
</tr>
<tr>
<td>CD 1%</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Mean of three replications ± standard deviation. Each replication consists of 25 plants. Values in parentheses indicate percentage inhibition of pathogen over the control. For each row values followed by a different lower case letter are significantly different at p < 0.01, according to Fishers LSD test.
### Table 4. Effect of seed coating formulation on growth of horticultural crops under greenhouse

<table>
<thead>
<tr>
<th>Crop</th>
<th>Treatment</th>
<th>Root length (cm)</th>
<th>Shoot length (cm)</th>
<th>Leaf area (cm²)</th>
<th>Root weight (g)</th>
<th>Shoot weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brinjal</td>
<td>VC with OTPB3+OTPB1</td>
<td>15.4</td>
<td>11.1</td>
<td>21.7</td>
<td>0.37</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>8.8</td>
<td>6.3</td>
<td>8.9</td>
<td>0.14</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>42.86</td>
<td>43.24</td>
<td>58.99</td>
<td>62.16</td>
<td>64.20</td>
</tr>
<tr>
<td>Cabbage</td>
<td>VC with OTPB3+OTPB1</td>
<td>19.99</td>
<td>9.26</td>
<td>7.55</td>
<td>0.49</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>6.01</td>
<td>4.29</td>
<td>0.54</td>
<td>0.05</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>69.93</td>
<td>53.67</td>
<td>92.85</td>
<td>89.80</td>
<td>78.20</td>
</tr>
<tr>
<td>Chilli</td>
<td>VC with OTPB3+OTPB1</td>
<td>20.99</td>
<td>16.5</td>
<td>4.3</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12.42</td>
<td>11.9</td>
<td>3.0</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>40.83</td>
<td>28.16</td>
<td>31.72</td>
<td>55.56</td>
<td>32.41</td>
</tr>
<tr>
<td>Soybean</td>
<td>VC with OTPB3+OTPB1</td>
<td>14.44</td>
<td>20.51</td>
<td>16.82</td>
<td>0.67</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12.68</td>
<td>9.28</td>
<td>5.48</td>
<td>0.30</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>12.19</td>
<td>54.75</td>
<td>67.39</td>
<td>55.18</td>
<td>37.45</td>
</tr>
<tr>
<td>Beans</td>
<td>VC with OTPB3+OTPB1</td>
<td>15.6</td>
<td>19.4</td>
<td>23.2</td>
<td>1.5</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9.65</td>
<td>15.67</td>
<td>15.00</td>
<td>1.23</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>38.26</td>
<td>19.27</td>
<td>35.32</td>
<td>20.23</td>
<td>19.10</td>
</tr>
<tr>
<td>Cauliflower</td>
<td>VC with OTPB3+OTPB1</td>
<td>12.98</td>
<td>10.74</td>
<td>9.80</td>
<td>0.16</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7.82</td>
<td>8.18</td>
<td>4.43</td>
<td>0.06</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>39.77</td>
<td>23.85</td>
<td>54.79</td>
<td>59.24</td>
<td>45.86</td>
</tr>
<tr>
<td>Bitter gourd</td>
<td>VC with OTPB3+OTPB1</td>
<td>13.0</td>
<td>11.3</td>
<td>32.3</td>
<td>0.7</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>8.97</td>
<td>9.25</td>
<td>20.40</td>
<td>0.56</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>31.05</td>
<td>18.43</td>
<td>36.75</td>
<td>24.18</td>
<td>17.69</td>
</tr>
<tr>
<td>Ridge gourd</td>
<td>VC with OTPB3+OTPB1</td>
<td>15.2</td>
<td>12.5</td>
<td>19.9</td>
<td>0.8</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9.68</td>
<td>9.54</td>
<td>0.51</td>
<td>2.68</td>
<td>11.51</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>36.19</td>
<td>23.50</td>
<td>42.06</td>
<td>36.62</td>
<td>15.49</td>
</tr>
<tr>
<td>Bottle gourd</td>
<td>VC with OTPB3+OTPB1</td>
<td>17.0</td>
<td>16.2</td>
<td>16.7</td>
<td>0.8</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9.57</td>
<td>11.36</td>
<td>9.82</td>
<td>0.60</td>
<td>2.38</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>43.67</td>
<td>29.70</td>
<td>41.21</td>
<td>26.17</td>
<td>19.60</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>VC with OTPB3+OTPB1</td>
<td>16.0</td>
<td>13.3</td>
<td>12.4</td>
<td>0.7</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>11.89</td>
<td>11.19</td>
<td>9.19</td>
<td>0.40</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>25.73</td>
<td>15.80</td>
<td>25.91</td>
<td>39.33</td>
<td>14.71</td>
</tr>
<tr>
<td>Papaya</td>
<td>VC with OTPB3+OTPB1</td>
<td>13.28</td>
<td>9.97</td>
<td>10.58</td>
<td>0.25</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>7.20</td>
<td>6.79</td>
<td>5.14</td>
<td>0.15</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>Increase over control (%)</td>
<td>45.78</td>
<td>31.90</td>
<td>51.45</td>
<td>38.87</td>
<td>54.77</td>
</tr>
</tbody>
</table>

### Table 5. Effect of seed coating formulation on growth and yield of potato at UAS, Hassan and IIHR, Bangalore

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant height (cm)</th>
<th>Leaf area (cm²)</th>
<th>Yield (tonnes/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC with OTPB3+OTPB1</td>
<td>58.60</td>
<td>29.90</td>
<td>30.5</td>
</tr>
<tr>
<td>Control</td>
<td>38.15</td>
<td>19.89</td>
<td>22.4</td>
</tr>
<tr>
<td>Increase over control (%)</td>
<td>34.89</td>
<td>34.48</td>
<td>26.6</td>
</tr>
<tr>
<td>CD (5%)</td>
<td>8.6</td>
<td>9.9</td>
<td>6.3</td>
</tr>
</tbody>
</table>

### Table 6. Effect of seed coating formulation on growth and yield of ginger at Kasaragod

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tillages</th>
<th>Soft rot disease inhibition (%)</th>
<th>No. of fingers</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC with OTPB3+OTPB1</td>
<td>26±1.02a</td>
<td>80.76a</td>
<td>16±4.25a</td>
<td>508.8±153.6a</td>
</tr>
<tr>
<td>Control</td>
<td>16±1.84c</td>
<td>11.14±5.06b</td>
<td>151.8±122.9c</td>
<td></td>
</tr>
<tr>
<td>CD (5%)</td>
<td>2.11</td>
<td>2.01</td>
<td>67.22</td>
<td></td>
</tr>
</tbody>
</table>

### Table 7. Effect of seed coating formulation on growth and yield of turmeric at Kasaragod

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of fingers</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC with OTPB3+OTPB1</td>
<td>27.73±3.44a</td>
<td>1083.3±288.26a</td>
</tr>
<tr>
<td>Control</td>
<td>17.93±3.88b</td>
<td>490±194.75b</td>
</tr>
<tr>
<td>CD (5%)</td>
<td>4.2</td>
<td>162.0</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENT

We thank Indian Council of Agricultural Research, New Delhi, for financial support in the form of ALCCERA, an outreach programme on Alternaria, Colletotrichum and Cercospora diseases of field and horticultural crops.

REFERENCES


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Study Report

Study No. : 1205453
Title : Acute Oral Toxicity/Pathogenicity Study with *Trichoderma harzianum* (OTPB3) (Primary culture) in Wistar rats
Study Director : O. Kumar Babu
Date of Submission : 14.08.2012

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Regulatory Guidelines

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We certify that the work reported here is a true and authentic report of the study entitled "ACUTE ORAL TOXICITY/PATHOGENICITY STUDY WITH Trichoderma harzianum (OTPB3) (Primary culture) IN WISTAR RATS", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

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SUMMARY

Acute oral toxicity/pathogenicity of *Trichoderma harzianum* (OTPB3) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum challenge dose level of 2.0ml containing $3.6 \times 10^{10}$ CFU (1.8 $\times 10^{10}$ CFU/ml) of *Trichoderma harzianum* (OTPB3) (Primary culture) was administered orally to the individual overnight fasted experimental group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity, bodyweight, feed consumption and rectal temperature (twice a day). Haematological and biochemical parameters were analysed on 0th and 21st day. Animals were necropsied at the end of 21 day observation period; gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism: i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls. ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms *Trichoderma harzianum*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

- Behavioural changes, mortality and general health condition
- Body weight
- Feed consumption
- Rectal temperature
- Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, Clotting time and Differential count)
- Biochemical parameters (Glucose, BUN, ALT, Total protein and Albumin)
- Gross pathology and organ weight
- On cultural examination, no *Trichoderma harzianum* organisms were detected in the samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Trichoderma harzianum* (OTPB3) (Primary culture) in rats following single oral administration.

Hence, the test substance, *Trichoderma harzianum* (OTPB3) (Primary culture) was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute oral toxicity / pathogenicity rat: $>3.6 \times 10^{10}$ CFU (1.8 $\times 10^{10}$ CFU/ml) of *Trichoderma harzianum* (OTPB3) (Primary culture) per rat.
Study Report

Study No. : 1205455
Title : Acute Pulmonary Toxicity/Pathogenicity Study with Trichoderma harzianum (OTPB3) (Primary culture) in Wistar rats
Study Director : O. Kumar Babu
Date of Submission : 14.08.2012

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SUMMARY

Acute pulmonary toxicity/pathogenicity of *Trichoderma harzianum* (OTPB3) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum dose level of 0.5 ml containing $0.9 \times 10^{10}$ 

(1.8 $\times 10^{10}$ CFU/ml) of *Trichoderma harzianum* (OTPB3) (Primary culture) was administered as such intranasally to the experimental group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with 0.5 ml normal saline. The animals were observed for 21 days for mortality, clinical signs of toxicity, body weight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, uterus, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Trichoderma harzianum*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

- Behavioural changes, mortality and general health condition
- Body weight
- Feed consumption
- Rectal temperature
- Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, Clotting time and Differential count)
- Biochemical parameters (Glucose, BUN, ALT Total Protein and Albumin)
- Gross pathology and organ weight
- On cultural examination, no *Trichoderma harzianum* organisms were detected on examination in brain, heart, liver, lungs, spleen, kidneys, uterus, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Trichoderma harzianum* (OTPB3) (Primary culture) in rats following single intranasal administration.

Hence, the test substance, *Trichoderma harzianum* (OTPB3) (Primary culture) was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute pulmonary toxicity / pathogenicity rat: > $0.9 \times 10^{10}$ (1.8 $\times 10^{10}$ CFU/ml) of *Trichoderma harzianum* (OTPB3) (Primary culture) per rat.
Study Report

Study No. : 1205454

Title : Acute Oral Toxicity/Pathogenicity Study with Trichoderma harzianum (OTPB3) (Primary culture) in CD 1 Mouse

Study Director : O. Kumar Babu

Date of Submission : 14.08.2012

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Regulatory Guidelines

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CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE ORAL TOXICITY/PATHOGENICITY STUDY WITH *Trichoderma harzianum (OTPB3) (Primary culture) IN CD 1 MOUSE*, based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology and Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

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SUMMARY

Acute oral toxicity/pathogenicity of *Trichoderma harzianum* (OTPB3) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female CD 1 Mouse. The maximum challenge dose level of 1.0ml containing $1.8 \times 10^{10}$ CFU of *Trichoderma harzianum* (OTPB3) (Primary culture) was administered orally to the individual overnight fasted experimental group of mice (10/sex). Control group of mice (10/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity, bodyweight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of control. ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual mice for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, urinary bladder, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Trichoderma harzianum*. From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition  
b. Body weight  
c. Feed consumption  
d. Rectal temperature  
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, Clotting time and Differential count)  
f. Biochemical parameters (Glucose, BUN, ALT, Total Protein and Albumin)  
g. Gross pathology and organ weight  
h. On cultural examination, no *Trichoderma harzianum* organisms were detected in the samples of liver, lungs, kidneys, brain, heart, spleen, urinary bladder, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Trichoderma harzianum* (OTPB3) (Primary culture) in mice following single oral administration.

Hence, the test substance, *Trichoderma harzianum* (OTPB3) (Primary culture) was non-toxic and non-virulent to CD 1 Mouse under the experimental conditions tested. **Acute oral toxicity / Pathogenicity mouse: > $1.8 \times 10^{10}$ CFU of *Trichoderma harzianum* (OTPB3) (Primary culture) per mouse**
Study Report

Study No. : 1205456
Title : Acute Intraperitoneal Toxicity / Pathogenicity Study with Trichoderma harzianum (OTPB3) (Primary culture) in Wistar rats
Study Director : O. Kumar Babu
Date of Submission : 14.08.2012

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* [A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]
SUMMARY

Acute intraperitoneal toxicity / pathogenicity of *Trichoderma harzianum* (OTPB3) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum dose level of 0.5ml containing $0.9 \times 10^{10}$ (1.8 x $10^{10}$ CFU/ml) of *Trichoderma harzianum* (OTPB3) (Primary culture) was injected intraperitoneally to the group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with 0.5ml sterile normal saline alone. The experimental animals were observed for 21 days for mortality, clinical signs of toxicity, bodyweight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Trichoderma harzianum*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Feed consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)
f. Biochemical parameters (Glucose, BUN, ALT, Total Protein and Albumin)
g. Gross pathology and organ weight recordings
h. On cultural examination, no *Trichoderma harzianum* organisms were detected in brain, heart, lungs, spleen, liver, kidneys, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Trichoderma harzianum* (OTPB3) (Primary culture) in rats following single intraperitoneal injection.

Hence, the test substance, *Trichoderma harzianum* (OTPB3) (Primary culture) was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute intraperitoneal toxicity / pathogenicity rat: > $0.9 \times 10^{10}$ (1.8 x $10^{10}$ CFU/ml) of *Trichoderma harzianum* (OTPB3) (Primary culture) per rat
Study Report

Study No. : 1205457
Title : Acute Dermal Toxicity / Pathogenicity Study with *Trichoderma harzianum* (OTPB3) (Primary culture) in New Zealand White Rabbits
Study Director : O. Kumar Babu
Date of Submission : 14.08.2012

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SUMMARY

Acute dermal toxicity / pathogenicity of *Trichoderma harzianum* (OTPB3) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female New Zealand white rabbits. The maximum challenge dose level of 1.0ml containing $1.8 \times 10^{10}$ CFU of *Trichoderma harzianum* (OTPB3) (Primary culture) was evenly applied to the shaven sites of experimental group of rabbits (6/sex) under a patch. Control group of rabbits (6/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity and rectal temperature (twice a day). Haematological and biochemical parameters were analyzed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological evaluations of lesions were recorded. Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rabbit for weight determination.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rabbit for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Trichoderma harzianum*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Food consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)
f. Clinical chemistry parameters (Glucose, BUN, ALT, Total Protein and albumin)
g. Gross pathology and organ weight recordings
h. On cultural examination, no *Trichoderma harzianum* organisms were detected in the sample of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Trichoderma harzianum* (OTPB3) (Primary culture) in rabbits following single dermal application.

Hence, the test substance *Trichoderma harzianum* (OTPB3) (Primary culture) was non-toxic and non-virulent to New Zealand white rabbits under the experimental conditions tested.

Acute dermal toxicity / pathogenicity rabbit: $> 1.8 \times 10^{10}$ CFU of *Trichoderma harzianum* (OTPB3) (Primary culture) per rabbit
Study Report

Study No. : 1205461
Title : Acute Intraperitoneal Toxicity / Pathogenicity Study with Bacillus subtilis (OTPB1) (Primary culture) in Wistar rats
Study Director : K. Madurapathi
Date of Submission : 13.08.2012

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CERTIFICATE

We certify that the work reported here is a true and authentic report of the project entitled "ACUTE INTRAPERITONEAL TOXICITY / PATHOGENICITY STUDY WITH BACILLUS SUBTILIS (OTPBI) (PRIMARY CULTURE) IN WISTAR RATS", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

* [A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

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G. SELVAM, M.V.Sc.
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P. BALAKRISHNA MURTHY, Ph.D., D.Sc.
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** Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute intraperitoneal toxicity / pathogenicity of *Bacillus subtilis* (OTPBI) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum dose level of 0.5ml containing $1.05 \times 10^9$ (2.1 x $10^8$ CFU/ml) of *Bacillus subtilis* (OTPBI) (Primary culture) was injected intraperitoneally to the group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with 0.5ml sterile normal saline alone. The experimental animals were observed for 21 days for mortality, clinical signs of toxicity, bodyweight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21\textsuperscript{th} day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Bacillus subtilis*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition  
b. Body weight  
c. Feed consumption  
d. Rectal temperature  
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)  
g. Biochemical parameters (Glucose, BUN, ALT, Total Protein and Albumin)  
h. Gross pathology and organ weight recordings  

On cultural examination, no *Bacillus subtilis* organisms were detected in brain, heart, lungs, spleen, liver, kidneys, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Bacillus subtilis* (OTPBI) (Primary culture) in rats following single intraperitoneal injection.

Hence, the test substance, *Bacillus subtilis* (OTPBI) (Primary culture) was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute intraperitoneal toxicity / pathogenicity rat: $> 1.05 \times 10^9$ (2.1 x $10^8$ CFU/ml) of *Bacillus subtilis* (OTPBI) (Primary culture) per rat
Study Report

Study No. : 1205460

Title : Acute Pulmonary Toxicity/Pathogenicity Study with *Bacillus subtilis* (OTPBI) (Primary culture) in Wistar rats

Study Director : K. Madurapathi

Date of Submission : 13.08.2012

Sponsor

M/s. Indian institute of Horticultural Research,
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Regulatory Guidelines

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

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Original 1 (2)
CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE PULMONARY TOXICITY / PATHOGENICITY STUDY WITH BACILLUS SUBTILIS (OTPBI) (PRIMARY CULTURE) IN WISTAR RATS", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

* [A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

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DIRECTOR

** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute pulmonary toxicity/pathogenicity of *Bacillus subtilis* (OTPBI) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum dose level of 0.5ml containing 1.05 x 10^8 (2.1 x 10^6 CFU/ml) of *Bacillus subtilis* (OTPBI) (Primary culture) was administered as such intranasally to the experimental group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with 0.5 ml normal saline. The animals were observed for 21 days for mortality, clinical signs of toxicity, body weight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, uterus, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Bacillus subtilis*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition  
b. Body weight  
c. Feed consumption  
d. Rectal temperature  
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, Clotting time and Differential count)  
f. Biochemical parameters (Glucose, BUN, ALT Total Protein and Albumin)  
g. Gross pathology and organ weight  
h. On cultural examination, no *Bacillus subtilis* organisms were detected on examination in brain, heart, liver, lungs, spleen, kidneys, uterus, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Bacillus subtilis* (OTPBI) (Primary culture) in rats following single intranasal administration.

Hence, the test substance, *Bacillus subtilis* (OTPBI) (Primary culture) was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute pulmonary toxicity / pathogenicity rat: > 1.05 x 10^8 (2.1 x 10^6 CFU/ml) of *Bacillus subtilis* (OTPBI) (Primary culture) per rat.
Study Report

Study No. : 1205462
Title : Acute Dermal Toxicity / Pathogenicity Study with \textit{Bacillus subtilis} (OTPBI) (Primary culture) in New Zealand White Rabbits

Study Director : K. Madurapathi

Date of Submission : 13.08.2012

Sponsor

M/s. Indian institute of Horticultural Research,
Hessaraghatta Lake Post,
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Regulatory Guidelines

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

Test Facility

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We certify that the work reported here is a true and authentic report of the study entitled "ACUTE DERMAL TOXICITY / PATHOGENICITY STUDY WITH BACILLUS SUBTILIS (OTPBI) (PRIMARY CULTURE) IN NEW ZEALAND WHITE RABBITS" based on the study conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

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SUMMARY

Acute dermal toxicity / pathogenicity of *Bacillus subtilis* (OTPBI) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female New Zealand white rabbits. The maximum challenge dose level of 1.0ml containing $2.1 \times 10^8$ CFU of *Bacillus subtilis* (OTPBI) (Primary culture) was evenly applied to the shaved sites of experimental group of rabbits (6/sex) under a patch. Control group of rabbits (6/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity and rectal temperature (twice a day). Haematological and biochemical parameters were analyzed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological evaluations of lesions were recorded. Brain, heart, liver, lungs, spleen, adenals, kidneys and gonads were isolated from individual rabbit for weight determination.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adenals, kidneys and gonads were isolated from individual rabbit for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Bacillus subtilis*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

- a. Behavioural changes, mortality and general health condition
- b. Body weight
- c. Feed consumption
- d. Rectal temperature
- e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)
- f. Clinical chemistry parameters (Glucose, BUN, ALT, Total Protein and albumin)
- g. Gross pathology and organ weight recordings

h. On cultural examination, no *Bacillus subtilis* organisms were detected in the sample of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Bacillus subtilis* (OTPBI) (Primary culture) in rabbits following single dermal application.

Hence, the test substance *Bacillus subtilis* (OTPBI) (Primary culture) was non-toxic and non-virulent to New Zealand white rabbits under the experimental conditions tested.

Acute dermal toxicity / pathogenicity rabbit: $>2.1 \times 10^8$ CFU of *Bacillus subtilis* (OTPBI) (Primary culture) per rabbit
Study Report

Study No. : 1205458
Title : Acute Oral Toxicity/Pathogenicity Study with *Bacillus subtilis* (OTPBI) (Primary culture) in Wistar rats
Study Director : K. Madurapathi
Date of Submission : 13.08.2012

Sponsor

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Regulatory Guidelines

CIB Guidelines for registration of Bio-Pesticides
(Anagonistic Bacteria / Fungi & Entomopathogenous Fungi)

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CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE ORAL TOXICITY/PATHOGENICITY STUDY WITH BACILLUS SUBTILIS (OTPBI) (PRIMARY CULTURE) IN WISTAR RATS", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

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** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute oral toxicity/pathogenicity of *Bacillus subtilis* (OTPBI) (Primary culture) sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum challenge dose level of 2.0ml containing $4.2 \times 10^8$ CFU ($2.1 \times 10^8$ CFU/ml) of *Bacillus subtilis* (OTPBI) (Primary culture) was administered orally to the individual overnight fasted experimental group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity, bodyweight, feed consumption and rectal temperature (twice a day). Haematological and biochemical parameters were analysed on 0th and 21th day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism. i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls. ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e *Bacillus subtilis*.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition  
b. Body weight  
c. Feed consumption  
d. Rectal temperature  
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, Clotting time and Differential count)  
f. Biochemical parameters (Glucose, BUN, ALT, Total protein and Albumin)  
g. Gross pathology and organ weight  
h. On cultural examination, no *Bacillus subtilis* organisms were detected in the samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Bacillus subtilis* (OTPBI) (Primary culture) in rats following single oral administration.

Hence, the test substance, *Bacillus subtilis* (OTPBI) (Primary culture) was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute oral toxicity / pathogenicity rat: $> 4.2 \times 10^8$ CFU ($2.1 \times 10^8$ CFU/ml) of *Bacillus subtilis* (OTPBI) (Primary culture) per rat.
Study Report

Study No. : 1205459
Title : Acute Oral Toxicity/Pathogenicity Study with *Bacillus subtilis* (OTPBI) (Primary culture) in CD 1 Mouse
Study Director : K. Madurapathi
Date of Submission : 13.08.2012

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Regulatory Guidelines

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

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CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE ORAL TOXICITY/PATHOGENICITY STUDY WITH BACILLUS SUBTILIS (OTPBI) (PRIMARY CULTURE) IN CD 1 MOUSE", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology and Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenic Fungi) and the report provides a true and accurate record of the results obtained during the study.

* [A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

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SUMMARY

Acute oral toxicity/pathogenicity of *Bacillus subtilis* (OTPBI) (Primary culture) sponsored by M/s. Indian Institute of Horticultural Research, Bangalore, India was tested in male and female CD 1 Mouse. The maximum challenge dose level of 1.0 ml containing 2.1 x 10^9 CFU of *Bacillus subtilis* (OTPBI) (Primary culture) was administered orally to the individual overnight fasted experimental group of mice (10/sex). Control group of mice (10/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity, body weight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism
i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of control. ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual mice for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, urinary bladder, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. *Bacillus subtilis*. From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Feed consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, Clotting time and Differential count)
f. Biochemical parameters (Glucose, BUN, ALT, Total Protein and Albumin)
g. Gross pathology and organ weight
h. On cultural examination, no *Bacillus subtilis* organisms were detected in the samples of liver, lungs, kidneys, brain, heart, spleen, urinary bladder, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Bacillus subtilis* (OTPBI) (Primary culture) in mice following single oral administration.

Hence, the test substance, *Bacillus subtilis* (OTPBI) (Primary culture) was non-toxic and non-virulent to CD 1 Mouse under the experimental conditions tested.

Acute oral toxicity / Pathogenicity mouse: > 2.1 x 10^9 CFU of *Bacillus subtilis* (OTPBI) (Primary culture) per mouse
Study Report

Study No. : 05-2014-027

Study Title : Acute Contact toxicity of Seed Pro (Trichoderma harzianum) (OTPB3) to honeybee, Apis cerana indica.

Study Director : T. Jeyalakshmi

Date of Submission : 04 June 2014

Sponsor
M/s. Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore - 560 089, India.

Regulatory Guideline
Gaitonde Committee Guideline (No. 6.6.0)

Test Facility
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CERTIFICATE

We certify that the work reported herein is a true and authentic report of the study entitled "Acute contact toxicity of Seed Pro (Trichoderma harzianum) (OTPB3) to honeybee, Apis cerana indica" based on the work conducted by the Department of Ecotoxicology at International Institute of Biotechnology And Toxicology (IIBAT), Padappai - 601 301, Tamil Nadu, India. The study has been conducted as per Gaitonde Committee Guideline No. (6.6.0) and the results presented here are faithful reflection of data collected during the study.

T. Jeyalakshmi, Ph.D.  
Study Director  
Date: 04 June 2014

A. Ramiah, Ph.D., D.Sc., CChem., MRSC.  
Test Facility Manager  
Date: 04 June 2014
Acute contact toxicity of Seed Pro (Trichoderma harzianum) (OTPB3) to honeybee, *Apis cerana indica*, supplied by M/s. Indian Institute of Horticultural Research, Bangalore, India was studied under laboratory condition by the Department of Ecotoxicology, International Institute of Biotechnology and Toxicology, Padappai, Tamil Nadu, India. Dimethoate 30EC was used as a standard check for comparison.

The maximum attainable concentration in distilled water was found to be 38.46% w/v. The maximum dose that could be tested was found to be 1538.52 µg/bee, with dosing volume 4 µL/bee. Active adult foraging worker honeybees were exposed to Seed Pro (Trichoderma harzianum) (OTPB3) at the tested dose of 1538.52 µg/bee along with control group (distilled water) and Standard check (Dimethoate 30 EC).

In the present experiment Seed Pro (Trichoderma harzianum) (OTPB3) recorded an LD₅₀ of >1538.52 µg/bee to honeybee *Apis cerana indica* at 24 hours after dosing.

However, the standard check, Dimethoate 30 EC registered an LD₅₀ of 0.120 µg/bee to honeybee *Apis cerana indica* at 24 hours after dosing, with 95 per cent fiducial limits ranging from 0.098 - 0.151 µg/bee.
Study Report

Study No. : 05-2014-026
Study Title : Acute Toxicity of Seed Pro (Trichoderma harzianum) (OTPB3) to the Earthworm, Eisenia fetida in Artificial soil
Study Director : Sweatha S. Mohan
Date of Submission : 04 June 2014

Sponsor
M/s. Indian Institute of Horticultural Research,
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Bangalore - 560 089, India.

Regulatory Guidelines
OECD No.207 (4th April, 1984) and ISO 11268-1, (2012)

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Original 1 (2)
CERTIFICATE

We certify that the work reported herein is a true and authentic report of the project entitled “Acute Toxicity of Seed Pro (Trichoderma harzianum) (OTP83) to the Earthworm, Eisenia fetida in artificial soil” based on the work conducted by the Department of Ecotoxicology at International Institute of Biotechnology and Toxicology (IIBAT), Padappai - 601 301, Tamil Nadu, India. The study has been conducted as per OECD 207; Guidelines for testing of chemicals and ISO 11268-1 and the results presented here are faithful reflection of data collected during the study.

Sweatha S. Mohan, M.Sc.  
Study Director

Date: 04 June 2014

A. Rajesh, Ph.D., D.Sc., CCchem., MRSC.  
Test Facility Manager

Date: 04 June 2014
3.0 SUMMARY

Test System:
The acute toxicity of Seed Pro (*Trichoderma harzianum*) (OTPB3) to the earthworm, *Eisenia fetida* (Savigny 1826), was estimated in a 14-day soil exposure laboratory study conducted as per OECD Guideline 207 (1984) and ISO-Guidelines 11268-1 (2012) during February-March 2014. Four replicates of ten clitellated adult earthworms were each exposed to nominal concentrations of 62.5, 125, 250, 500 and 1000 mg Seed Pro (*Trichoderma harzianum*) (OTPB3)/kg dry soil. The untreated control (Quartz sand) was replicated four times, with ten earthworms in each replicate.

Earthworms were assessed for mortality after 7 and 14 days of exposure, behavioral abnormalities on 0, 7 and 14 days of exposure and earthworm body weights were assessed on day 0 and day 14.

The toxic reference, 2-Chloroacetamide is tested twice a year. The 14-day LC₅₀ of the most recent test conducted in February-March, 2014 was 22.2 mg Chloroacetamide/kg dry soil (Annexure 1).

Findings:

### TABLE 1 SUMMARY OF RESULTS

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Control (Quartz sand)</th>
<th>Seed Pro (<em>Trichoderma harzianum</em>) (OTPB3) [mg/kg dry soil]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>62.5 125 250 500 1000</td>
</tr>
<tr>
<td>Mortality after 14 days [%]</td>
<td>0.00 0.00 0.00 0.00 0.00</td>
<td></td>
</tr>
<tr>
<td>Biomass change after 14 days [%]</td>
<td>2.90 0.80 0.20 2.69 2.19</td>
<td></td>
</tr>
<tr>
<td>14 day LC₅₀</td>
<td>greater than 1000 mg/kg dry soil</td>
<td></td>
</tr>
</tbody>
</table>

Fiducial Limits (95% confidence): NA

NOEC related to mortality: 1000 mg/kg dry soil

NOEC related to biomass: 1000 mg/kg dry soil

*Not significantly different compared to the control; †Dunn's test (α = 0.05); NA = Not Applicable

Conclusions:

According to the results of this study, the 14-day LC₅₀ of Seed Pro (*Trichoderma harzianum*) (OTPB3) was considered as greater than 1000 mg/kg dry soil. The 14-day No-Observed-Effect Concentration (NOEC) with respect to biomass and mortality was determined to be 1000 mg Seed Pro (*Trichoderma harzianum*) (OTPB3)/kg dry soil respectively.
Study Report

Study No. : 05-2014-025
Study Title : Toxicity of Seed Pro (Trichoderma harzianum) (OTPB3) to Silkworm, Bombyx mori under laboratory conditions
Study Director : R. Shanmugasundaram
Date of Submission : 04 June 2014

Sponsor
M/s. Indian Institute of Horticultural Research,
Hessaraghatta Lake Post,
Bangalore - 560 089, India.

Regulatory Guidelines
Following JMAFF Guideline No. 2-8-2 (2000) and Standard Protocol

Test Facility
International Institute of Biotechnology And Toxicology (IIBAT),
Padappai - 601 301, Kancheepuram District, Tamil Nadu, India.
Ph: 91 - 44 - 27174246 / 27174266
Fax: 91 - 44 - 27174455
E-Mail: director@iibat.com
CERTIFICATE

We certify that the work reported herein is a true and authentic report of the study entitled "Toxicity of Seed Pro (Trichoderma harzianum) (OTPBJ) to Silkworm, Bombyx mori under laboratory conditions" based on the research conducted by the Department of Eco-toxicology at International Institute of Biotechnology And Toxicology, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides. The report under reference provides a true and accurate record of the results obtained during the study.

R. Shanmugasundaram, M.Sc., Dip. (Ag.). Date: 04 June 2014
Study Director

A. Ramesh, Ph.D., D.Sc., CChem., MRSC. Date: 04 June 2014
Test Facility Manager
SUMMARY

In order to assess the toxicity of Seed Pro (Trichoderma harzianum) (OTPB3) supplied by M/s. Indian Institute of Horticultural Research, Bangalore, India to silkworm larvae, Bombyx mori, a laboratory experiment was carried out at Department of Ecotoxicology, International Institute of Biotechnology And Toxicology, during March 2014. Various concentrations of the test product ranging from 0.05 to 1.60% w/v were evaluated on the early 3rd instar larvae of silkworm by following leaf dip method. Observation on larval mortality was done on 1, 3, 5, and 7th day after treatment. Dimethoate 30% EC was used as reference standard evaluated with concentrations viz., 0.0125, 0.025, 0.05, 0.1, 0.2 and 0.4% w/v to the silkworm by following the procedure mentioned above. Observation on larval mortality was done on 24 and 48 hour after treatment.

The results of the present investigation revealed that, Seed Pro (Trichoderma harzianum) (OTPB3) showed no mortality even at the highest concentration of 1.60% w/v and the LC50 of the test item is greater than 1.60% w/v. The standard check, Dimethoate 30% EC recorded a LC50 of 0.124 % w/v (with 95% confidence limits of 0.074 - 0.21% w/v). Hence it is concluded that the test item is non-toxic to silkworm larvae, Bombyx mori, under the test condition employed.
Study Report

Study No. : 05-2014-024
Title : Acute Toxicity Study of Seed pro (Trichoderma harzianum) (OTPB3) Formulation to Freshwater Fish, Cyprinus carpio
Study Director : S. Ayyappan
Date of Submission : 02.06.2014

Sponsor

M/s. Indian Institute of Horticultural Research,
Hessaraghatta Lake Post,
Bangalore - 560 089
India.

Regulatory Reference

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

Test Facility

International Institute of Biotechnology and Toxicology (IIBAT),
Padappai - 601 301, Kancheepuram District, Tamil Nadu, India
Ph: 91-44 - 27174246/27174266
Fax: 91-44 – 27174455
E-Mail: director@iibat.com
CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE TOXICITY STUDY OF Seed pro (Trichoderma harzianum) (OTPB3) Formulation ** TO FRESH WATER FISH, CYPRINUS CARPIO" based on study conducted by the Department of Ecotoxicology at International Institute of Biotechnology and Toxicology *, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) / Gaitonde Committee guidelines. The report provides a true and accurate record of the results obtained during the study.

** [A Product of M/s, Indian Institute of Horticultural Research, Bangalore, India]

S. AYYAPPAN, Ph.D.,
STUDY DIRECTOR

Date: 02.06.2014

A. RAMESH, Ph.D., D.Sc. CChem, MRSC,
TEST FACILITY MANAGER

Date: 02.06.2014

* - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

A study on the acute toxicity of Seed pro \textit{(Trichoderma harzianum)} (OTPB3) Formulation supplied by M/s. Indian Institute of Horticultural Research, Bangalore, India to freshwater fish, \textit{Cyprinus carpio} was conducted by the Department of Ecotoxicology, International Institute of Biotechnology and Toxicology, Padappai.

The static bioassay procedure was followed to determine the 96 hours LC50. Group of ten fish each was exposed to control, 25, 50 and 100 mg/l Seed pro \textit{(Trichoderma harzianum)} (OTPB3) Formulation corresponding to 0, 0.64×10^6, 1.27×10^6 and 2.54×10^6 CFU, respectively and were observed for mortality and abnormal behaviour for the entire experimental period (i.e. 96 hours).

No abnormal behaviour or mortality was observed in any fish belonging to the treated Vs control group throughout the experimental period.

On the basis of the findings of the above study, the 96 hours LC50 of Seed pro \textit{(Trichoderma harzianum)} (OTPB3) Formulation to freshwater fish, \textit{Cyprinus carpio} was determined as >100 mg/l (~2.54×10^6 CFU). Hence, it may be concluded that the test product is practically non-toxic to freshwater fish, \textit{Cyprinus carpio}. 
Study Report

Study No. : 05-2014-023
Title : Acute Oral Toxicity / Pathogenicity Study with Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation in Pigeon

Study Director : R.A. Gopi
Date of Submission : 02.06.2014

Sponsor

M/s. Indian Institute of Horticultural Research,
Hesaraghatta Lake Post,
Bangalore - 560 089
India.

Regulatory Reference

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

Test Facility

International Institute of Biotechnology and Toxicology (IIBAT),
Padappai - 601 301, Kancheepuram District, Tamil Nadu, India
Ph: 91-44 - 27174246/27174266
Fax: 91-44 - 27174455
E-Mail: director@iibat.com
CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled “ACUTE ORAL TOXICITY / PATHOGENICITY STUDY WITH Seed pro (Trichoderma harzianum) (OTP3) Formulation ** IN PIGEON”, based on study conducted by the Department of Ecotoxicology at International Institute of Biotechnology and Toxicology *, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) / Gaitonde Committee guidelines. The report provides a true and accurate record of the results obtained during the study.

** [A Product of M/s. Indian Institute of Horticultural Research, Bangalore, India]

R.A. GOPI, M.Sc.
STUDY DIRECTOR

Date: 02.06.2014

G. SELVAM, M.V.Sc., DICVP.
STUDY PATHOLOGIST

Date: 02.06.2014

A. RAMESH, Ph.D., D.Sc., CChem, MRSC,
TEST FACILITY MANAGER

Date: 02.06.2014

* - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute oral toxicity / pathogenicity potential of Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation supplied by M/s. Indian Institute of Horticultural Research, Bangalore, India was tested in Pigeon. The maximum challenge dose level of 5.0 ml containing 2.54 x 10^7 CFUs/g of Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation was administered orally to the overnight fasted experimental group of 3 pigeon. Control group of 3 pigeon was similarly treated but with distilled water alone. The birds were observed for 21 days for Physical appearance, Behavioural changes, Toxic signs, Body weight (growth) and Mortality. Birds were necropsied at the end of 21 day observation period, gross pathological evaluations of lesions, if any were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) birds were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Samples of liver, lungs, kidney, brain, spleen, and blood were collected aseptically for microbial evaluation from the treated vs control birds at the end of the experimental period and cultured on selective medium for *Trichoderma harzianum* for the presence/multiplication, if any, of the test organisms i.e. *Trichoderma harzianum*.

From the experimental results it is concluded that the test substance did not have any effect on the following parameters investigated:

- Behavioural changes and general health
- Body weight gain
- Rectal temperature
- Gross pathology

No *Trichoderma harzianum* organisms were detected in the sample of liver, lungs, kidney, brain, spleen and blood. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of *Trichoderma harzianum* in pigeon following single oral ingestion.

Hence, the test substance, Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation was considered as non-toxic and non-virulent to Pigeon under the experimental conditions tested.

**Acute Oral Toxicity / Pathogenicity in Pigeon:** > 2.54 x 10^7 CFUs/g of Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation per Pigeon
Study Report

Study No. : 05-2014-022
Title : Acute Oral Toxicity / Pathogenicity Study with Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation in Chicken
Study Director : R.A. Gopi
Date of Submission : 02.06.2014

Sponsor
M/s. Indian Institute of Horticultural Research,
Hessaraghatta Lake Post,
Bangalore - 560 089
India.

Regulatory Reference
CIB Guidelines for registration of Bio-Pesticides
(© antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

Test Facility
International Institute of Biotechnology and Toxicology (IIBAT),
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INTERNATIONAL INSTITUTE OF BIOTECHNOLOGY AND TOXICOLOGY (IIBAT)  
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Ph: 91 - 44 – 27174246 / 27174266, Fax: 91 - 44 - 27174455  
E-Mail: director@iibat.com

CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled “ACUTE ORAL TOXICITY / PATHOGENICITY STUDY WITH Seed pro (Trichoderma harzianum) (OTPB3) Formulation ** IN CHICKEN”, based on study conducted by the Department of Ecotoxicology at International Institute of Biotechnology and Toxicology *, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) / Gaitonde Committee guidelines. The report provides a true and accurate record of the results obtained during the study.

** [A Product of M/s. Indian Institute of Horticultural Research, Bangalore, India]

R.A. GOPI, M.Sc.,  
STUDY DIRECTOR  
Date: 02.06.2014

G. SELVAM, M.V.Sc., DICVP.  
STUDY PATHOLOGIST  
Date: 02.06.2014

A. RAMESH, Ph.D., D.Sc., CChem, MRSC,  
TEST FACILITY MANAGER  
Date: 02.06.2014

* - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute oral toxicity / pathogenicity potential of Seed pro (Trichoderma harzianum) (OTPB3) Formulation supplied by M/s. Indian Institute of Horticultural Research, Bangalore, India was tested in Chicken. The maximum challenge dose level of 5.0 ml containing $2.54 \times 10^7$ CFUs/g of Seed pro (Trichoderma harzianum) (OTPB3) Formulation was administered orally to the overnight fasted experimental group of 3 chicken. Control group of 3 chicken was similarly treated but with distilled water alone. The birds were observed for 21 days for Physical appearance, Behavioural changes, Toxic signs, Body weight (growth) and Mortality. Birds were necropsied at the end of 21 day observation period, gross pathological evaluations of lesions, if any were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) birds were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Samples of liver, lungs, kidney, brain, spleen and blood were collected aseptically for microbial evaluation from the treated Vs control birds at the end of the experimental period and cultured on selective medium for Trichoderma harzianum for the presence/multiplication, if any of the test organisms i.e. Trichoderma harzianum.

From the experimental results it is concluded that the test substance did not have any effect on the following parameters investigated:

- Behavioural changes and general health
- Body weight gain
- Rectal temperature
- Gross pathology

No Trichoderma harzianum organisms were detected in the sample of liver, lungs, kidney, brain, spleen and blood. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of Trichoderma harzianum in chicken following single oral ingestion.

Hence, the test substance, Seed pro (Trichoderma harzianum) (OTPB3) Formulation was considered as non-toxic and non-virulent to chicken under the experimental conditions tested.

Acute Oral Toxicity / Pathogenicity in Chicken: $> 2.54 \times 10^7$ CFUs/g Seed pro (Trichoderma harzianum) (OTPB3) Formulation per Chicken
# Study Report

<table>
<thead>
<tr>
<th>Study No.</th>
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<tr>
<td>Title</td>
<td>Acute Intraperitoneal Toxicity / Pathogenicity Study with Seed pro (<em>Trichoderma harzianum</em>) (OTPBR) Formulation in Wistar rats</td>
</tr>
<tr>
<td>Study Director</td>
<td>K. Madurapathi</td>
</tr>
<tr>
<td>Date of Submission</td>
<td>31.05.2014</td>
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</tbody>
</table>

**Sponsor**

M/s. Indian Institute of Horticultural Research, 
Hessaraghatta Lake Post, 
Bangalore - 560 089 
India.

**Regulatory Guidelines**

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

**Test Facility**

International Institute of Biotechnology and Toxicology (IIBAT), 
Padappai, 601 301, Kancheepuram District, Tamil Nadu, India.

Ph: 91 - 44 – 27174246 / 27174266 
Fax: 91 - 44 - 27174555 
E-Mail: director@iibat.com
CERTIFICATE

We certify that the work reported here is a true and authentic report of the project entitled "ACUTE INTRAPERITONEAL TOXICITY / PATHOGENICITY STUDY WITH Seed pro (Trichoderma harzianum) (OTPB3) Formulation IN WISTAR RATS", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology and Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

[A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

K. MADURAPATHI, M.Sc.
STUDY DIRECTOR
Date: 31.05.2014

G. SELVAM, M.V.Sc, DICVP.
STUDY PATHOLOGIST
Date: 31.05.2014

A. RAMESH, Ph.D., D.Sc., CChem, MRSC,
TEST FACILITY MANAGER
Date: 31.05.2014

** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute intraperitoneal toxicity / pathogenicity of Seed pro (Trichoderma harzianum) (OTPB3) Formulation sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum dose level of 0.5ml containing 2.54 x 10^6 (5.08 x 10^6 CFU/ml) of Seed pro (Trichoderma harzianum) (OTPB3) Formulation was injected intraperitoneally to the group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with 0.5ml sterile normal saline alone. The experimental animals were observed for 21 days for mortality, clinical signs of toxicity, bodyweight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21 day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. (Trichoderma harzianum).

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Feed consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)
f. Biochemical parameters (Glucose, BUN, ALT, Total Protein and Albumin)
g. Gross pathology and organ weight recordings
h. In cultural examination, no Trichoderma harzianum organisms were detected in brain, heart, lungs, spleen, liver, kidneys, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of Seed pro (Trichoderma harzianum) (OTPB3) Formulation in rats following single intraperitoneal injection.

Hence, the test substance, Seed pro (Trichoderma harzianum) (OTPB3) Formulation was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute intraperitoneal toxicity / pathogenicity rat: > 2.54 x 10^6 (5.08 x 10^6 CFU/ml) of Seed pro (Trichoderma harzianum) (OTPB3) Formulation per rat
Study Report

Study No. : 05-2014-020
Title : Acute Oral Toxicity/Pathogenicity Study with Seed pro (Trichoderma harzianum) (OTPB3) Formulation in Wistar rats
Study Director : O. Kumar Babu
Date of Submission : 31.05.2014

Sponsor
M/s. Indian Institute of Horticultural Research,
Hessaraghatta Lake Post,
Bangalore - 560 089
India.

Regulatory Guideline
CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

Test Facility
International Institute of Biotechnology and Toxicology (IIBAT),
Padalampet - 601 301, Kancheepuram District, Tamil Nadu, India.
Ph: 91 - 44 - 27174246 / 27174266
Fax: 91 - 44 - 27174455
E-Mail : director@iibat.com
CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE ORAL TOXICITY/PATHOGENICITY STUDY WITH Seed pro (Trichoderma harzianum) (OTPB3) Formulation IN WISTAR RATS", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

[A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

O. KUMAR BABU, M.Sc.
STUDY DIRECTOR
Date: 31.05.2014

G. SELVAM, M.V.Sc., DICVP.
STUDY PATHOLOGIST
Date: 31.05.2014

A. RAMESH, Ph.D., D.Sc., CCChem, MRSC,
TEST FACILITY MANAGER
Date: 31.05.2014

** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute oral toxicity/pathogenicity of Seed pro (Trichoderma harzianum) (OTPB3) Formulation sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum challenge dose level of 2.0 ml containing 5.08 x 10⁶ CFU (2.54 x 10⁷ CFU/g) of Seed pro (Trichoderma harzianum) (OTPB3) Formulation was administered orally to the individual overnight fasted experimental group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity, body weight, feed consumption and rectal temperature (twice a day). Haematological and biochemical parameters were analysed on 0th and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism, i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls. ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination, iii) Samples of liver, lungs, kidneys, brain, heart, spleen, uterus, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e Trichoderma harzianum.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Feed consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and differential count)
g. Biochemical parameters (Glucose, BUN, ALT, Total protein and Albumin)
h. Gross pathology and organ weight
i. On cultural examination, no Trichoderma harzianum organisms were detected in the samples of liver, lungs, kidneys, brain, heart, spleen, uterus, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of Seed pro (Trichoderma harzianum) (OTPB3) Formulation in rats following single oral administration.

Hence, the test substance, Seed pro (Trichoderma harzianum) (OTPB3) Formulation was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute oral toxicity / Pathogenicity rat: > 5.08 x 10⁶ CFU (2.54 x 10⁷ CFU/g) of Seed pro (Trichoderma harzianum) (OTPB3) Formulation per rat.
Study Report

Study No. : 05-2014-019
Title : Acute Oral Toxicity/Pathogenicity Study with Seed pro (Trichoderma harzianum) (OTPB3) Formulation in CD 1 Mouse
Study Director : K. Madurapathi
Date of Submission : 31.05.2014

Sponsor

M/s. Indian Institute of Horticultural Research,
Hessaraghatta Lake Post,
Bangalore - 560 089
India.

Regulatory Guideline

CIB Guidelines for registration of Bio-Pesticides (Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

Test Facility

International Institute of Biotechnology and Toxicology (IIBAT),
Padappai - 601 301, Kancheepuram District, Tamil Nadu, India.
Ph: 91 - 44 - 27174246 / 27174266
Fax: 91 - 44 - 27174455
E-Mail: director@iibat.com
CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE ORAL TOXICITY/PATHOGENICITY STUDY WITH Seed pro (Trichoderma harzianum) (OTPB3) Formulation IN CD 1 MOUSE", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology and Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

[A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

K. MADURAPATHI, M.Sc.
STUDY DIRECTOR

Date: 31.05.2014

G. SELVAM, M.V.Sc., DICVP.
STUDY PATHOLOGIST

Date: 31.05.2014

A. RAMESH, Ph.D., D.Sc., CChem, MRSC,
TEST FACILITY MANAGER

Date: 31.05.2014

** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute oral toxicity/pathogenicity of Seed pro (Trichoderma harzianum) (OTPB3) Formulation sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female CD 1 Mouse. The maximum challenge dose level of 1.0ml containing $5.08 \times 10^6$ CFU of Seed pro (Trichoderma harzianum) (OTPB3) Formulation was administered orally to the fastest experimental group of mice (10/sex). Control group of mice (10/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity, bodyweight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of control. ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual mice for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, urinary bladder, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e (Trichoderma harzianum).

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Feed consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)
f. Biochemical parameters (Glucose, BUN, ALT, Total Protein and Albumin)
g. Gross pathology and organ weight
h. On cultural examination, no (Trichoderma harzianum) organisms were detected in the samples of liver, lungs, kidneys, brain, heart, spleen, urinary bladder, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of Seed pro (Trichoderma harzianum) (OTPB3) Formulation in mice following single oral administration.

Hence, the test substance, Seed pro (Trichoderma harzianum) (OTPB3) Formulation was non-toxic and non-virulent to CD 1 Mouse under the experimental conditions tested.

Acute oral toxicity / Pathogenicity mouse: > $5.08 \times 10^6$ CFU/ml of Seed pro (Trichoderma harzianum) (OTPB3) Formulation per mouse
Study Report

Study No. : 05-2014-018

Title : Acute Pulmonary Toxicity/Pathogenicity Study with Seed pro
(Trichoderma harzianum) (OTPB3) Formulation in Wistar rats

Study Director : O. Kumar Babu

Date of Submission : 31.05.2014

Sponsor

M/s. Indian Institute of Horticultural Research,
Hassaraghatta Lake Post,
Bangalore - 560 089
India.

Regulatory Guideline

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

Test Facility

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CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE PULMONARY TOXICITY / PATHOGENICITY STUDY WITH Seed pro (Trichoderma harzianum) (OTP83) Formulation in Wistar Rats", based on the experiment conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines of standard protocols approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

[A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

O. KUMAR BABU, M.Sc.,
STUDY DIRECTOR
Date: 31.05.2014

G. SELVAM, M.V.Sc., DICVP,
STUDY PATHOLOGIST
Date: 31.05.2014

A. RAMESH, Ph.D., D.Sc., CCChem, MRSC,
TEST FACILITY MANAGER
Date: 31.05.2014

** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute pulmonary toxicity/pathogenicity of Seed pro (Trichoderma harzianum) (OTPB3) Formulation sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in male and female Wistar rats. The maximum dose level of 0.1 ml of the test substance containing 0.0508 × 10⁷ (2.54 × 10⁷ CFU/ml) of Seed pro (Trichoderma harzianum) (OTPB3) Formulation was administered as such intranasally to the experimental group of rats (10/sex). Control group of rats (10/sex) were similarly treated but with 0.1 ml normal saline. The animals were observed for 21 days for mortality, clinical signs of toxicity, body weight, feed consumption and rectal temperature. Haematological and biochemical parameters were analysed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological lesions were recorded.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rat for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, ovaries, uterus, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e Trichoderma harzianum.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Feed consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)
g. Biochemical parameters (Glucose, BUN, ALT Total Protein and Albumin)

h. On cultural examination, no Trichoderma harzianum organisms were detected in the samples of liver, lungs, kidneys, brain, heart, spleen, ovaries, uterus, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of Seed pro (Trichoderma harzianum) (OTPB3) Formulation in rats following single intranasal administration.

Hence, the test substance, Seed pro (Trichoderma harzianum) (OTPB3) Formulation was non-toxic and non-virulent to Wistar rats under the experimental conditions tested.

Acute pulmonary toxicity / pathogenicity rat: > 0.0508 × 10⁷ (2.54 × 10⁷ CFU/ml) of Seed pro (Trichoderma harzianum) (OTPB3) Formulation per rat
Study Report

Study No. : 05-2014-017
Title : Acute Dermal Toxicity / Pathogenicity Study with Seed pro
(Trichoderma harzianum) (OTP3) Formulation in New
Zealand White Rabbits
Study Director : S.M. Kesavan
Date of Submission : 31.05.2014

Sponsor

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Regulatory Guideline

CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

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CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled "ACUTE DERMAL TOXICITY / PATHOGENICITY STUDY WITH Seed pro (Trichoderma harzianum) (OTPB3) Formulation IN NEW ZEALAND WHITE RABBITS" based on the study conducted by the Department of Toxicology at International Institute of Biotechnology and Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

*A Product of M/s. Indian Institute of Horticultural Research, Bangalore, India*

S.M. KESAVAN, M.Sc.
STUDY DIRECTOR

Date: 31.05.2014

G. SELVAM, M.V.Sc., DICVP
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Date: 31.05.2014

A. RAMESH, Ph.D., D.Sc., CChem, MRSC,
TEST FACILITY MANAGER

Date: 31.05.2014

** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Acute dermal toxicity / pathogenicity of Seed pro (Trichoderma harzianum) (OTPB3) Formulation sponsored by M/s. Indian Institute of Horticultural Research, Bangalore, India was tested in male and female New Zealand white rabbits. The maximum challenge dose level of 1.0 ml containing $0.51 \times 10^7$ CFU of Seed pro (Trichoderma harzianum) (OTPB3) Formulation was evenly applied to the shaved sites of experimental group of rabbits (6/sex) under a, . Control group of rabbits (6/sex) were similarly treated but with distilled water alone. The animals were observed for 21 days for mortality, clinical signs of toxicity and rectal temperature (twice a day). Haematological and biochemical parameters were analyzed on 0 and 21st day. Animals were necropsied at the end of 21 day observation period, gross pathological evaluations of lesions were recorded. Brain, heart, liver, lungs, spleen, kidneys and gonads were isolated from individual rabbit for weight determination.

To determine the infectivity (pathogenicity/multiplication) potential of the test organism i) Rectal temperature (as an index of pyrogenicity/infectivity) measurement of the treated (test) animals were recorded twice daily (morning/evening) for the entire duration of the experiment and were compared with that of controls (vehicle treated), ii) Brain, heart, liver, lungs, spleen, adrenals, kidneys and gonads were isolated from individual rabbit for weight determination. iii) Samples of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces were collected aseptically for microbial evaluation from the treated and control animals at the end of the experimental period and cultured on selective medium for the presence/multiplication, if any, of the test organisms i.e. Trichoderma harzianum.

From the experimental results, it is concluded that the test substance did not have any effect on the following parameters investigated:

a. Behavioural changes, mortality and general health condition
b. Body weight
c. Feed consumption
d. Rectal temperature
e. Haematological parameters (RBC, WBC, Hb, HCT, Prothrombin time, clotting time and Differential count)
f. Clinical chemistry parameters (Glucose, BUN, ALT, Total Protein and albumin)
g. Gross pathology and organ weight recordings
h. On cultural examination, no Trichoderma harzianum organisms were detected in the sample of liver, lungs, kidneys, brain, heart, spleen, blood, urine and faeces. Thus, there was no microbiological evidence for pathogenicity (infectivity) potential of Seed pro (Trichoderma harzianum) (OTPB3) Formulation in rabbits following single dermal application.

Hence, the test substance Seed pro (Trichoderma harzianum) (OTPB3) Formulation was non-toxic and non-virulent to New Zealand white rabbits under the experimental conditions tested.

Acute dermal toxicity / pathogenicity rabbit: $> 0.51 \times 10^7$ CFU of Seed pro (Trichoderma harzianum) (OTPB3) Formulation per rabbit
Study Report

Study No. : 05-2014-016
Title : A Study on Irritation of Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation to mucous membrane in New Zealand white rabbits
Study Director : O. Kumar Babu
Date of Submission : 31.05.2014

Sponsor
M/s. Indian institute of Horticultural Research,
Hessaraghatta Lake Post,
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Regulatory Guideline
CIB Guidelines for registration of Bio-Pesticides
(Antagonistic Bacteria / Fungi & Entomopathogenous Fungi)

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We certify that the work reported here is a true and authentic report of the study entitled "A STUDY ON IRRITATION OF Seed pro (Trichoderma harzianum) (OTP3) Formulation TO MUCOUS MEMBRANE IN NEW ZEALAND WHITE RABBITS", based on the study conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

[A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

O. KUMAR BABU, M.Sc.
STUDY DIRECTOR

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TEST FACILITY MANAGER

Date: 31.05.2014

** - Registered (No.31/1999/CPCSEA dated 11.03.99) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.
SUMMARY

Mucous membrane irritation potential of Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in female New Zealand white rabbits. Initially a quantity of 0.1gm of the test substance containing $2.54 \times 10^6$ CFU (2.54 x 10^7 CFU/gm) of Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation was applied into the conjunctival sac of the left eye of one rabbit. The right eye of the rabbit was served as the control. Test substance treated eye (left) of initial test animal did not exhibit any ocular lesions at 24th hour.

To confirm the findings of the initial test, a confirmatory test was conducted with two additional animals and the same procedure as initial test was followed. Similarly, confirmatory test animals also did not exhibit lesions as initial test animal.

The eyes were examined and lesions were graded after application of the test substance at 24, 48, 72 and thereafter on daily basis for 14 days. The ocular irritation potential was evaluated by Draize's method.

None of the animals presented any ocular lesion throughout the observation period.

The maximum mean score for ocular lesions observed in the experiment was 0. Hence, the test substance, Seed pro (*Trichoderma harzianum*) (OTPB3) Formulation was considered as Non-Irritating (N) to the mucous membrane of eye of the rabbits.
A study on Primary Skin Irritation of Seed pro (Trichoderma harzianum) (OTPB3) Formulation in New Zealand White Rabbits

O. Kumar Babu

31.05.2014

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CIB Guidelines for registration of Bio-Pesticides
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We certify that the work reported here is a true and authentic report of the study entitled "A STUDY ON PRIMARY SKIN IRRITATION OF Seed pro (Trichoderma harzianum) (OTPB3) Formulation IN NEW ZEALAND WHITE RABBITS", based on the study conducted by the Department of Toxicology at International Institute of Biotechnology And Toxicology**, Padappai - 601 301, Tamil Nadu, India. The study has been conducted in accordance with the guidelines approved by the Central Insecticide Board and Registration Committee (CIB-RC) for Bio-Pesticides (Antagonistic Bacteria/Fungi & Entomopathogenous Fungi) and the report provides a true and accurate record of the results obtained during the study.

[A Product of M/s. Indian institute of Horticultural Research, Bangalore, India]

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SUMMARY

Primary Skin Irritation potential of Seed pro (Trichoderma harzianum) (OTPB3) Formulation sponsored by M/s. Indian institute of Horticultural Research, Bangalore, India was tested in New Zealand white rabbits. A quantity of 0.5 gm of the test substance containing $1.27 \times 10^7$ CFU ($2.54 \times 10^7$ CFU/g) of test organism was moistened with minimum volume of the distilled water and the paste thus prepared was evenly applied to the clipped intact skin of the rabbits (3 six) under a patch. At the end of 24h, the patch was removed and the application site was wiped off with distilled water to remove the residual test substance. The untreated side was kept as control area.

Treated and untreated skin sites of the animals were examined for erythema, edema and eschar at 24 and 72h after the application of the test substance.

Animals treated with the test substance exhibited no clinical signs of toxicity.

No dermal reactions were observed at 24 or 72h following the application of the test substance.

Hence, it is concluded that Seed pro (Trichoderma harzianum) (OTPB3) Formulation was non-irritant to the skin of rabbits under the experimental conditions tested.