MOLECULAR CHARACTERIZATIONS OF MICROBIAL ANTAGONISTS AND DEVELOPMENT OF BIOFORMULATIONS FOR MANAGEMENT OF BACTERIAL WILT OF NAGA CHILLI (*Capsicum chinens Jacq.*) IN ASSAM

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ABSTRACT

Aggressive strains of five different saprophytic antagonists *Trichoderma parareesei* TPJ-S-1, *Trichoderma viride* TVJ-S-1, *Paecilomyces variotii* Isolate-1, *Bacillus thuringiensis* BTJ-S-1 and *Citrobacter farmeri* CTJ-S-1 and their consortial formulations were evaluated during 2012-14, for their effectiveness in management of bacterial wilt disease (c. o. *Ralstonia solanacearum*) of *Naga chilli* (*Capsicum chinens* Jacq.). The molecular characterization of selected antagonists was undertaken to determine their distinctiveness from their close relatives through sequencing of the 18S & 28S region of ribosomal DNA in case of fungal antagonists and 16S region in case of bacterial antagonists along with its phylogenetic analysis. The antagonistic potential of the five microbes were tested *in vitro* singly and in consortia against *R. solanacearum* adopting dual culture method. Altogether 31 treatment combinations were compared; the inhibition zones (mm) and percent inhibitions were recorded and analyzed. The highest inhibition (91.47%) against *R. solanacearum* was recorded in consortia of *T. parareesei, T. viride* and *B. thuringiensis* followed by the consortia of *T. parareesei, T. viride, P. variotii, B. thuringiensis* and *C. farmeri* (82.22%). Quantitative aspect of population dynamics of selected antagonists in three different substrate carrier *viz.* vermicompost, talcum powder (TP) and mustard oil cake (MOC) were compared to evaluate their shelf – life at different days of storage.

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Naga chilli

Phylogenetic analysis

All substrate based formulations contained carboxy methyl cellulose (CMC), mannitol and chitin as standard additives. A steady population count was recorded in all different substrate based bio-formulations up to 270 days of storage at room temperature. However, highest population recovery (112.43×10^7 cfu/g) was found in vermicompost based formulation *T. viride* followed by vermicompost based *T. parareesei* (108.83×10^7 cfu/g) at 270 days of storage. The best three consortial bio-formulations having all desired characteristics were selected for field evaluation and applied in plant as combination of seed treatment, soil application and sprays at 30 days after transplanting which showed significant reduction in bacterial wilt incidence and enhanced economic yield of *Naga chilli*. Maximum disease reduction (83.87%) was recorded in consortial formulation of *T. parareesei, T. viride, P. variotii, B. thuringensis* and *C. farmeri*. The treatment also recorded significantly higher yield (9.68 ton/ha) as compared to other treatment combinations.

1 Introduction

India needs about 294 Mt food grains for a projected population of around 1.3 billion by 2020. Furthermore, the projected world population till 2020 will be 7.5 billion. The present chemical intensive high input agriculture is increasingly recognized as environmental degrading and people are now seriously concerned with the protection of the environment and more about safe-guarding their health. Therefore, work on the development of technologies based on natural resources, particularly exploration of the potentials of natural biodiversity has immense scope as alternative for sustainable agriculture.

Plant beneficial microbes (PBM), a component of extensive microbial biodiversity, affect the plant health and development. Some PBM are well known antagonists against soil borne plant pathogens and attempts have been made throughout the world to explore the possibilities of using these saprophytic antagonists for crop disease management (Burr et al., 1978; Papavizas, 1985; Anuratha & Gnanamanickam, 1990; Bora, 2011). Therefore, to explore the techniques for mass culture and enhance survivability of these antagonists in different organic substrates and develop effective biological formulations are becoming priority research for most of the agricultural scientists associated with biological management of crop diseases.

Vegetables and spices have a vital importance in a balanced diet and are the cheapest source of carbohydrates, vitamins, minerals and proteins. Amongst spices, *Naga chilli* (*bhut jolokia*) an interspecies hybrid chilli cultivated in Assam, Nagaland and Manipur region of North East India. Results of molecular characterization study revealed that the chilli is an interspecies hybrid of *C. chinense* ans *C. frutescens* (Bosland & Baral, 2007). The Guinness Book of World Records certified *Naga chilli* (*bhut jolokia)* as the hottest chilli pepper with a heat level of 577,000 SHUs (Bosland & Baral, 2007). The chemical responsible for such high SHU in *Naga chilli* is due to presence of high quantity (3-5%) of powerful alkaloid capsaicin and dihydrocapsaicin. It is the most ideal chilli variety of India for extraction of oleoresin and capsaicin.

Naga chilli crop is highly susceptible for various plant diseases such as bacterial wilt, die-back, anthracnose, leaf curl and leaf necrosis. The intensity of these diseases are higher in Assam as well as other parts of the North-Eastern India. Among these, the bacterial wilt caused by *Ralstonia solanacearum* (Smith) is one of the most serious diseases of crops in tropics, subtropics and warm temperate regions of the world (James et al., 2003). This disease is endemic to all the agro-climatic zones of the North Eastern states of the India and becomes severe when environmental factor favors disease outbreak. Different management practices such as chemicals, antibiotics, soil amendments, soil solarization *etc* used for disease management. However, these methods have limitations and do not give results up to the desirable level. Chemicals are costly, besides of posing a risk of environmental hazards. Therefore, there is a growing concern to find out alternative methods by exploiting bioactive antagonists which results in the reduction of the inoculums density or disease producing activities of pathogens in their active or dormant state (Weller & Cook, 1983). Various biological agents have been employed for management of the plant diseases. *Pseudomonas fluoresens* recognized as a major bacterial antagonist, which has the ability to suppress various seed, soil and air-borne fungal and bacterial pathogens (Burr et al., 1978; Manoranjitham et al., 2000). Among the antagonistic fungi, *Trichoderma* sp. have the ability to exert influence on most of the soil borne pathogens through competition, antibiosis, hyper-parasitism, lysis or other forms of antagonism (Chet, 1987). Many entomopathogenic fungi like *Verticillium lecani* have high potential to inhibit the growth of different plant pathogens besides having lytic properties against insect pests (Askary et al., 1997). Similarly other entomopathogenic fungus *Metarhizium anisopliae* have ability to produces a family of cyclic peptide toxins, destruxins (DTX). Destruxins are highly effective bioactive compound which worked against a large group of Lepidopteran, Orthopteran and Coleopteran insect pests (Kershaw et al., 1999).

With the above view points, the present study was made to evaluate a management strategy of *Naga chilli* bacterial wilt by using bioformulations of established and newly isolated microbial bioagents like *Trichoderma parareesei, T. viride,*

Paecilomyces variotii, Bacillus thuringiensis and *Citrobacter farmeri* as well as their consortia formulations.

2 Materials and Methods

Various experiments were conducted under laboratory and fields condition at the Department of Plant Pathology, and Assam Agricultural University, Jorhat, during the cropping season of 2012 to 2014.

2.1 Isolation of pathogen and antagonists agent from the experimental site

The bacterial wilt pathogen (*R. solanacearum*) was isolated from diseased *Naga chilli* plants showing typical symptoms of wilting by using Triphenyl Tetrazolium Chloride (TTC) medium (Kelman 1954). The antagonists required for the study *viz., T. parareesei, T. viride, P. variotii, B. thuringiensis* and *C. farmeri* were isolated from soils collected from various regions of Assam following the serial dilution plate technique of Waksman (1922). The medium used for isolation of the antagonists are King's B agar medium (King et al., 1954) for *P. fluorescens, Trichoderma* Selective Agar (TSA) medium for *Trichoderma* sp. (Elad et al., 1981), and for remaining bioagents Potato Dextrose Agar (PDA) and Nutrient agar (NA) media was used for fungal and bacterial antagonists, respectively. The different strains obtained were characterized based on "Bergey's Manual of Determinative Bacteriology". The cultures were routinely maintained on slants at 4°C for further studies.

2.2 Molecular analysis of bio agents

2.2.1 Isolation of genomic DNA

The total genomic DNA was extracted from fungal bioagents following the extraction method as described by Samuels & Ismaiel (2009) whereas, genomic DNA of bacterial bioagents was extracted and purified according to the method illustrated by Wang et al. (2001). The isolated DNA was quantified and its purity was estimated with the help of Nanodrop 1000 (Thermo Scientific). The absorbance of genomic DNA was measured at 260 nm to find out the concentration of DNA in solution. The absorbance at 280 nm was also used to estimate the extent of protein contamination in the extracted DNA.

2.2.2 Primer sets used for PCR study

The primer sets used in present investigations were LROR and LR7 (Vilgalys & Hester, 1990) for amplification of 28S rDNA gene fragment of *T. parareesei and T. viride;* ITS1 and ITS4 for 18S rDNA in case of *P. variotii;* 8F and 1492R for 16S rDNA gene fragment of bacterial bio agents *B. thuringiensis* and *C. farmeri.*

2.3.2 28S, 18S and 16S ribosomal DNA gene sequencing

Purified fungal and bacterial bio agents were initially identified using rDNA based partial sequencing technique. For direct cycle sequencing, fragments were purified with QI Aquick PCR purification kit (Qiagen) by following manufacturer's instructions. PCR amplification for 28S and 18S ribosomal DNA fragment was performed in a 50 µl reaction volume following the PCR protocol described by Xiaoke et al. (2013) and Hermosa et al.(2000) respectively. For 18S rDNA amplification the reaction mixture was 10 ng template DNA, 1 µL dNTP mix (2.5 mM each), 100 ng/µL each of forward and reverse primer, 1X Taq DNA polymerase assay buffer (10X), 3U Taq DNA polymerase (Bangalore Genei Ltd., Bangalore, India), and made up 50 µL reaction volume. All reactions were done with 5 min initial denaturation at 94°C, followed by 35 cycles: 30 s at 94°C, 30 s at 54°C, 1 min at 72°C and a final extension of 10 min at 72°C. The reactions were carried out in GeneAmp PCR System (Applied Biosystems model 9700). Quality of amplified PCR products were visualized on a 1.0% agarose gel and purified to remove contaminants using the MinElute PCR purification kit (Qiagen, India), before sequencing. Forward and reverse DNA sequencing was done with respective primer sets used in PCR amplification using BigDye® Terminator v3.1 Cycle Sequencing Kit on ABI 3730xl Genetic Analyzer (Applied Biosystems). The generated gene sequences were used to carry out homology search against previously reported sequences using NCBI BLASTn search engine with default parameters. Based on maximum identity score first ten sequences were selected and the phylogenetic tree was constructed using MEGA 4 software.

2.3.3 Multiple sequence alignments and phylogenetic analysis

Phylogenetic analysis of two bioagents *viz. T. parareesei* TPJ-S-1 and *B. thuringiensis* BTJ-S-1 were done only, as the revolutionary analysis of remaining three bioagents was done earlier by the author. Multiple sequence alignment of the fungal and bacterial bio agents was analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) Software ver. 5.05 (Tamura et al., 2011) using Clustal W function with default parameters. The partial gene sequences of the closest identified relatives (identity > 90%) of the test bacterial (*B. thuringiensis* BTJ-S-1 *C*) and fungal (*T. parareesei* TPJ-S-1) antagonists were manually imported from NCBI GenBank sequence database and aligned against each 28S, 18S and 16S rDNA gene sequences separately using this software. From these aligned sequences phylogenetic tree was constructed using the MEGA software through neighbour-joining tree building algorithm (Tajima & Nei, 1984) the data sets were subjected to 100 bootstraps replicates.

2.4 *In vitro* evaluation of the efficacy of the isolated antagonists against *R. solanacearum*

The antagonistic potential of the five antagonist's *viz., T. parareesei, T. viride, P. variotii, B. thuringiensis* and *C. farmeri* and their consortia were *in vitro* tested against *R. solanacearum* by agar plug method in dual culture assays (Aspiras & Della, 1985). The initial inoculums concentration

of the pathogen was always adjusted to 1 x 10^8 colony forming units per milliliter (cfu/ml) for a bacterial population. Altogether 31 treatment combinations were tested and the antagonistic potential was evaluated on the basis of inhibition zones (mm) and percent inhibitions. A pairs of untreated control plate are also be maintained for comparison. The best four antagonist consortia was selected on the basis of their *in vitro* antagonistic performance and used for further evaluation of their ability to multiply in different growth substrates so that the bioformulation so obtained could be used to suppress wilt incidence of *Naga chilli in vivo*.

2.5 Evaluations of growth and multiplication of antagonists in different substrates

Three types of substrates were evaluated with an objective to select the best substrate for mass multiplication of the antagonists. These evaluated substrates were Vermicompost (VC), Talcum powder (TP) and Mustard oil cake (MOC). The substrates were air dried and passed through 350 mesh sieves to obtain fine powders. These were filled into polypropylene bags separately, sealed with non-absorbent cotton plugs and sterilized at 121°C for 30 minutes. Mass culture of bacterial antagonists *B. thuringiensis* and *C. farmeri* were prepared by transferring aseptically its 24h old growth in NA into 1000 ml nutrient broth and incubated at 28°C for 48h. Similarly, mass cultures of fungal antagonists *T. parareesei, T. viride, P. variotii,* was prepared by transferring aseptically their 72h old growth in PDA to 1000ml PD broth and incubated at 28°C for 120h. From these, 10ml of each bacterial antagonist viz *B. thuringiensis* (10^{7}cfu/ml), *C. farmeri* cells (10^{7}cfu/ml) and fungal antagonist viz *T. parareesei, T. viride* and *P. variotii* with 10^{7}cfu/ml respectively were added to the sterilized substrates contained in the polypropylene bags.

Into all the substrates 10 ml of each 1% Carboxy-Methyl Cellulose (as standard sticker) and 3% Mannitol (as standard osmoticant) were added in order to impart greater adherence and higher moisture retaining property respectively to the substrates. The inoculated substrates were then mixed properly and incubated at 28°C for 72h. The bags were stored at room temperature after incubation.

For determination of the population of the antagonists in different substrate formulation after different days of storage as prepared above, shelf life study experiment was designed following CRD with 3 replications. The 16 different treatment combinations were: Vermicompost + *T. parareesei;* Talc powder + *T. parareesei;* Mustard oil cake + *T. parareesei;* VC + *T. viride;* TP + *T. viride;* MOC + *T. viride;* VC + *P. variotii;* TP + *P. variotii;* MOC + *P. variotii;* VC + *B. thuringiensis;* TP + *B. thuringiensis;* MOC + *B. thuringiensis;* VC + *C. farmeri;* TP + *C. farmeri;* MOC + C. *farmeri;* and control. The viable population of *T. parareesei, T. viride, P. variotii, B. thuringiensis* and *C. farmeri* in different substrates was determined after 90, 180, 270, 360 and 450 days of inoculation following dilution plate technique to ascertain the best antagonist + substrate combination for highest shelf life of the

formulation in storage at room temperature. On the basis of the highest recovery of the population of these antagonists (cfu/g), the best substrate was selected for development of bioformulations consortia and further experimentations under field conditions.

2.6 Field evaluation of the substrate based bio-formulations against bacterial wilt of *Naga chilli*

The best three consortial bioformulations were applied *in planta* as combination of seed treatment, soil application at transplanting and spray at 30 days after transplanting to evaluate their efficacy to reduce wilt incidence *vis a vis* enhancement of crop yield. The consortial formulation of *T. parareesei, T. viride* and *B. thuringiensis; T. parareesei, T. Viride, P. variotii, B. thuringiensis* and *C. farmeri* was used for seed treatment, seedling treatment, soil application and their combinations. All together 6 (six) treatment combinations were compared (Table 3), arranged in RBD and each treatment was replicated four times.

2.6.1 Seed treatment

Naga chilli seeds were first surface sterilized with 1 % sodium hypo chloride then rinsing them twice with sterile water and treated with each of the substrate based bioformulations @10g/100g of seed along with a sticker/adhesive like rice glue for easy adherence. The coated seeds were then spread over a clean paper and dried overnight.

2.6.2 Root treatment

Substrate based bioformulations were mixed with clean water to form a fine slurry and the roots of the seedlings were dipped into the each slurry of each formulation for about 30 minutes. For 1000 seedlings 1kg of bioformulations was required. The treated roots were dried for 1 hour under shade before transplanting.

2.6.3 Soil treatment

At the time of transplanting, 1kg each of substrate based bioformulation was mixed with 10 kg of vermicompost and was applied to the soil near the root zone of plants @ 100g/plant. Pot grown *Naga chilli* (var. *Bor* Bhoot) plants were inoculated with *R. solanacearum* (10^{9}cfu/ml) by root inoculation technique (Winstead & Kelman, 1952).

The control plants were treated with sterile distilled water before inoculation with the pathogen. Observations on percent wilting after 7days of inoculation were recorded and record of crop yield (kg) per plant harvested (pods plucked) 7 times at an interval of 10 days till crop maturity.

3 Results and Discussion

3.1 Isolation of pathogen and antagonists

All the isolated microbial antagonists were identified on the basis of morphological, physiological and biochemical characteristics. The key identification characters and identified species on the behalf of these characteristics are as follows:

3.1.1 *Bacillus thuringiensis* BTJ-S-1

Gram-positive, spore-forming, rod-shaped bacteria, approx 1 x 5 µm in size with a diamond-shaped crystal; white to off-white colour colonies with smooth edges and slightly raised elevation (Fig. 1 G). The isolated bacterial strain showed mesophiles nature with temperature ranging from 30-45°C, aerobic, chemoheterotrophs; found endospore and the parasporal bodies after staining with Coomassie Brilliant Blue R-250, hydrolysis of esculin and lecithin, utilization of sucrose, starch hydrolysis, gelatin hydrolysis and glucose and fructose fermentation test positive, production of indole and H_2S and fermentation of galactose and lactose negative (Hyakumachi et al., 2013)

3.1.2 *Trichoderma parareesei* TPJ-S-1

On PDA medium mycelium covers the plate within 3 days on incubation at 25 ± 2°C. Secondary hyphae formed a dense reticulum. After 6 days of incubation at $25^0C\pm 2°C$ on PDA medium, at first colonies appear white, becoming light to dark green as conidia develop, production of conidia is abundant and variably superposed bright yellow-green to dark green pustules and give rise to a powdery to granular texture (Fig. 1 D). A bright yellow pigment diffuses into the PDA medium which is clearly visible in reverse side of the culture. Fungal conidia are ovoid to uniformly ellipsoidal, ellipsoidal to cylindrical at 37°C more variable, (3.3-6.2 X 2.5-3.5) µm in size, phialide lageni form or ampulli form, often with a cylindrical neck, usually with distinct widening at or above the middle (Fig. 1 A-C), length 4.5-11µm, width at widest point 2.5-3.8 µm, width at base 1.4-3.2 µm (Atanasova et al., 2010). The fungus are Mesophiles in nature (25°C to 30°C) and typical aerobic.

3.1.3 *Citrobacter farmeri* CTJ-S-1

Gram negative, bacilli, 1.0 x 2.0- 6.0 µm, found singly or in pairs, frequently motile, peritrichous flagella, usually not encapsulated, nonspore forming, oxidase negative, utilization of acetic acid positive, Simmon's citrate negative, indole production positive, ornithine decarboxylase positive, malonate negative, KCN growth positive, sucrose positive, gelatinase positive, urease positive, fermentation of melibiose positive, utilization of Tween80 and Tween40 negative, utilization of D-arabitol negative, utilization of sorbitol positive, methyl red positive, arginine dehydrolase positive, facultative anaerobes (Adams & Boopathy, 2005).

3.1.4 *Trichoderma viride* TVJ-S-1

On PDA medium colonies are fast growing, conidiation are compact tufts like or more effuse, dark bluish-green (Fig. 1 F), typically uncoloured on reverse side, distinctly aromatic odour as of coconut. Conidiophores having a relatively loose arrangement with less branching, most often paired or single or three verticillate, often appeating flexous, Phialides arising singly or paired or 3-verticillate or narrowly logeniform, size (8-14 X 2.4-3.0) µm. Conidal globose to ellipsoidal, often conspicuously warted, size (4.0-4.8 X 3.5-4.0) µm, bluish to dark green in colour (Rifai, 1969). This is also a mesophilic (25°C to 30°C) and typical aerobic fungi.

3.1.5 *Paecilomyces variotii* Isolate-1

Colonies of growing *Paecilomyces variotii* Isolate-1 rapidly growing on PDA medium and produced powdery to floccose, funiculose or tufted, yellow-brown or sand colored mycelium of the medium (fig 1.E). Conidiophores are verticillately arranged, branches with phialides up to 150 µm in length, 3.5-6.5 µm width. Phialides are cylindrical or ellipsoidal, tapering abruptly into a long, thin, cylindrical neck. Conidia of the fungi are subspherical, ellipsoidal to fusiform in shape and hyaline 3-5 x 2-4 µm, arising in long, divergent chains. Black pigment visible in reverse side of the culture (Samson 1974).

3.2 Molecular characterization of antagonists

Identification of the antagonists by using ribosomal DNA based partial sequencing tool clearly validated that the five microbial strains are member of potential microbial antagonist's community based on their nucleotide homology and phylogenetic analysis. The 16S rRNA gene sequence of the tested bacterial antagonist BTJ-S-1 was identified as *B. thuringiensis* which showed 100% sequence identity with 16S rRNA gene sequence of *B. thuringiensis* strains Et10/1 (Accession: KJ722441.1) and EAPL02 (Accession: JX500174.1). The other bacterial antagonist (CTJ-S-1) was diagnosed as *C. farmeri* where 100% sequence similarity was observed with 16S rDNA gene sequence of *C. farmeri*, strain: GTC 01319 (Accession: AB741662.1). Submission of 18S rDNA partial sequence segment of the fungal antagonist (Isolate-1) into NCBI BLASTn indicated that the isolate is *P. variotii* which exhibited 99 % sequence identity to 18S ribosomal RNA gene sequence of *P. variotii* strain KUC5015 (Accession: GQ241284.1) and isolate SUMS0303 (Accession: FJ011547.1). The BLAST search of 28S rDNA gene fragment of two tested fungal antagonists TPJ-S-1 and TVJ-S-1 in NCBI BLASTn indicated that the isolates are *T. parareesei* and *T. viride* respectively. TPJ-S-1 isolate showed 100% sequence similarity with *T. parareesei* strain ATCC MYA-4777 (Accession: JN874490.1) whereas 99% sequence identity was observed in tested fungal antagonist TVJ-S-1 with *T. viride* strain KSAP113 (Accession: GQ408917.1).
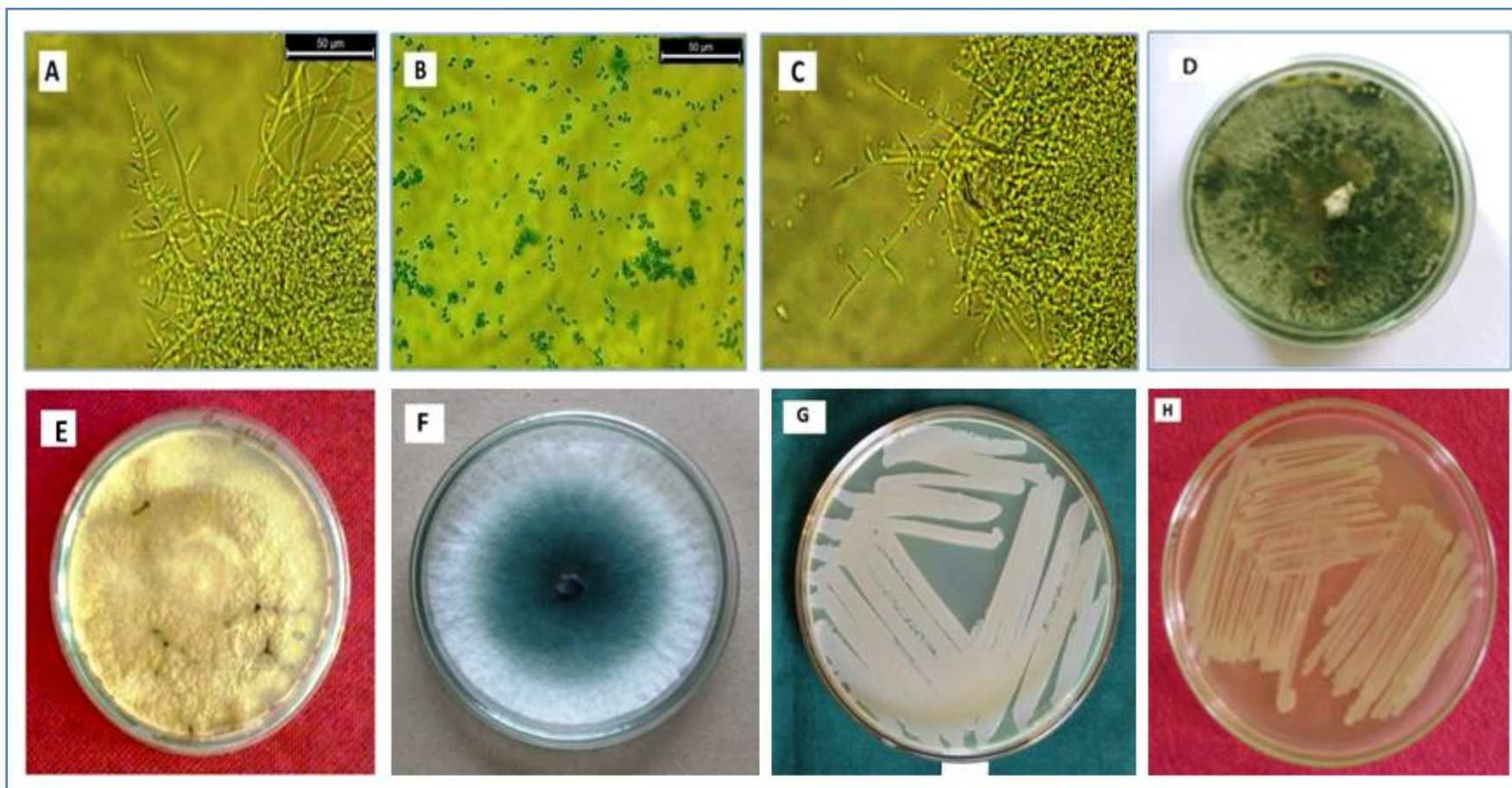
Figure 1 Micrograph and pure culture of tested antagonists. A: conidiophores/phialides of *T. parareesei* TPJ-S-1 with conidia at 48 hr; B: young conidia of *T. parareesei* TPJ-S-1 at 48 hr; D, E and F: pure culture of *T. parareesei* TPJ-S-1; *Paecilomyces variotii* Isolate-1 and *Trichoderma viride* TVJ-S-1 respectively on PDA medium after 7 days incubation; G and H: pure culture of *Bacillus thuringiensis* BTJ-S-1 and *Citrobacter farmeri* CTJ-S-1 respectively.
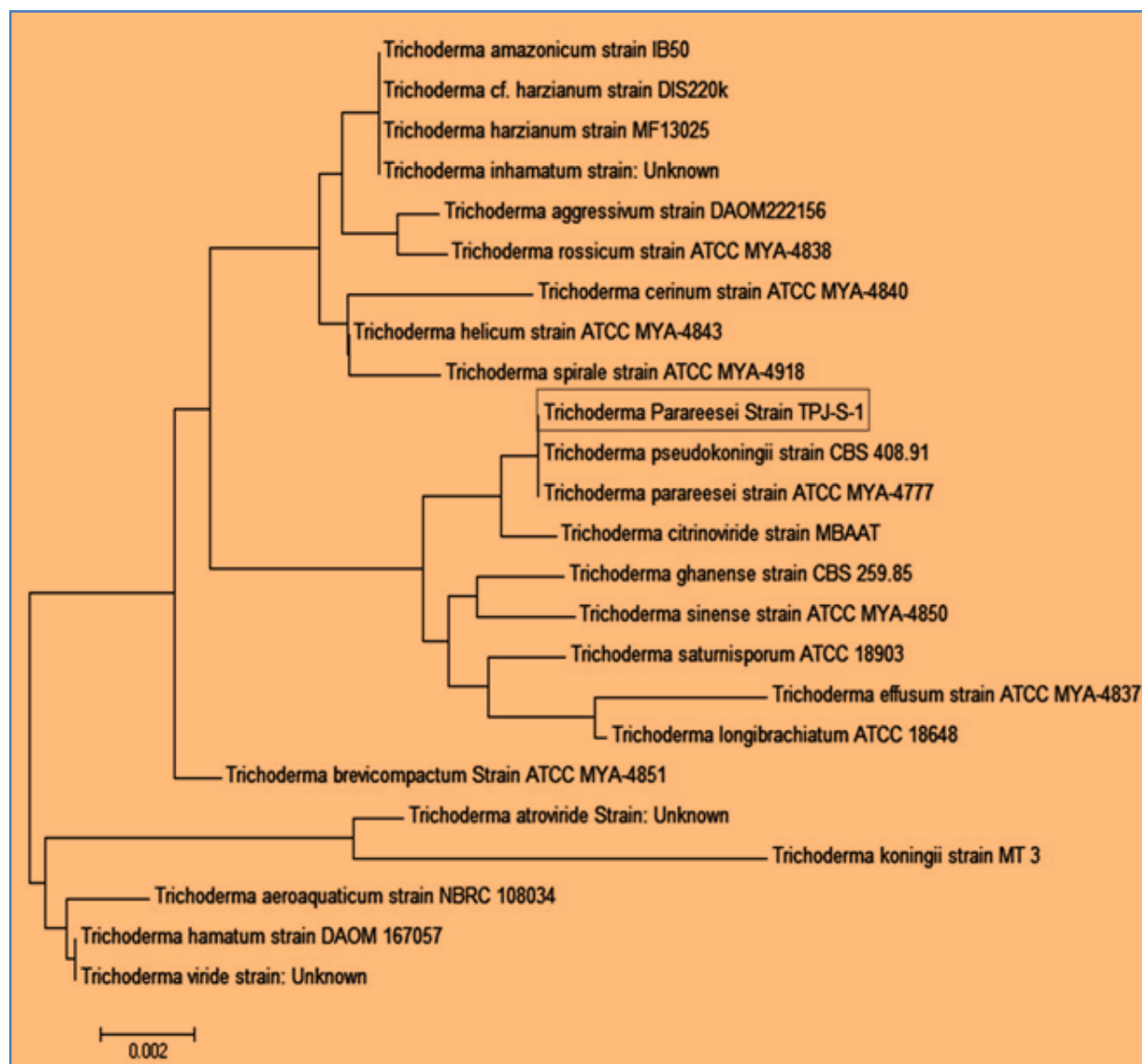
Figure 2 28S rDNA gene sequence based neighbor-joining phylogenetic tree illustrating genetic relationship of tested antagonist *T. parareesei* TPJ-S-1with few members of the genera *Trichoderma*.

3.3 Nucleotide sequences accession numbers

All the sequences have been deposited in GenBank database under accession no KF439054 (*Bacillus thuringiensis* BTJ-S-1), KF439053 (*Paecilomyces variotii* Isolate-1), KF439052 (*Trichoderma parareesei* TPJ-S-1), KF439055 (*Trichoderma viride* TVJ-S-1), KF439051 (*Citrobacter* sp.).

3.4 Multiple sequence alignments and phylogenetic analysis

Phylogenetic analysis of *T. parareesei* TPJ-S-1 and *B. thuringiensis* BTJ-S-1 at nucleotide level showed close relationship of these bioagents to their close antagonistic

relatives reported previously. This will help to understand the molecular basis of their antagonistic properties and to determine phylogenetic relationships with pathogenic species. 28S rDNA gene fragment based multiple sequence alignments and phylogenetic analysis of *T. parareesei* TPJ-S-1 yielded higher resolution discrimination between other closely related *Trichoderma* species. Phylogenetic tree analysis showed 100% identity of *T. parareesei* TPJ-S-1(Accession: KF439052) with *T. pseudokoningii* CBS 408.91(Accession: AF400740.1) and *T. parareesei* strain ATCC MYA-4777 and distance relationship with previously reported successful antagonist species *T. viride, T. koningii, T. harzianum etc* (Fig. 2). The tested antagonist *T. parareesei* TPJ-S-1 can be placed in same

cluster with *T. citrinoviride* strain MBAAT (Accession: KF380830.1), *T. ghanense* strain CBS 259.85 (Accession: AF399234.1), *T. sinense* strain ATCC MYA-4850 (Accession: KC171318.1), *T. saturnisporum* ATCC 18903 (Accession: AF399246.1), *T. effusum* strain ATCC MYA-4837 (Accession: KC171306.1). Whereas in 16S rDNA based neighbour-joining phylogenetic tree analysis, *B. thuringiensis* BTJ-S-1 showed close relationship with *B. thuringiensis* strain Et10/1, B. cereus strain Bactisubtil and formed a cluster with *B. muralis* strain LMG 20238, *B. simplex* strain LMG 11160, *B. psychrosaccharolyticus* strain unknown, *B. acidiceler* CBD 119, *B. isronensis* strain B3W22, *B. coahuilensis* strain m4-4, *B. mycoides* strain unknown (Fig. 3).
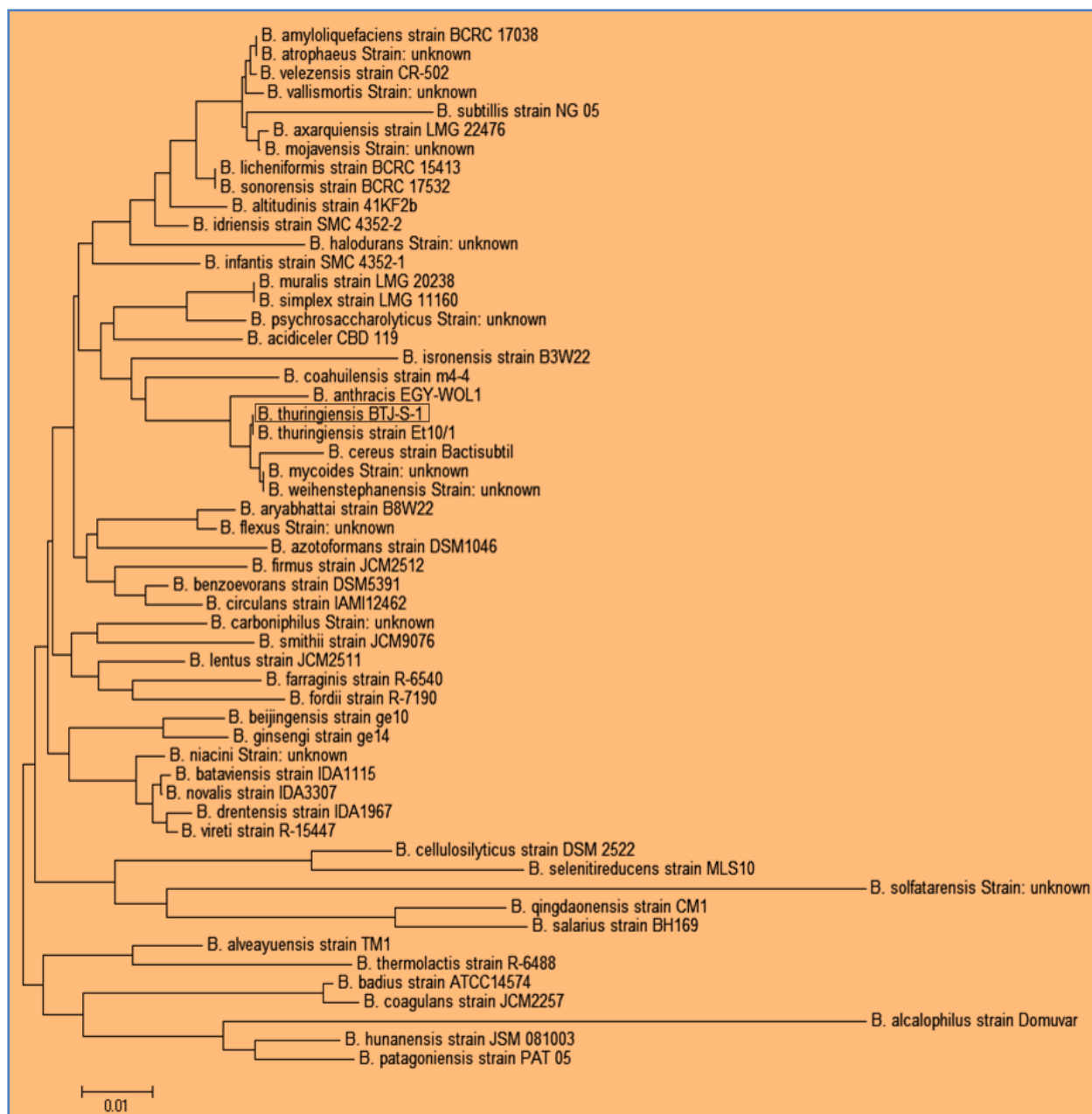

Figure 3 16S rDNA gene sequence based neighbour-joining phylogenetic tree illustrating genetic relationship of tested antagonist *Bacillus thuringiensis* BTJ-S-1 with few members of the genera *Bacillus*.

3.5 Inhibitions of *R. solanacearum* by antagonists and their consortia *in vitro*

The data representing inhibitions by the five antagonists and their consortia against *R. solanacearum* in TTC media were recorded and are presented in Table 1. The highest inhibition of *R. solanacearum* was recorded by consortia of *T. parareesei + T. viride + B. thuringensis* (91.47%) which was followed by the consortia of *T. parareesei + T.viride + P. variotii + B. thuringensis + C. farmeri* (82.22%); and consortia of *T. parareesei + T. viride + P. variotii* (79.24%). The inhibition zone produced by *C. farmeri* in all the treatment combination consortia was found to be less as compared to other antagonists. Based on the ability of the consortia to inhibit the growth of *R. solanacearum in vitro,* these four consortia were selected for further experimentations.

Table 1. Inhibition Zones produced by antagonists and their combinations against *R. solanacearum* dual cultured in TTC media.

Treatments	Inhibition Zone (mm)*	Inhibition of *R. solanacearum* (%)
T. parareesei + R. solanacearum	27.33	30.36
T. viride + R. solanacearum	41.50	46.11
P. variotii + R. solanacearum	25.00	27.77
B. thuringensis + R. solanacearum	22.33	24.81
C. farmeri + R. solanacearum	17.66	19.62
T. parareesei + T. viride + R. Solanacearum	64.00	71.11
T. parareesei + P. variotii + R. Solanacearum	62.00	68.88
T. parareesei + B. thuringensis + R. Solanacearum	47.34	52.60
T. parareesei + C. farmeri + R. Solanacearum	56.83	63.14
T. viride + P. variotii + R. Solanacearum	49.84	62.22
T. viride+ B. thuringensis + R. Solanacearum	50.83	56.47
T. viride+ C. farmeri + R. Solanacearum	48.00	53.33
P. variotii + B. thuringensis + R. solanacearum	42.16	46.84
P. variotii + C. farmeri + R. solanacearum	39.99	44.43
B. thuringensis + C. farmeri + R. Solanacearum	31.99	35.54
T. parareesei + T. viride + P. variotii + R. Solanacearum	71.32	79.24
T. parareesei + T. viride + B. thuringensis + R. Solanacearum	82.33	91.47
T. parareesei + T. viride + C. farmeri + R. solanacearum	67.83	75.36
T. parareesei + P. variotii + B. thuringensis + R. solanacearum	62.17	69.07
T. parareesei + P. variotii + C. farmeri + R. solanacearum	67.16	74.62
T. parareesei + B. thuringensis + C. farmeri + R. solanacearum	55.33	61.47
T. viride + P. variotii + B. thuringensis + R. solanacearum	57.16	63.51
T. viride + P. variotii + C. farmeri + R. solanacearum	56.49	62.76
T. viride + B. thuringensis + C. farmeri + R. solanacearum	44.33	49.25
P. variotii + B. thuringensis + C. farmeri +R. solanacearum	26.16	29.06
T. parareesei + T. viride + P. variotii + B. thuringensis + R. solanacearum	52.56	58.50
T. parareesei + T. viride + P. variotii + C. farmeri + R. solanacearum	67.98	75.53
T. parareesei + P. variotii + B. thuringensis + C. farmeri + R. solanacearum	48.49	53.87
T. viride + P. variotii + B. thuringensis + C. farmeri + R. solanacearum	48.32	53.68
T. parareesei + T. viride + P. variotii + B. thuringensis + C. farmeri + R.	74.00	82.22
Control (R. solanacearum alone)	0.00	0.00
SEd (±) **CD (P=0.05)**		2.63 5.24

3.6 Mass multiplication of the antagonists on various substrates

The mean population (64.55 x 10^7 cfu/g) of all the five antagonists in formulations of three substrates significantly increased up to 270 days of storage after which it showed declining trend (Table-2). The highest population of *T. viride* (112.43 X 10^7cfu/g) was recovered from vermicompost based formulation, which was followed by population of *T. parareesei* (108.83 X 10^7 cfu/g) after 270 days of storage. Among different antagonists, the recovery of *T. viride* was significantly higher in all the three substrates evaluated, while the recovery of *Citobacter farmeri* was significantly lower after different days of storage, the lowest recovery being (1.50 X 10^7 cfu/g) after 450 days of storage. Similarly, amongst different days of storage, least mean population of all antagonists (5.20 X 10^7 cfu/g) was recovered after 450 days of storage. On an average, vermicompost among the substrates appeared to be the best nutrient source to support the antagonists for maximum multiplication and subsequently better disease reduction. Earlier, Suslow & Schroth (1982) reported that bacterial antagonists like *P. fluorescens* could survive 7 months to 1 year with a higher population level when it was incorporated into carrier materials *viz*., talc or peat along with CMC. The higher population of bacterial antagonists like *B. thuringiensis* and *C. farmeri* might be due to high nutrient content of vermicompost, which is a good source of humus, Vitamin-B, auxin and antibiotics. Moreover, it contains 2.5-3.5 per cent nitrogen, 1.5-2.0 per cent phosphorous and 2.0-3.5 per cent potassium (Kohli et al., 1988). Vermicompost causes a shift of pH towards neutral, a reduction in electrical conductivity, and as most of the antagonists which prefers neutral to alkaline pH tends to exhibit higher population shift in vermicompost (Alexander, 1997). CMC was used in the formulation as an adhesive, which might have also played a role of preservative for the long-term viability of the antagonist. Moreover, mannitol used as osmoticant, has the ability to protect the antagonist from desiccation and thereby increases their survival (Vidhyasekaran & Muthamilan, 1995). The fungal antagonist *T. viride, T. parareesei* and *P. variotti* also higher population count when vermicompost was used as its substrate, where these might have congenially favored by nutrients for better sporulation and production of colony forming units. Other attributes of fungal antagonists like, production of enzymes like β-1-3 glucanase and chitinase might have higher in vermicompost and thus have helped the fungi during utilization of cellulose and chitin present in different substrates (Hadar et al., 1979). *Trichoderma* spp multiply faster at higher concentration of CO_2, a condition favoured by different substrates. Similarly, their multiplication might also been favored by humic acid present in the substrates (Ushasree et al., 1989).

3.7 Efficacy of the substrate based formulations of antagonists and their consortia in reduction of bacterial wilt incidence of *Naga chilli*

The formulations of antagonist consortia which showed comparatively better *in vitro* inhibition of the wilt pathogen *R. solanacearum viz.,* consortia of *T. parareesei + T. viride + B. thuringiensis; T. parareesei + T.viride + P. variotii + B. thuringiensis + C. farmeri*; *T. parareesei + P. variotii + B. thuringiensis* and *T. parareesei + T. viride + P. variotii* were further evaluated for their ability to suppress wilt disease in *Naga chilli* under field condition by seed, root and soil treatment/application methods. A mixture of COC (0.2%) and Streptocycline (0.1%) was also applied similarly for comparison. The data representing disease suppression (%) and yield (kg/plant) are presented in Table 3.

All the antagonist consortia as well as mixture of COC and Streptocycline were significantly effective in lowering the wilt incidence of *Naga chilli* (Table 3). However, a consortium of *T. parareesei + T.viride + P. variotii + B. thuringiensis + C. farmeri* was significantly most effective in reducing disease incidence. Data also depicted that the mixture of COC and Streptocycline was least effective in reducing disease incidence.

Better disease reduction in tomato caused by *R. solanacearum* was earlier recorded by using an antagonistic bacterium like *P. fluorescens* as seed and seedling inoculation, and the antagonistic ability and subsequent reduction of plant disease was attributed to its ability to produce siderophores (Aspiras & Della, 1985), antibiotics and rapid colonization (Kloepper & Schroth, 1981). Fungal antagonists like *T. viride* along with vermicompost as substrate, was reported to be highly effective in reduction of disease incidence in ginger (Bora et al., 2013). Vermicompost might have helped the fungal antagonists like *T. viride, T. parareesei* and *P. variotti* for better sporulation, production of colony forming units and subsequent higher reduction of wilt incidence in *Naga chilli*. Furthermore, Danielson & Davey (1973) reported that *Trichoderma* spp multiply faster at higher concentration of CO_2 and substrates like vermicompost containing high carbon. From above discussion, it is evident that substrate like vermicompost enhances the activity of antagonists, which complete with the soil borne plant pathogen for nutrient and space.

The addition of antagonists along with different substrates might have influenced of soil organic carbon. Earlier, Hoitink & Fahy (1986) tried to establish positive correlation between C:N ratio of residues of organic carbon and disease severity. Similarly, an increase in the available phosphorus content of soil has been reported to be effective in suppressing the disease incidence. The substrates particularly vermicompost increases available phosphorus content in the soil in contrast to the other treatment, which could provide maximum protection to the plants from the disease. The increased availability of phosphorus and potash in soil might have contributed towards the resistance of the plants to the diseases as have been recorded in many other pathogens (Sharif Hossain et al., 2003).

Table 2 Population of *Trichoderma parareesei, Trichoderma viride, Paecilomyces variotii, Bacillus thuringensis* and *Citrobacter farmeri* assayed from substrate based bioformulations after different days of storage.

Treatments	Population of antagonists (x 10^7 cfu/g) after different days of storage[*]					
	90	180	270	360	450	Mean
Vermicompost (VC) + *T. parareesei*	62.83 (1.80)	91.17 (1.96)	108.83 (2.04)	71.50 (1.85)	7.50 (0.87)	68.37 (1.83)
Talc powder (TC) + *T. parareesei*	44.55 (1.65)	67.50 (1.81)	79.66 (1.66)	75.27 (1.87)	22.15 (1.33)	57.83 (1.76)
Mustard oil cake (MOC) + *T. parareesei*	40.50 (1.61)	48.50 (1.68)	59.33 (1.77)	65.33 (1.81)	58.00 (1.76)	54.33 (1.73)
VC + *T. viride*	64.33 (1.80)	90.50 (1.96)	112.43(2.05)	86.10 (1.93)	16.48 (1.21)	73.97 (1.87)
TC + *T. viride*	43.50 (1.64)	59.50 (1.77)	71.17 (1.85)	39.06 (1.59)	14.50 (0.87)	45.55 (1.65)
MOC + *T. viride*	27.33 (1.44)	43.00 (1.63)	61.66 (1.79)	36.50 (1.56)	24.33 (1.39)	38.56 (1.59)
VC + *P. variotii*	34.50 (1.54)	60.00 (1.77)	72.88 (1.86)	57.87 (1.76)	7.45 (0.87)	46.54 (1.66)
TC + *P. variotii*	25.99 (1.41)	49.50 (1.69)	51.50 (1.71)	33.10 (1.52)	1.50 (0.17)	32.32 (1.51)
MOC + *P. variotii.*	15.66 (1.19)	45.66 (1.66)	47.33 (1.67)	20.66 (1.31)	0.83 (0.08)	26.03 (1.41)
VC + *B. thuringensis*	16.50 (1.22)	26.50 (1.42)	27.89 (1.44)	22.17 (1.35)	5.30 (0.72)	19.67 (1.29)
TC + *B. thuringensis*	11.27 (1.05)	22.40 (1.35)	38.17 (1.58)	25.50 (1.40)	11.50 (1.06)	21.77 (1.34)
MOC + *B. thuringensis*	13.50 (1.13)	21.66 (1.33)	32.33 (1.51)	18.33 (1.26)	6.00 (0.78)	18.36 (1.26)
VC + *C. farmeri*	21.27 (1.33)	52.40 (1.72)	48.17 (1.68)	35.50 (1.55)	1.55 (0.19)	31.78 (1.50)
TC + *C. farmeri*	15.50 (1.19)	16.50 (1.22)	34.80 (1.54)	9.30 (0.96)	1.50 (0.17)	15.52 (1.19)
MOC + *C. farmeri.*	12.66 (1.10)	15.33 (1.18)	31.00 (1.49)	5.50 (0.74)	1.50 (0.18)	13.20 (1.12)
	29.99 (1.48)	**45.57 (1.66)**	**58.48 (1.77)**	**40.11 (1.60)**	**12.01 (1.08)**	

S.Ed. ± = For treatment = 0.022, for days = 0.016, for treatment x days = 0.042; CD (P=0.05) = For treatment = 0.044, for days = 0.032, for treatment x days = 0.084; *Figures in the parentheses are logarithm-transformed values.

Table 3 Efficacy of substrate based bioformulations of antagonists and their consortia on reduction of bacterial wilt incidence and yield of *Naga chilli.*

Treatment	Disease incidence (%)*	Disease reduction (%)	Yield (qt/ha)
Consortia of *T. parareesei, T. viride,* and *P. variotii*	63.20 (53.72)	26.16	30.80
Consortia of *T. parareesei, T. viride* and *B. thuringiensis*	49.6 (45.10)	42.05	58.80
Consortia of *T. parareesei, P. variotii* and *B. thuringiensis*	31.8 (34.36)	62.85	74.20
Consortia of *T. parareesei, T.viride, P. variotii, B. thuringiensis* and *C. farmeri*	13.8 (19.20)	83.87	96.80
COC + Streptocycline	57.60 (50.96)	21.02	17.06
Control	85.60 (68.81)		3.70
S.ed (±)	6.82		3.65
CD (P=0.05)	11.68		6.24

*Figures in the parentheses are angular transformed values.

3.8 Efficacy of the substrate based formulations of antagonists and their consortia on yield enhancement of *Naga chilli* (t/ha)

The yield of the *Naga chilli* significantly increased when plants were treated with *T. parareesei, T.viride, P. variotii, B. thuringiensis* and *C. farmeri* based consortia bioformulation, following different methods (Table 3).

Successful biological control agents *T. viride and T. parareesei* have the ability to compete with other members of the soil microflora and also to produce antibiotics or induce a response in the host that favors the growth of the plant beneficial microbes while inhibiting the growth of the pathogen like *R.*

solanacearum. Simultaneous application of two or more compatible antagonists led to their higher population densities in the rhizosphere and the density dependent mechanism might have prevailed by rapid rhizosphere colonization, and thereby led to rhizosphere niche exclusion in terms of space and nutrients for the pathogen and ultimately reduction of wilt incidence and corresponding enhancement of crop yields. Such mechanisms have been demonstrated by Bull (1987), and explained that the wheat take-all disease control and enhancement of crop yield by biological means was directly related to the rapid root colonization by antagonist like *P. fluorescens.* Moreover, the efficiency of biocontrol agents with organic substrates is depended on method of introduction, rate

of inoculum density of the bioagents applied against the pathogens (Papavizas, 1985). Parakhia & Vaishnav (1986) observed that seed treatment with wheat husk-bran culture of *T. harzianum* followed by soil drenching could successfully control *R. bataticola* infection in chick pea and increase crop yield. Similarly, Vidya (1995) used combination of talc-based formulation of *T. harzianum* + vermiculite-wheat bran formulation for soil application and effectively managed *M. phaseolina* root rot disease of mung bean resulting higher crop yield. Biological control of plant diseases by using plant associated microorganisms is an eco friendly approach (Bargabus et al., 2003; Tjamos et al., 2005). Mandal et al. (2013) reported that Gram-negative genera *Citrobacter* and *Enterobacter* belonging to the family *Enterobacteriaceae* could produce multiple antimicrobial lipopeptides like fengycin analogues and isomer of iturins, fengycins, kurstakins surfactins etc active against *P. aeruginosa*, *Micrococcus luteus and Staphylococcus aureus.* Several antagonistic bacterial species from the family *Enterobacteriaceae* have been reported to suppress root pathogens of various plants. For example root associated bacteria *Citrobacter freundii and Enterobacter cloacae* have been found to reduce root associated diseases in lemon plants (Lima et al., 1994; Araújo et al., 2001). Xue et al. (2009) reported that, *Enterobacter* sp. (family *Enterobacteriaceae*) could show good bio control potential against bacterial wilt pathogen *R. solanacearum* with a control efficacy up to 65% under field condition. They also mentioned that this antagonist bacteria possessed plant growth promoting properties and could enhance plant biomass by 23.0%.

The naturally abundant Gram-positive bacteria species of the genus *Bacillus* are known to produce large number of antimicrobial lipopeptides (Nybroe & Sørensen, 2004; Ongena & Jacques, 2008). The cyclic lipopeptide biosurfactants like iturin, mycosubtilin, surfactin and kurstakin produced by species of *Bacillus* have strong antimicrobial activity (Rodrigues et al., 2006; Raaijmakers et al., 2010). The well-known bio-insecticide *B. thuringiensis* (Roh et al., 2007) recently, attracted considerable attention as a potential biological control agent for the suppression of plant diseases. This species can be characterized by the production of crystal proteins (d-endotoxins) with a specific activity against insect pests, nematodes, mites and protozoa (Beegle & Yamamoto 1992; Feitelson et al., 1992). Besides this, they also produces a number of extracellular compounds including phospholipases, chitinases, proteases (Lovgren et al., 1990), β exotoxins, and antibiotic compounds with antifungal activity (Stabb et al., 1994). Most recently Hyakumachi et al. (2013) reported that, root treatment of tomato plants with *B. thuringiensis* culture could considerably reduce the bacterial wilt (c.o. *Ralstonia solanacearum*) of tomato to less than one third of the control. They attributed this phenomenon to systemic activation of plant defence system and expression of defence-related genes such as PR-1, acidic chitinase, and β-1, 3 glucanase in stem and leaf tissues. Similar report also made by Takahashi et al. (2014) where they mentioned that SA-dependent signalling pathway and suppression of JA-dependent signalling pathway

seem to play key roles in inducing resistance in tomato plants against *R. Solanacearum.*

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