Evaluation of *Trichoderma asperellum* biofortified with vermi compost against *Fusarium oxysporum* f.sp. lycopersici

Nikhil Raman. K, Vipul Kumar*, Prahlad Masurkar and Angel Jemima

ABSTRACT

Morphological features are not adequate to accurately categorize different species of the genus *Trichoderma*. Molecular characters, in combination with morphological characters, are used to identify *Trichoderma* at the species or subspecies level. The present study was focused on characterising *Trichoderma asperellum* based on morphology and molecular analysis using genes such as ITS. Light microscopy results showed that *Trichoderma asperellum* regularly branched and typically paired conidiophores with straight phialides and globose to subglobose shaped conidia Sequence similarity analysis with reference *T. asperellum* isolates available in the NCBI database showed 100 percent nucleotide similarity for ITS4 and ITS5. A dual culture test clearly showed that *Trichoderma asperllum* inhibited the tested fungal plant pathogen, *Fusarium oxysporum* f. sp. *lycopersici* (87.38 percent). Population dynamics of *Trichoderma asperellum* in compost were found to be stable until 21 days after mixing.

Keywords: *Trichoderma asperellum, Fusarium oxysporum* f.sp*lycopersici,* Light microscopy population dynamics

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INTRODUCTION

Certain species of genus *Trichoderma* have several beneficial characters and used as a potent producer of enzymes (Kumar *et al.,* 2008; Seiboth *et al*., 2012), growth promoter (Harman *et a*l., 2011) and as well as a potential antagonist of phytopathogens (Marra *et al.,* 2019; Rivera Mendej *et al* 2020, Xiao *et al.,*2023). Simultaneously, some species have been reported as human pathogen (Hatvani*et al.,* 2013) or pathogen of green mould disease in mushrooms (Komoń-Zelazowska*et al.,* 2007, Šašić Zorić *et al.,* 2023), Owing to hybridization (Olson, Å et al., 2002; Hughes *et al.,* 2013), cryptic speciation (M. J. *et al.,* 2013, Lücking, R *et al.,* 2014), convergent evolution (Brun, *et al.,* 2010), complexity in taxonomy, biological restrictions (Susila *et al.,* 2023) other multiple reasons (Summerbell, 2003; Hermosa *et al.,* 2004), morphological based identification alone is challenging and inadequate at the species level (Druzhinina *et al*. 2010; Jaklitsch *et al*., 2013). Therefore, correct identification of species should be the first step and molecular methods must be combined with parameters for correct identification for phylogenetic comparisons based on target sequences (Bissett *et al*., 2015). To investigate genetic diversity within the genus, several molecular methods have been used (Hassan *et al.,* 2019) which are based on DNA sequence analysis. These methods are now routinely used in species identification. Among the molecular methods of identification, sequence analysis of ITS region is one of the most robust methods which exhibited the highest probability of correct identification (Schoch *et al.,* 2012, Susila *et al.,* 2023). Therefore, it is now possible to identify each isolate of *Trichoderma* at species level (Druzhinina *et al.,* 2015) by combined use of morphological and molecular methods.

Tomato is one of the most agriculturally important vegetable which is consumed by humans in various ways (Abdel Karim *et al.,* 2006). Many soil borne plant pathogens cause diseases and reduce economic yield in tomatoes (Babalola and Glick, 2012). Among the Plant pathogens, *Fusariumoxysporum*

f.sp. *lycopersici* is one of the most devastating and challenging pathogens in the terms of management (Pasco *et al.,* 2017, El-Aswad *et al.,*2023). Numerous strategies of plant diseases management are developed such as resistant varieties, agronomical practices and fungicide control (Abo-Elyousr *et al.,* 2009). Fungicides have been found to be an effective management strategy, but at the same time it has proven health hazards (Maitlo *et al.,* 2014). Resistant variety is another successful method for management of *Fusarium* wilt (Amini, 2009), but new races of the pathogen appear to overcome resistance genes in currently grown cultivars (Lücking *et al.,* 2014). Therefore, the aim of the present research work was to molecular identification of *Trichoderma* isolate and assessing the antifungal potential of *Trichoderma asperellum*, the effect of vermicompost on its growth and bio control activity.

MATERIALS AND METHODS

Microbial cultures

Three biocontrol agents and one pathogen namely, *T. asperellum, Bacillus subtilis, Pseudomonas aeruginosa* and *Fusarium oxysporum* f. sp *lycopersici* were tested in the current study at Trichy Research Institute of Biotechnology, Tiruchirapalli, Tamil Nadu, India.Out of three, *Bacillus subtilis* and *Pseudomonas aeruginosa* are provided by the research laboratory of Trichy Research Institute whereas *T. asperellum* was isolated from rhizospheric soil.

Isolation of *Trichoderma*

Rhizospheric soil samples were collected from the ecological habitat of coconut plant from two different villages (Trichy and Kulumani) of district Tiruchiraipalli, south India, for isolation of *Trichoderma* spp. Soil samples were brought in the laboratory and kept in freeze at 4^0C until used. Soil samples were serially diluted in sterilized distilled water till 10^{-5} and 100µl diluted samples were uniformly spread on *Trichoderma* selective Medium (TSM) poured Petri plate (Elad *et al.,* 1982). Petri plates were kept in the BOD incubator at 28 ± 2^0C temperature for five days. After incubation for five days, morphologically different fungal colonies appeared on the TSM plates, which were pulled out by sterilized needle and purified by single spore isolation technique (Wah Cho *et al.,* 1999) on potato dextrose agar medium (PDA).The purified *Trichoderma* isolate was preserved at 4^0C for further studies .

Morphological identification of *Trichoderma* isolate

For morphological identification of *Trichoderma* species, certain characters were studied such as colony color, chlamydo spores formation, conidiophores, phialides characters, and shape of the conidia (Gams and Bisset, 1998).

Molecular identification:

Fungal Growth Conditions and Extraction of DNA: The *Trichoderma* isolate was grown on potato dextrose agar medium (PDA) wrapped with parafilm tape on 28^0C for 96 hours. Two bits of seven days old culture were inoculated in potato dextrose broth (PDB) and kept in the BOD incubator for 3 days. Mycelia mat was collected on filter paper, washed with millipore water for 2–3 times and homogenized in liquid nitrogen for DNA extraction. Genomic DNA was extracted using the method of Raeder et al., 1985 with slight modifications. DNA resuspended in 50µl of TE buffer and quantified by using dye ethidium bromide fluorescence.

Quantification of DNA

DNA concentration and purity was quantified through UV spectrophotometer using the optical density (OD) at 260 and 280 nm absorbance values.

PCR amplification and Sequencing of Amplification Product: PCR amplification of ITS4 and ITS5 internal transcribed spacer regions generated from the total genomic DNA extractions were done by the methods described by White *et al.* (1990), Guigón-López *et al.,* (2010). The thermo cycling program was used: 1 cycle at 94 °C for 2 min, 30 cycles at 94 °C for 30 s, 30 cycles at 50 °C cycles for 45s, 30 cycles at 72 °C for 1 min and a final 72 °C cycle for 15 min. PCR products were evaluated on 2% agarose gel at 100 V for 30 minutes

DNA Sequencing

Template DNA (50 µLl) was directly prepared from PCR-products by purifying it with PCR Purification Kit. Purified PCR products (30 µl) were sent to GCC Biotech New Delhi India for the direct sequencing. DNA sequence for the internal transcribed spacer region (ITS) of the rDNA was obtained. After

collecting these query sequences, they were submitted to the NCBI GenBank for their accession number

Phylogenetic analysis

The Basic Local Alignment Search Tool (BLASTN) was used to find the regions of local similarities between *Trichoderma* nucleotide sequences obtained from NCBI, anopen database resource providing genome sequences of various organisms. Phylogenetic tree was constructed using the FASTA format of various *Trichoderma* species. Construction of the Phylogenetic tree was prepared by using Mega 5.2 software.

Pathogen

The fungal plant pathogen *F. oxysporum* f. sp. *lycopersici* causing vascular wilt in tomatoes was isolated from a naturally infected plant of Tamil nadu agriculture farm India by using standard isolation technique with some modifications (Riker and Riker, 1936). Pathogens were identified macroscopically and microscopically (Leslie and Summerell, 2006). The pathogen was tested for pathogenicity (Venkatesh *et al.,* 2013). Pathogen was grown and maintained on a PDA medium for further experiments.

Antagonistic activity *Trichoderma* against *F. oxysporum* f.sp. *lycopersici*

Antagonistic activity of *Trichoderma asperellum* was tested against soil borne pathogenic fungi *F. oxysporum* f. sp*lycopersici* (FOL) by using dual culture technique proposed by (Yassin *et al.,* 2021), which was the part of microbial collection in this research study. Five mm discs of seven day's old *Trichoderma* culture were kept one cm away from the margin on the PDA Petri plate and the same size of disc of pathogen was placed at the reverse side of *Trichoderma* isolate. Bacterial isolates were streaked at the opposite side of the test pathogen around 40mm away from the centre followed by (Islam *et al.,* 2018). A medium inoculated only with the same size of pathogen disc, served as control. The experiment was conducted in three replications and inoculated Petri plates were kept in a BOD incubator at 28 ± 2^0C for seven days. The percentage inhibition of radial growth was determined according to Ezziyyani *et al.* (2004) by using the following formula:

I = (C-T)/C X100,

Where C is mycelial growth in control plate, T is mycelial growth of test organisms in inoculated plate and I is inhibition of mycelial growth. Preparation of formulation: Identified *T. asperellum* was grown in Potato dextrose broth (PDB) medium for seven days and culture mixed with sterilized talc powder in the ratio of 1:2 and dried to 8% moisture under shade condition. Tomato seeds were treated at the rate of @3-4gm/ kg seed.

Microbial population assessment in vermi compost fortified with Bio-inoculants

The sterilized vermi compost was inoculated separately with three bio inoculants such as *B. subtilis, P. aeruginosa, T. asperellum.* Twenty five kg vermi compost were measured and filled in sterilized tray and mixed with 1L of 2 days old pure culture of *T. asperellum* broth (CFU count approximately 1.8×10^6). Another, 25 kg vermi compost were taken in each two trays separately and mixed *Bacillus subtilis* and *Pseudomonas aeruginosa* respectively. After mixing, these trays were covered with white polythene sheet and incubated under shady conditions for 15 days. Microbial population was assessed from the fortified compost on 7th and 14^{th} day by using serial dilution technique. 1g of sample was taken from each tray and diluted till 10^{-6} and transferred on Potato dextrose medium (PDA). The total colony count of each biocontrol agent were assessed by using the following formula given below and designated as CFU/gm of dry weight (Subashini *et al.,* 2021).

Colony Forming Units (CFU) is = No of colonies X Dilution factor/Volume of taken sample

In vitro evaluation to check efficacy of biofortified vermincompost against *F. oxysporum* f.sp. *lycopersici*

Effect of fortified vermicompost against *F. oxysporum* f.sp. *lycopersici* were accessed by using agar well diffusion given by (Perez *et al.,*1990) in which 6 wells are made in PDA containing 9mm agar plate four wells are considered for taking the readings for vermicompost and plant extracts which are in the order from higher concentration to lower *viz.,* 500µl, 250µl, 100µl, and 50µl respectively, In remaining two wells one is negative control where nothing is used there and remaining well is

considered for Antibiotic control (PC) where 500µl of amphotericin is used. For 500µl of fortified vermi compost, mixture preparation add 5ml of DMS to 0.05 grams of fortified vermicompost .after inserting this in all the six wells allow to settle down in wells for 30 min inside laminar chamber and incubated at 27±2ºc for 3 days.

Results

Isolation and morphological Characterization

Trichoderma isolate was collected from coconut rhizospheric soils of district Tamil nadu, South India. The isolate was grown on Potato dextrose medium (PDA) for observation of morphological characters and colony color. The mycelia of isolated *Trichoderma* appeared as light greenish. The morphology appears as coarse and light to dark green colour mycelia and preliminary observation confirms it as *Trichoderma*. However, morphological characteristics were insufficient to confirm *Trichoderma* isolate at species level. Therefore, molecular identification was needed to differentiate the complex and overlapping.

Trichoderma isolate. *T. asperellum* mycelia were white and dark green, and arranged in concentric rings, each of either white or dark green colour when grown for three days in PDA media (Fig. 1). Conidia were sub globose to globose or ovoidal to ellipsoid, and some ovoid conidia, ampliform phialides, were described (Samuels *et al.,* 2010). Most of the conidiophores of *T. asperellum* were symmetrically paired along the main branches and axis. Phialides were characteristically elongate divergent lageniform and can be ampuliform in dense area in shapes. The terminal phialides were usually more elongated than another side of phialides

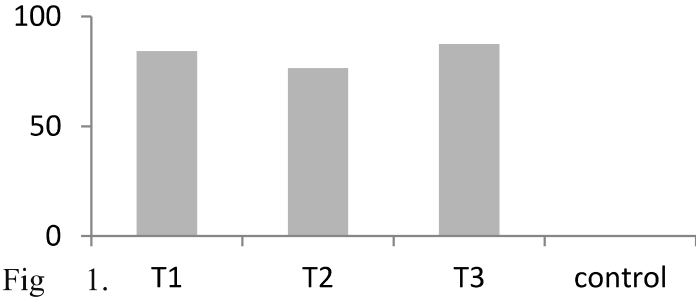


Fig 1. Antagonistic effect of bio control agents *Fusarium oxysporum* f.sp. *lycopersici*

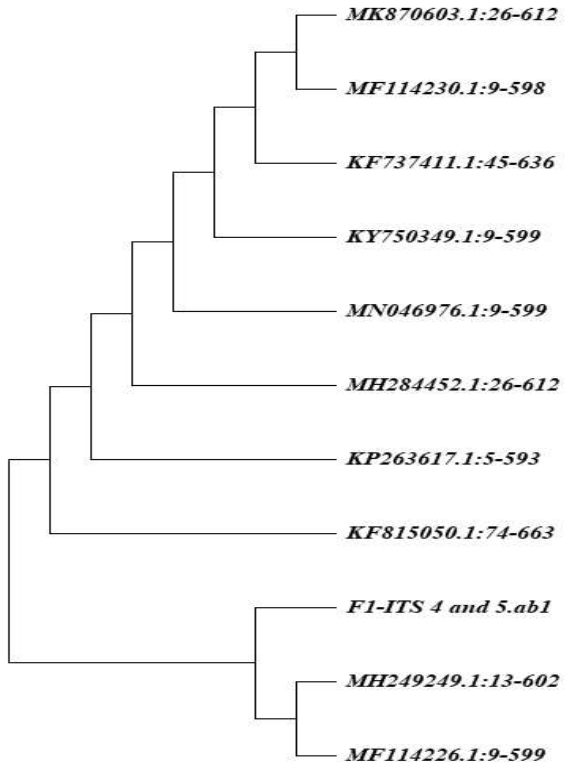
Molecular characterization of *T. asperellum*

The isolated genomic DNA after analyses of ITS regions in nucleotide PCR amplification at ITS4 and ITS5 region resulted in a band size of 650 bp.

Sequence analyses of 5.8S-ITS region

Pure genomic DNA of *Trichoderma* isolate was isolated and used for nucleotide sequencing. The ITS-rDNA partial gene was successfully amplified using universal primers ITS 4 and ITS 5 and resulted in a band size of 650 bp. The sequences obtained were subjected to BLAST analysis to identify the new isolate and were submitted to the National Center for Biotechnology Information (NCBI) GenBank The tested fungal stain showed 100% sequence similarity homology with *T. asperellum* and clustered with similar groups. The results of phylogenetic analysis of *T. asperellum* is given in Fig 2.

Fig 2. Phylogenetic analysis



Antagonistic activity of bio control agents

For *in vitro* antagonism assay, three bio control agents were tested against *Fusarium*. Percentages of mycelial growth inhibition of bio control agents were analyzed by analysis of statistical variance, with significant variation ($p \leq 0.001$), when paired were against *F. oxysporum* f.sp *lycopersici,* 76.50 to 87.38

of inhibition. Among the three microbial bio agents, *T. asperellum* was found superior which showed 87.38 % mycelium inhibition. The second highest antagonistic activity was observed 84.24% in *B. subtilis* followed by *P. aeruginosa* (76.50%). *T. asperellum* showed significant suppression of mycelium wth of *F. oxysporum.*

The CFU count was higher after a few days of application, while it was decreasing further. This may have occurred because after the digestion of organic material, vermin compost was formed, providing a large quantity of material to decompose and a large surface area for microbes to adhere to the substrate. In this study total microbial dynamics of all three biocontrol agents were estimated and shown in Figure 3.

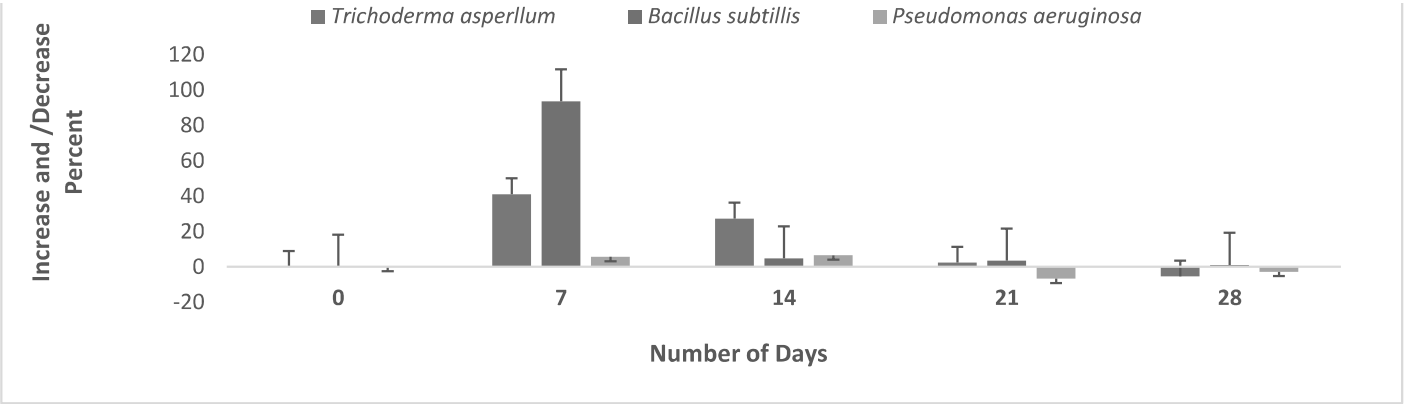


Fig 3. Population dynamics of *T. asperllum, B. subtilis* and *P. aeruginosa* in different days from 0 to 28-days

After fortification of vermicompost with fungal bioinoculants, *T. asperllum* on the first day, CFU values were recorded (3.24 x 10^6 cfu/g) and after 14 days, they were recorded 5.82 x 10^6cfu/g) Similarly, the microbial population of *B. subtilis,* (3.75 x 10^6cfu/g)was recorded, whereas the *P. aeruginosa* population was recorded at 1.96 (1.96x10^6cfu/g). Vermicompost is rich in organic matter and beneficial microorganisms, including bacteria, fungi, and actinomycetes. When vermi compost is applied to a new environment or substrate, the initial microbial colonization occurs rapidly because. Microbes present in the vermi compost start adapting to the new conditions and uptake nutrients this abundance of nutrients and proliferate, resulting in an increase in CFU count. After the initial colonization and proliferation, a process known as microbial succession takes place. Different microbial species compete for resources, and some species may outcompete others, leading to a decline in their CFU count. As the microbial community establishes a dynamic equilibrium, certain species become dominant and may suppress the growth of others, resulting in a decrease in overall CFU count.

A comparative investigation was conducted to assess the inhibitory effects of two distinct concentrations, namely 500 µg/ml and 400 µg/ml, of fortified vermincompost using the plate zone of inhibition technique. It was observed that the concentration of 500 µg/ml exhibited the highest level of inhibition in terms of mycelial growth of the pathogenic organism to the new conditions and uptake nutrients this abundance of nutrients and proliferate, resulting in an increase in CFU count. After the initial colonization and proliferation, a process known as microbial succession takes place. Different microbial species compete for resources, and some species may outcompete others, leading to a decline in their CFU count. As the microbial community establishes a dynamic equilibrium, certain species become dominant and may suppress the growth of others, resulting in a decrease in overall CFU count. A comparative investigation was conducted to assess the inhibitory effects of two distinct concentrations, namely 500 µg/ml and 400 µg/ml, of fortified vermincompost using the plate zone of inhibition technique. It was observed that the concentration of 500 µg/ml exhibited the highest level of inhibition in terms of mycelial growth of the pathogenic organism.

Characterization based on ITS region

Sequence analyses of the ITS region in nucleotide PCR amplification at the ITS4 and ITS5 regions produced 650bp size bands. ITS region is a commonly used target for DNA sequencing and molecular identification in many organisms, including fungi. It lies between the highly conserved rRNA genes (18S, 5.8S, and 28S) and exhibits considerable sequence variation, making it suitable for species-level identification and phylogenetic analyses. The ITS4 and ITS5 primers are well-established and widely used in PCR amplification of the ITS region. The length of the amplified product can vary among different species due to differences in the sequence composition and size of the ITS region. The ITS region is known to exhibit size polymorphism, with variations in the number of repetitive elements, insertions, and deletions. Therefore, observing a 650-bp size band suggests that the target species or organisms being analyzed possess an ITS region with a length of approximately 650 base pairs. Blast homology revealed that following *T. asperellum* MH249249.1:13602, KF815050.1:74-663, MF114226.1:9-599, KF815, KF737411.1:45-636, MF1144226:1:9-599, KP263617.1:5-593, KF737411.1:45-636, MK870603.1:26612, (MN046976.1:9-599, MH284452.1:26-612,MF114230.1:9-59 showed 100 percent similarity and was confirmed as *Trichoderma asperellum.*

DISCUSSION

Biocontrol agents are an alternative to chemical fungicides needed to control the vascular wilt pathogen, *F. oxysporum* f. sp. *lycopersici* (FOL), which is a major limiting factor of tomato production worldwide (Larkin and Fravel, 1998).

In a dual culture test, *Trichoderma asperellum* showed antagonistic activity against the tested pathogen. In this research, the percentage of inhibition was recorded at 87.38%, which is the highest among all the tested biocontrol agents. An important advantage of an antagonist is its ability to grow rapidly and compete with pathogens for space and food (Benítez *et al.*, 2004). These results are in agreement with numerous previous studies where *T. asperellum* isolates showed high capabilities for being versatile biocontrol agents (Cotxarrera *et al.*, 2002; Gromovykh *et al.,* 2002; de los SantosVillalobos et al., 2013). The similar results are in agreement with many other works that indicate that *T. asperellum* is a successful biocontrol agent against *F. oxysporum*. f. sp. *lycopersici* (El Komy *et al.*, 2015), *Rhizoctonia solani*, *Sclerotium rolfsii* (Hamed *et al.,* 2015), and Gibberellafugikorai (Watanabe *et al.,* 2007). Molecular identification and phylogenetic analysis: Accurate morphological identification at the species level is of utmost importance for the safe and effective use of *Trichoderma* spp. But overlapping morphological features among the closely related species have created difficulty in precise identification (Prabhakaran *et al.,* 2015). Few commercial *Trichoderma* strains have been reclassified that were commonly used (Mukherjee *et al.,* 2013; Chaverri *et al.,* 2015). Many of the strains in the culture collections and gene sequences in the NCBI database need to be updated. In the present classification scheme, in addition to morphological characterization, molecular confirmation is also routinely performed to identify and categorise the species (Atanasova *et al.,* 2013). Fungal taxonomy has evolved as a result of advances in molecular biology, particularly in DNA analysis. Using techniques of molecular identification, (Hermosa *et al.,* 2013) obtained bands between 560 and 600 bp when they amplified ITS regions in order to identify different isolates of *Trichoderma* spp., among these, one isolate was confirmed as *Trichoderma asperellum*. Similarly, Menezes *et al.* (2010) also identified three isolates of *Trichoderma* spp using the same techniques and confirmed *Trichoderma* species. The results described by these authors are similar to those found in this work, where primers ITS amplified sequences of approximately 500 base pairs for the studied strains of the genus *Trichoderma*.

In this research, we studied the suitability of ocompost as a 1carrier medium for microbial bioagents. Sterilised carrier material (vermicompost) was estimated to have the effect of compost biology on biocontrol agents such as *T. sperllum, Bacillus subtillis* and *P. aeruginosa.* Population dynamics were quantified for all compost treatments. Here, *Trichoderma* and *Bacillus* spp populations declined but became stable after 14 days. This indicates that *Trichoderma* spp could maintain a secure population in compost after a certain period, which is a necessary requirement for compost to be used as a carrier medium. Similar results were found by Nandini *et al.* (2014) reported that the population of

microbes was highest on the 14th day in the enriched vermi in comparison to the population at zero days of enrichment in vermicompost. The CFU count was higher after a few days of application, while it was decreasing further. Which may have happened because, after the digestion of organic material, vermin compost formed; providing a large quantity of material to decompose and a large surface area for microbes to adhere to the substrate. In this study, the total microbial dynamics of the three biocontrol agents were estimated. After fortification of vermicompost with fungal bioinoculants, *T. asperllum on the* first day, CFU values were recorded (3.24 x 106 cfu/g) and after 14 days, they were recorded $5.82x10^6$cfu/g) Similarly, the Trichoderma isolate was identified at the species level based on ITS sequence analysis. The results obtained suggest that *Trichoderma asperellum* promising suppressor of *Fusarium* growth, suggesting that they could be valuable biocontrol agents for the management of diseases in tomatoes. Additionally, our results demonstrate the population dynamics of *Trichoderma* isolates in compost. Our results suggest that this stain can be used as a potential biocontrol agent.

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Nikhil Raman, K.,Vipul Kumar*, Prahlad Masurkar and Angel Jemima

[1]School of Agriculture, Lovely Professional University, Punjab, India -144411

[2]Trichy Research Institute of Biotechnology Pvt. Ltd, Tiruchirappalli, Tamil Nadu 620018, India

*Corresponding author

E-mail: vipul.19845@lpu.co.in