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**Research Article**

# Characterization of novel strains of *Trichoderma* spp. and their utilization in management of damping off disease in tomato

**ATHIRA NAIR[1], G. V. SIBLE[2*], ANIT CYRIAC[1], SUSHA S. THARA[1], JOY MICHAL JOHNSON[3], N. S. RADHIKA[4] and K. B. SONI[5]**

*[1]Department of Plant Pathology, [5]Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram - 695522, Kerala, India*

*[2]Regional Agricultural Research Station, Kumarakom, Kottayam - 686563, Kerala, India*

*[3]Farming Systems Research Station, Sadanandapuram, Kollam - 691531, Kerala, India*

*[4]Department of Plant Pathology, College of Agriculture, Padannakkad, Kasaragod - 671314, Kerala, India*

**Correspondence author email:* sible.gv@kau.in

**ABSTRACT:** Chemical fungicides used in plant disease management may have deteriorative effects on humans, animals, and the environment. The use of native strains of *Trichoderma* spp. against plant diseases may help to reduce the dependence on chemical fungicides. In this study, eleven novel isolates of *Trichoderma* spp. from virgin forest soils of different agro-climatic zones of Kerala were characterized and evaluated for their efficacy against damping off disease of tomato caused by *Pythium aphanidermatum* under *in vitro* and *in vivo*; and also, against wilt pathogen, *Fusarium oxysporum* under *in vitro* conditions. Dual culture assay showed that all the *Trichoderma* isolates were found to inhibit the growth of *P. aphanidermatum* and *F. oxysporum* under *in vitro* conditions with multiple modes of action. The mycelial colour, texture, and conidial characters varied among all the isolates. The volatile metabolites by isolates of *Trichoderma* spp. also showed *in vitro* inhibition of the pathogens. Seed treatment (20 g $kg^{-1}$) and potting medium addition @ 2 % (w/w) of isolates TRMW-2, TRKR-2, TRPN-3, TRPN-11 and TRPN-17 could effectively reduce pre- and post-emergence damping off of tomato. Among them, isolates TRMW-2, TRKR-2, and TRPN-11 were the most effective ones in reducing pre- and post-emergence damping off to about 72 and 90 percent respectively. Molecular identification of the isolates of *Trichoderma* spp. using ITS universal primers revealed similarity with certain reference strains of the NCBI Genbank database.

**KEY WORDS:** Biocontrol, damping off, *Fusarium oxysporum*, *Pythium aphanidermatum, Trichoderma,* wilt

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## INTRODUCTION

Seed and soil-borne diseases are thought to be more limiting the crop production than other types of plant diseases since they accurately change the quantity as well as the quality of production in different crops, resulting in 10 to 20 percent yearly yield loss globally (Ray *et al.,* 2017). In India, 50 percent of commercially important crops are lost each year due to the aggressiveness and destructive activity of soil-borne phytopathogenic fungi. Fungal species such as *Pythium aphanidermatum*, *Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium rolfsii*, and *Fusarium oxysporum* appear to be the most important among the phytopathogens responsible for soil-borne diseases in plants.

*Pythium aphanidermatum,* an Oomycete fungus is an important soil-borne plant pathogen that causes huge losses in agricultural production. Young tissues and plants are infected much more severely by this pathogen (Agrios, 2005). It causes damping off disease in several plants including tomatoes; it affects the plant both in a pre- and post-emergence stage in nursery beds. Pre-emergence damping off is observed when the seed is infected prior to germination. This can result in poor or no germination and is observable as browning or rotting of the seed. Post-emergence damping off takes place after germination and results in a thin, water-soaked stem near the plant collar, which eventually causes the collapse of the plant. Similarly, Fusarium wilt in vegetable cowpea caused by *Fusarium oxysporum* pv. *tracheiphilum* is a destructive disease that causes considerable reduction in yield. Infection occurs through wounds or direct penetration of the roots and is followed by colonization of the xylem (MacHardy and Beckman, 1981). Infected plants exhibit basal stem swelling, leaf chlorosis and leaf drop, wilting, vascular discoloration,

and death. Since the pathogens are soil-borne, fungicide drenches are expensive, impractical, and cause undesirable effects on the environment.

Soil-borne pathogens survive in the soil for long periods making their management very difficult. To manage the diseases caused by these soil-borne pathogens, there is an increased use of chemical fungicides nowadays, which although helps to reduce the disease but on the other hand, pollutes the environment and is harmful to other non-target species. Hence, there is a need for a better, efficient, and environment-friendly method to manage these diseases which can be done with the help of biological control agents (BCAs).

Among the various bioagents that are available worldwide, the filamentous fungus belonging to the genus *Trichoderma* is more predominant. *Trichoderma* spp. has been successfully used as biocontrol agents for the control of various soil-borne diseases. Strains of *Trichoderma* show high variability in their biocontrol traits. Characterization of various strains of *Trichoderma* is very important to understand their biocontrol traits. This will help us to select highly potential strains or modify their ability to ensure better efficacy in the management of soil-borne diseases. The various species of *Trichoderma* employed in plant disease management include *T. asperellum, T. harzianum, T. atroviride, T. longibrachiatum, T. virens, T. viride,* etc.

Earlier, Cyriac *et al.* (2021) had studied the antagonistic efficacy of twelve *Trichoderma* isolates obtained from the Thiruvananthapuram district of Kerala against *P. aphanidermatum* and *R. solani*. The present study aims to test the combined efficacy of eight isolates obtained from that study along with five new isolates from different agro-climatic zones Kerala against *P. aphanidermatum* and *F. oxysporum*. The morphological and molecular characterization of the potent isolates and *in vivo* efficacy against damping off disease in tomatoes are also studied. The study also aims at developing formulations of *Trichoderma* spp. which can be utilized commonly for the management of soil-borne diseases of both tomatoes and cowpea.

## MATERIALS AND METHODS

### *Trichoderma* isolates

*Trichoderma* spp. isolated from virgin forest soils and collected from five agro-climatic zones of Kerala, India were used in the present study. Out of 31 isolates obtained from various locations, eight were selected on the basis of their antagonistic properties and inhibition percentage against the pathogens *P. aphanidermatum* causing damping-off of tomato and *R. solani* causing collar rot of cowpea (Cyriac *et al.,* 2021). The isolates were TRKR-2, TRPN-3, TRPN-7, TRPN-11, TRPN-14, TRPN-15, TRPN-17 and TRPN-18 from Southern zone. In addition, isolates TRSN-1 and TRMW-2 collected from Central zone; TRML-1 from the Northern zone; *Trichoderma* sp. (KAU strain), and *T. harzianum* (NBAIR strain) were also included in the study.

### Isolation of pathogens and proving Koch's postulates

*P. aphanidermatum* was isolated from tomato plants exhibiting symptoms of damping off from the College of Agriculture, Vellayani, Kerala. Pre-emergence damping off symptoms included the appearance of water-soaked lesions leading to complete rotting of the radicle. In case of post-emergence damping off, the collar region was affected and led to softening of the infected region resulting in the toppling down of seedlings. *F. oxysporum* was isolated from wilt-infected cowpea plants collected from the College of Agriculture, Vellayani. The characteristic symptoms included yellowing of the leaves and pods later leading to wilting. The basal stem portion showed swelling and small to elongated brownish pitting. When the basal portion was split, brownish discoloration of the vascular bundles could be seen.

Isolation of pathogens was carried out by cutting small pieces/bits of stem/basal portion with a margin of the healthy region. These pieces/bits were then surface sterilized in 0.1 percent mercuric chloride ($HgCl_2$) for 30 seconds followed by three washes with sterile water. Sterilized bits were then blot dried with sterilized tissue paper before being placed on a Potato Dextrose Agar (PDA) medium. The emerging mycelium from the tissues was then sub-cultured to another sterile Petri plate containing PDA medium and incubated at room temperature (28 ± 2°C). The cultural characters of the pathogen on PDA medium were recorded. Spore characters were observed under a microscope at 100X magnification.

Surface sterilized tomato seeds of damping-off susceptible variety Vellayani Vijay were sown in protrays @ three seed per hole. The pathogen was multiplied in the sand-maize mixture (19:1). Pathogen was incorporated in the potting mixture (soil:coir-pith:cow dung at 1:1:1 ratio) @ 0.5 percent (w/w) before sowing in case of pre-emergence damping off. For post-emergence damping off, the pathogen was incorporated in the potting mixture 5 Days After Sowing (DAS) (Karmel and Muthukumar, 2019). The plants that showed the symptoms were used for re-isolation of the pathogen for proving Koch's postulates.

The pathogenicity test of *F. oxysporum* was carried out on the cowpea variety Vellayani Jyothika. Surface sterilized seeds were sown in grow bags containing sterilized potting mixture as mentioned earlier @ 3 seeds per grow bag. The potting medium was artificially incorporated with *F. oxysporum* mass multiplied in the sand-maize mixture (9:1) at 20 DAS. Three grow bags containing only a sterilized potting

mixture were maintained as control. The plants that showed the symptoms were used for re-isolation of the pathogen for proving of Koch's postulates and the re-isolated pathogen was maintained in PDA medium for further studies.

**Colony characters of *Trichoderma* isolates**

All the 11 isolates and the two reference strains were studied for their cultural and morphological characters on PDA medium. Three replicates for each isolate were grown on PDA and incubated at room temperature (28 ± 2°C). The isolates were observed for their mycelial colour at 3 Days After Inoculation (DAI), colony texture and sporulation pattern. Spore size and shape of all the isolates were observed by slide culture technique (Riddell, 1950). The length and width of conidia (in microns) were measured using compound microscope with Zeiss 3.0 software.

***In vitro* screening of isolates of *Trichoderma* spp. against *P. aphanidermatum* and *F. oxysporum***

The 13 isolates of *Trichoderma* spp. were tested *in vitro* against *P. aphanidermatum* and *F. oxyporum* by dual culture assay which was performed based on the method given by Skidmore and Dickinson (1976). Petri plate containing PDA medium with 5 mm mycelial disc of pathogen alone at one end was kept as control. Five replicates were maintained. Radial growth of the pathogen (cm) in all the Petri plates was measured till fifth day when the pathogen attained full growth in control plate. Per cent inhibition of the pathogen by the *Trichoderma* isolates was calculated. The antagonism index (Campanille *et al.,* 2007) was calculated using the formulae as under;

$$\text{Antagonism Index} = \frac{(RM - rm)}{RM} \times 100$$

rm = ray of colony towards antagonist

RM = average of three rays of a colony in other directions

**Assessment of antagonistic properties of *Trichoderma* Isolates against the pathogens**

Antagonistic properties *viz.*, antibiosis, lysis and overgrowth were observed against the pathogens in dual culture assay. Antibiosis was observed by formation of pigmentation where the antagonist and the pathogen interacted. Overgrowth was identified as antagonist growing over the pathogen mycelium. Zone of lysis was also seen as clear regions in certain pathogen-antagonist interactions.

***In vitro* efficacy of volatile metabolites produced by isolates of *Trichoderma* spp.**

The production of volatile metabolites by different isolates of *Trichoderma* spp. against both the pathogens was assessed by following the method of Dennis and Webster (1971a) as described by Eziashi *et al.* (2006). The pathogens were grown on PDA medium in Petri plates for one day at 28 ± 2°C. The next day, the lids of each Petri plates were removed and replaced by the bottom part of the Petri plate containing PDA medium inoculated with *Trichoderma* spp. and the dishes were taped together with cling film. This was performed for all the *Trichoderma* isolates separately. The lids of Petri plates that served as control (pathogen alone) were also replaced in the same manner. Two replications were kept for all the isolates of *Trichoderma* spp. Mycelial growth of the pathogens in all the plates was measured till the pathogen covered the entire plate and the percent growth inhibition of the pathogen over control was calculated.

***In vivo* testing of the efficacy of the selected isolates of *Trichoderma* spp. against damping off of tomato**

Based on the percent inhibition and antagonistic characters of the different isolates of Trichoderma against *P. aphanidermatum* and *F. oxysporum*, the isolates *viz.*, TRMW-2, TRKR-2, TRPN-3, TRPN-11, TRPN-17 and *Trichoderma* sp. (KAU strain) were selected for the *in vivo* experiment against damping-off caused by *P. aphanidermatum.* Talc-based formulations were prepared for each of these *Trichoderma* isolates. For this, the isolates were grown in 300 ml potato dextrose broth for seven days and then thoroughly mixed with one-kilogram talc. To get the required consistency, the formulations were dried upto a moisture content of seven to eight percent. A minimum population count of 2 x $10^6$ cfu $g^{-1}$of *Trichoderma* spp. was assured in the formulations before use. The prepared formulations were tested in protray seedlings against pre-emergence and post-emergence damping off disease in tomato (*var.* Anagha) at College of Agriculture, Vellayani.

The experiment was laid out in Completely Randomized Design (CRD) with eight treatments and five replicates for each treatment. The *Trichoderma* isolates were applied as seed treatment with talc-based formulation @ 20 g $kg^{-1}$ and as additive in potting medium @ 2 percent (w/w). A standard check was maintained by drenching of copper oxychloride @ 3g $l^{-1}$ at the time of sowing of seeds. Inoculation of pathogen in the case of pre-emergence and post-emergence damping off was carried out as mentioned earlier. Pathogen-inoculated control was also maintained. The number of plants infected in each treatment was recorded.

**DNA barcoding of promising isolates of *Trichoderma* spp. using Universal Primers of ITS**

**DNA isolation and PCR Amplification**

DNA isolation was carried out using the Macherey-Nagel NucleoSpin® Plant II Kit. The quality of the DNA isolated was checked using agarose gel electrophoresis.

PCR analysis mixture consisted of 2X Phire master mix 5 µl, distilled water 4 µl, forward primer 0.25 µl, reverse primer 0.25 µl and DNA1 µl. Universal primers ITS-1 5'-TCCGTAGGTGAACCTGCGG-3' (forward) and ITS-4 5'-TCCTCCGCTTATTGATATGC-3' (reverse) were used for the analysis. (White et al., 1990). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, USA). The PCR amplification was carried out starting with an initial denaturation of 98°C for 30 s followed by 40 cycles of denaturation at 98°C for 5 s, annealing at 58°C for 10 s and extension at 72°C for 15 s. This was followed by final extension at 72°C for 60 s and holding at 4°C. The PCR products were checked in 1.2 percent agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. One microlitre of 6X loading dye was mixed with 4 µl of PCR products and was loaded and electrophoresis was performed at 75 V power supply with 0.5X TBE as electrophoresis buffer for about one to two hours, until the bromophenol blue front had migrated to three fourth of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). Five micro-litres of PCR product were mixed with 0.5 µl of ExoSAP-IT and incubated at 37 °C for 15 min. followed by enzyme inactivation at 85 °C for 5 min.

**Sequencing of ITS Regions, Post Sequencing PCR Clean-Up**

The sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) and then post-sequencing PCR clean-up following the manufacturer's protocol. The cleaned-up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems, USA).

**Sequence analysis**

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems, USA). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.,* 2010). The identity of the conserved region of ITS-rDNA of the *Trichoderma* isolates was established by performing a similarity search using Basic Local Alignment Search Tool (BLAST) at National Centre for Biotechnology Information (NCBI) database and the sequences were matched with the existing sequence database for species confirmation.

**Statistical analysis**

Analysis of Variance (ANOVA) was calculated based on the data obtained from the experiments. Critical Difference (CD) at five percent level of significance was calculated and used for comparison between the means of the treatments. Standard deviation and standard error of mean were also calculated for all the treatments. The analysis was performed using both OPSTAT and KAU GRAPES 1.0.0.

**RESULTS**

The cultural and morphological characteristics of the 13 isolates of *Trichoderma* spp. were studied on PDA medium (Table 1). It was observed that all the isolates of *Trichoderma* spp. showed white-coloured mycelial growth except for the isolate TRML-1 which showed white/creamy white mycelial growth. The texture of the colonies was sparse, spreading, and cottony or fluffy growth at centre. Moreover, the sporulation and growth pattern also varied among the isolates which showed spores with varying shades of green.

The different isolates of *Trichoderma* were found to produce conidia of different shapes which varied from globose to sub-globose and oval to ellipsoidal. The ellipsoidal shape was seen in isolates TRKR-2, TRPN-14 and TRML-1 while globose shape was seen in isolate TRPN-11. Similarly, the size of the spores also varied among the 13 isolates. The length of the spores ranged from 2.197 µm to 4.448 µm while the width ranged from 2.700 **µm** to 4.172 **µm**. *P. aphanidermatum* showed white-coloured mycelial growth in the Petri plate. *P. aphanidermatum* produced hyaline hyphae and lobed sporangia. Oospores which were round in shape were also produced. Pre-emergence damping off symptoms were produced 10 days after inoculation.

Symptoms of post-emergence damping off were produced after 13-15 days of inoculation. It was found that seed germination was inhibited due to the rotting of radicle in pre-emergence damping off while in case of post-emergence damping off the seedlings showed water-soaked lesions and later the collar region became rotted which resulted in toppling down of seedlings. The *in vitro* efficacy of the isolates of *Trichoderma* spp. was assessed by dual culture method. The production of volatile metabolites by the different isolates was also assessed. *In vitro* screening of different isolates of *Trichoderma* spp. against *P. aphanidermatum* was performed based on which the radial growth and percent inhibition were calculated (Plate 1, 2). The percent inhibition of *P. aphanidermatum* by different isolates of *Trichoderma* spp. and antagonistic index are presented in Table 2. The results revealed that all the isolates of *Trichoderma* brought about more than 57 per cent inhibition of the pathogen. The isolates that exhibited highest inhibition were TRPN-7, TRPN-18, and TRML-1 (70 %). The lowest inhibition of 58.88 percent was seen in the interaction between isolate TRSN-1 and the pathogen. *T. harzianum* (NBAIR strain) and *Trichoderma* sp. (KAU strain) caused inhibition of 61.11 percent and 60

**Table 1.** Colony and conidial characters of isolates of *Trichoderma* spp. grown on PDA medium

| Sl. No. | Isolate | Colour of mycelium at 3 DAI | Texture of the colony | Sporulation pattern | Conidial size (µm)* | | Conidial shape |
|---|---|---|---|---|---|---|---|
| | | | | | Length | Width | |
| 1. | TRSN-1 | White | Sparse/Spreading | Olive green coloured spores at the centre with white mycelial periphery | 2.197±0.040$^{i}$ | 2.881±0.019$^{g}$ | Sub-globose |
| 2. | TRMW-2 | White | Cottony | Dark green circular ring like sporulation | 2.521±0.068$^{h}$ | 2.787±0.029$^{h}$ | Sub-globose |
| 3. | TRKR-2 | White | Greyish fluffy at centre and thin at periphery | No/ less sporulation | 3.755±0.039$^{b}$ | 2.921±0.008$^{fg}$ | Ellipsoidal |
| 4. | TRPN-3 | White | Sparse | Greenish at the centre | 2.961±0.054$^{f}$ | 3.100±0.017$^{e}$ | Sub-globose |
| 5. | TRPN-7 | White | Spreading/ Sparse | Light green spores at centre | 2.593±0.042$^{h}$ | 2.700±0.016$^{i}$ | Globose to sub-globose |
| 6. | TRPN-11 | White | Sparse | Light green spores at centre | 3.228±0.094$^{e}$ | 3.262±0.010$^{d}$ | Globose to ovoidal |
| 7. | TRPN-14 | White | Sparse | Dark green spores surrounding the inner light green spores at the centre | 2.929±0.026$^{f}$ | 3.805±0.006$^{b}$ | Ellipsoidal |
| 8. | TRPN-15 | White | Fluffy at centre and spreading | Dark green spores in concentric ring | 2.811±0.002$^{g}$ | 2.716±0.046$^{hi}$ | Sub-globose |
| 9. | TRPN-17 | White | Fluffy at centre and thin towards periphery | Light greenish centre | 2.565±0.047$^{h}$ | 2.960±0.047$^{f}$ | Sub-globose to ovoidal |
| 10. | TRPN-18 | White | Sparse | Dark green spores at centre | 3.484±0.108$^{d}$ | 3.349±0.097$^{c}$ | Sub-globose to ovoidal |
| 11. | TRML-1 | White/ Creamish white | Greyish fluffy centre with concentric zones | Light green spores surrounding dark green spores at the centre | 4.448±0.054$^{a}$ | 4.172±0.084$^{a}$ | Ellipsoidal |
| 12. | *T. harzianum* (NBAIR strain) | White | Spreading | Circular dark green sporulation | 3.268±0.011$^{e}$ | 3.081±0.041$^{e}$ | Sub-globose to ovoidal |
| 13. | *Trichoderma* sp. (KAU strain) | White | Spreading | Dark green spores | 3.652±0.044$^{c}$ | 3.875±0.014$^{b}$ | Sub-globose to ovoidal |

*Mean of three replications; Superscripts with same alphabets indicate on par values and those in different alphabets indicate significant difference at 5 per cent level of significance

**Table 2.** *In vitro* efficacy and antagonistic index of isolates of *Trichoderma* spp. against *P. aphanidermatum* and *F. oxysporum* in dual culture

| Sl. No. | Isolate | Against *P. aphanidermatum* | | | Against *F. oxysporum* | | |
|---|---|---|---|---|---|---|---|
| | | Radial growth of pathogen (cm)* | Inhibition (%)** | Antagonistic index | Radial growth of pathogen (cm)* | Inhibition (%)** | Antagonistic index |
| 1. | TRSN-1 | 3.70±0.042 | 58.88 (50.10) | 27.45±0.679 | 4.90 ± 0.057 | 45.56 (42.43) | 17.54 ± 0.113 |
| 2. | TRMW-2 | 3.40±0.071 | 62.22 (52.05) | 29.61±0.382 | 4.80 ± 0.057 | 46.67 (43.07) | 22.95 ± 0.368 |
| 3. | TRKR-2 | 2.80±0.028 | 68.88 (56.08) | 44.77±0.226 | 4.90 ± 0.057 | 45.56 (42.43) | 15.08 ± 0.113 |
| 4. | TRPN-3 | 3.00±0.057 | 66.66 (54.71) | 42.31±0.537 | 4.70 ± 0.071 | 47.78 (43.71) | 24.61 ± 0.396 |
| 5. | TRPN-7 | 2.70±0.071 | 70.00 (56.77) | 51.53±0.339 | 5.20 ± 0.057 | 42.22 (40.51) | 8.77 ± 0.226 |
| 6. | TRPN-11 | 3.20±0.042 | 64.44 (53.37) | 44.54±0.410 | 5.00 ± 0.085 | 44.44 (41.79) | 16.25 ± 0.156 |
| 7. | TRPN-14 | 3.00±0.085 | 66.66 (54.71) | 37.50±0.509 | 4.80 ± 0.099 | 46.67 (43.07) | 19.60 ± 0.085 |
| 8. | TRPN-15 | 3.50±0.057 | 61.11 (51.39) | 29.58±0.339 | 5.10 ± 0.028 | 43.33 (41.15) | 15.00 ± 0.141 |
| 9. | TRPN-17 | 2.80±0.085 | 68.88 (56.08) | 49.72±0.566 | 4.80 ± 0.099 | 46.67 (43.07) | 18.64 ± 0.552 |
| 10. | TRPN-18 | 2.70±0.028 | 70.00 (56.77) | 51.97±0.580 | 5.20 ± 0.113 | 42.22 (40.51) | 5.97 ± 0.226 |
| 11. | TRML-1 | 2.70±0.085 | 70.00 (56.77) | 52.63±0.198 | 4.90 ± 0.127 | 45.56 (42.43) | 15.52 ± 0.325 |
| 12. | *T. harzianum* (NBAIR strain) | 3.50±0.028 | 61.11 (51.39) | 33.59±0.509 | 5.10 ± 0.127 | 43.33 (41.15) | 15.00 ±0.085 |
| 13. | *Trichoderma* sp. (KAU strain) | 3.60±0.057 | 60.00 (50.75) | 32.08±0.269 | 5.60 ± 0.085 | 37.78 (37.91) | 5.56 ± 0.453 |
| 14. | Control | 9.00 | - | - | 9.00 | - | - |
| | CD (0.05) | 0.130 | 0.888 | 0.972 | 0.187 | 1.213 | 0.627 |

*Mean ± SD of five replications; **Values in parentheses are arcsine transformed.

percent respectively. The isolates that showed more than 50 percent antagonistic index were TRPN-7, TRPN-18, and TRML-1 closely followed by isolate TRPN-14 (49.72). The other isolates showed an antagonistic index from 27.45 to 44.77. Against *F. oxysporum,* isolates TRSN-1, TRMW-2, TRKR-2, TRPN-3, TRPN-14, TRPN-17, and TRML-1 displayed more than 45 per cent inhibition. This was followed by TRPN-11, *T harzianum* (NBAIR strain), and *Trichoderma* sp. (KAU strain) which resulted in 44.44, 43.33, and 37.78 percent inhibition respectively. Antagonistic index was found to be greatest for isolate TRPN-3 (24.61) followed by isolates TRMW-2 (22.95), TRPN-14 (19.60, and TRPN-17 (18.64).

Against *P. aphanidermatum*, antibiosis and overgrowth were the two antagonistic characteristics that were found to be prominent among all the interactions in moderate to high levels. Lysis of mycelium of *P. aphanidermatum* was seen only in interactions with *Trichoderma* isolates TRMW-2, TRKR-2, TRPN-15, and TRPN-18. *Trichoderma* isolates TRMW-2, TRKR-2, TRPN-15 and TRPN-18 exhibited all three antagonistic properties against *P. aphanidermatum.* Against *F. oxysporum*, overgrowth was the major antagonistic character shown by all the isolates of *Trichoderma* spp. in moderate to a high level. Isolate TRPN-7 showed moderate level of anitibiosis while isolates TRSN-1, TRMW-2, TRKR-2, TRPN-3, TRPN-11, TRPN-14, TRPN-15 and TRPN-17 showed low level of antibiosis. Isolates that showed moderate to high levels of lysis were *T. harzianum* (NBAIR strain), TRPN-17, *Trichoderma* sp. (KAU strain), and TRPN-18. Low level of lysis was seen in the interactions with isolates TRMW-2, TRPN-3, TRPN-11, and TRPN-15. Among all the isolates, TRMW-2, TRPN-3, TRPN-11, TRPN-15, and TRPN-17 exhibited all three antagonistic properties. Percent inhibition of the pathogens by volatile metabolites produced by different isolates of *Trichoderma* spp. are represented in Fig. 1 and 2. The isolates that showed comparatively better performance than others against *P. aphanidermatum* were TRPN-17 (27.78 %), TRPN-18 (24.44 %), TRKR-2 (17.78 %), TRMW-2 (16.67 %) and TRPN-14 (16.67 %). *T. harzianum* (NBAIR strain) and *Trichoderma* sp. (KAU strain)

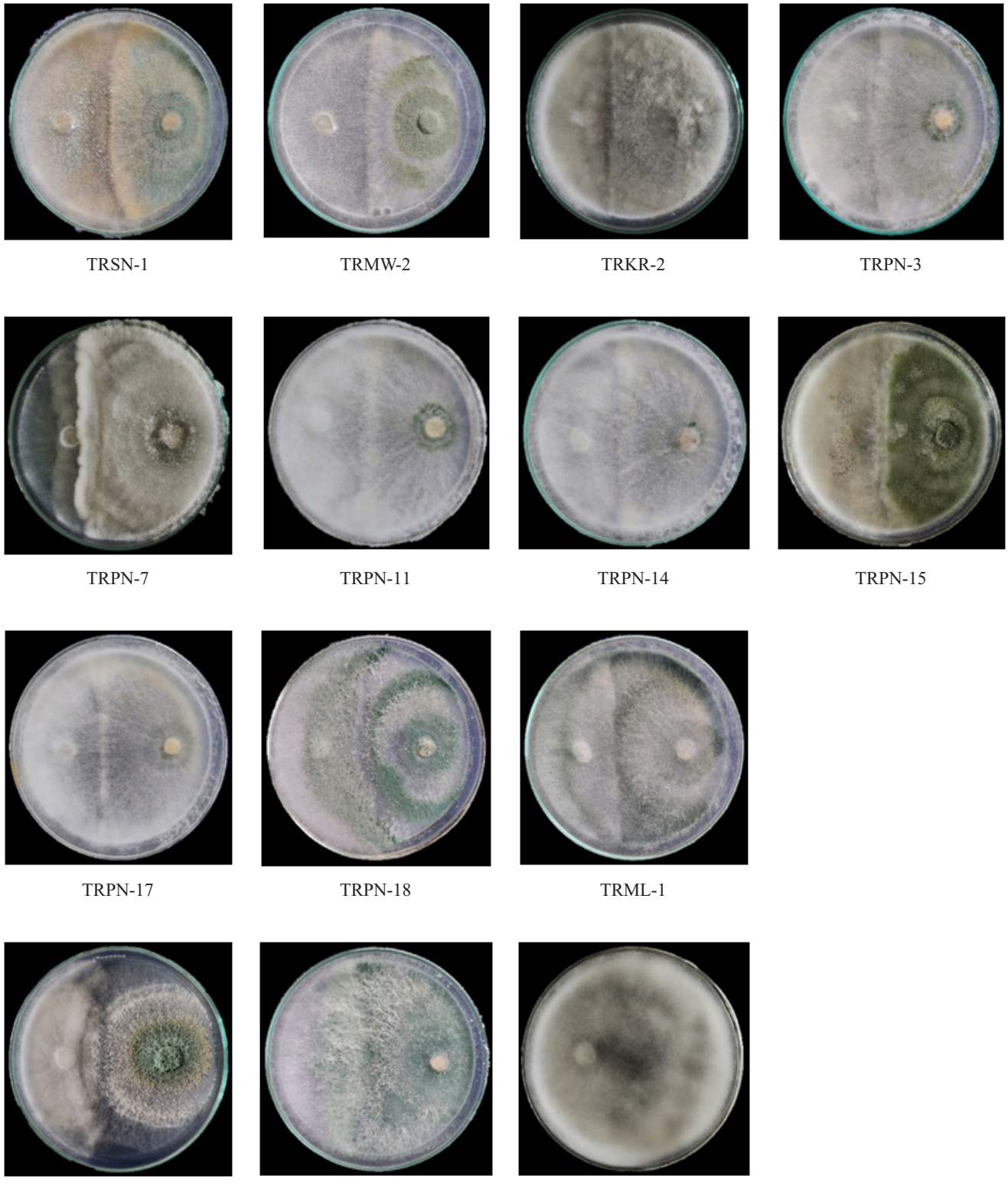



**Plate 1.** Dual culture assay of *Trichoderma* isolates against *P. aphanidermatum*.

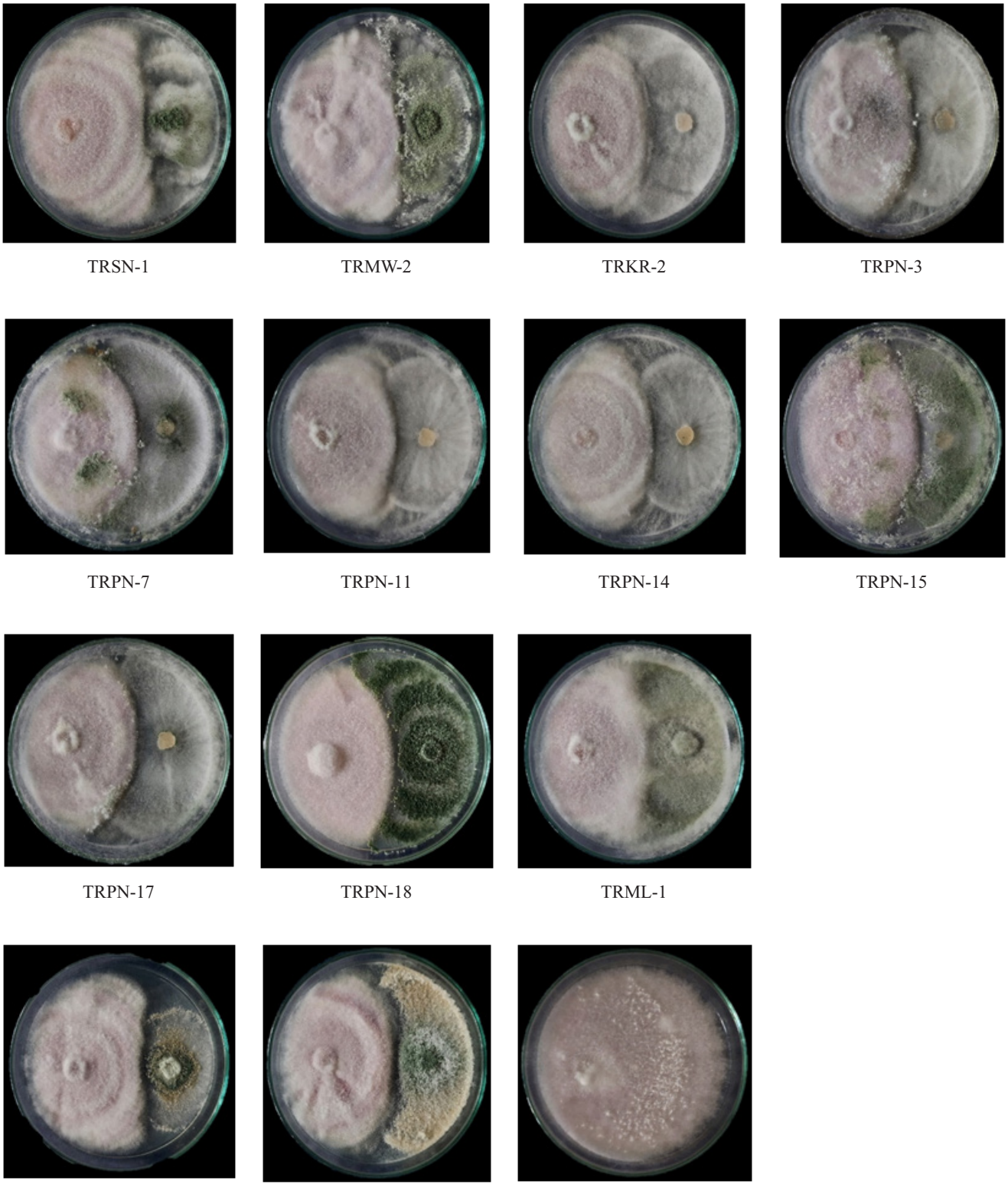



**Plate 2.** Dual culture assay of *Trichoderma* isolates against *F. oxysporum*.

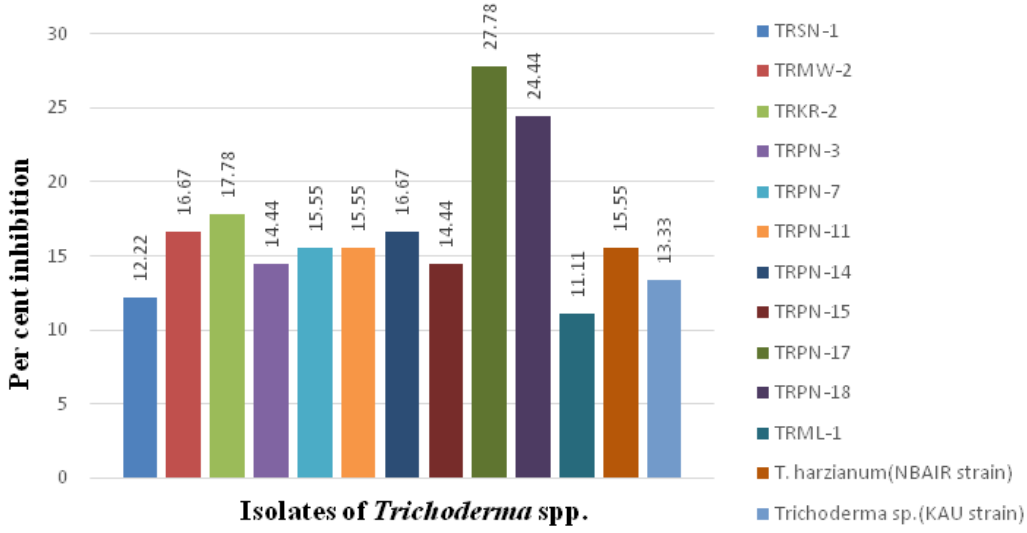



**Fig. 1.** Per cent inhibition of *P. aphanidermatum* by volatile metabolites produced by isolates of *Trichoderma* spp.

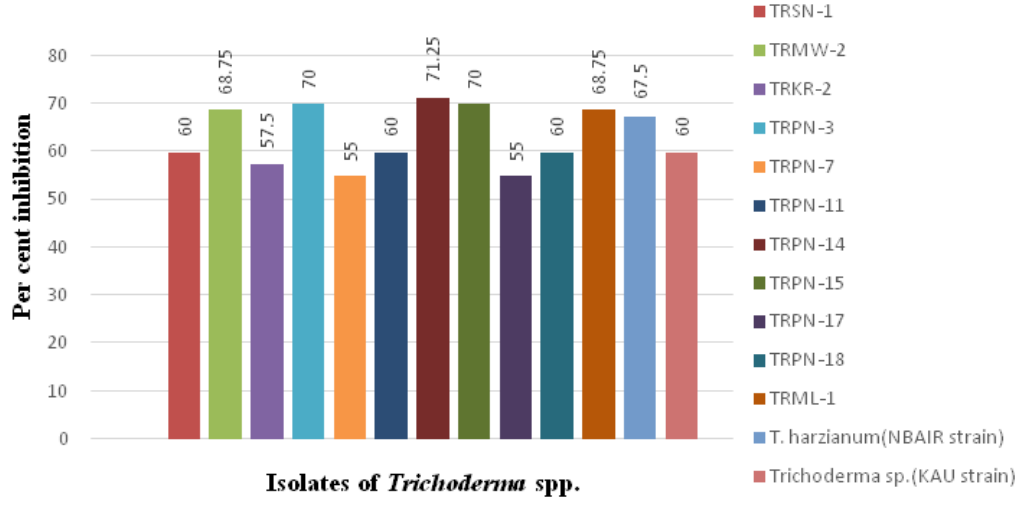



**Fig. 2.** Per cent inhibition of *F. oxysporum* by volatile metabolites produced by isolates of *Trichoderma* spp.

produced 15.55 and 13.33 percent inhibition respectively. Against *F. oxysporum*, the highest inhibition was exhibited by the isolate TRPN-14 (71.25 %) closely followed by isolates TRPN-3 and TRPN-15 (70 %).

Molecular characterization of 10 isolates of *Trichoderma* spp. was done using ITS-1 (forward) and ITS-4 (reverse) universal primers and the sequences were analyzed using NCBI BLAST. The isolates of *Trichoderma* spp. used in this study were found to share some similarities with certain reference strains of Genbank database (Table 3). It provided evidence on the identity of the isolates to be *T. harzianum* (TRMW-2, TRPN-3, TRPN-14, TRPN-17), *T. koningiopsis* (TRKR-2), *T. lixii* (TRPN-11, TRPN-15) and *T. asperellum* (TRPN-18).

*In vivo* testing of the selected isolates against pre-emergence damping off of tomato revealed that seed treatment and potting medium application of isolate TRMW-2 showed 72.01 percent reduction of pre-emergence damping off followed by treatment with isolates TRKR-2 (71.82 %) and TRPN-11 (71.36 %) (Table 4). Treatment with isolates TRPN-3 and TRPN-17 showed 68.12 percent and 64.85 percent reduction over control respectively. Treatment with *Trichoderma* sp. (KAU strain) and copper oxychloride displayed 64. 38 percent and 59.35 percent respectively. Notable reduction in post-emergence damping off was seen on treatment with isolates TRPN-11 (90.33 %), TRMW-2 (90.20 %), and TRKR-2 (90 %) followed by treatments with isolates TRPN-17 (89.88 %) and TRPN-3 (89.78 %) (Plate 3, 4).

**Table 3.** Molecular characterization of isolates of *Trichoderma* spp. by ITS-PCR

| Sl. No. | Isolate | Best match with reference strains in Genbank data base | Per cent similarity | Accession Number |
|---|---|---|---|---|
| 1 | TRSN-1 | *Trichoderma* sp. isolate SDAS204116 | 99.66 | MK870785.1 |
| 2 | TRMW-2 | *T. harzianum* strain CEN-257 | 97.03 | KC576680.1 |
| 3 | TRKR-2 | *T. koningiopsis* | 99.62 | MT102395.1 |
| 4 | TRPN-3 | *T. harzianum* clone HC-1 | 100.00 | MK552405.1 |
| 5 | TRPN-7 | Uncultured *Trichoderma* clone CHR1FC240 | 98.31 | KJ713225.1 |
| 6 | TRPN-11 | *T. lixii* strain F-2 | 100.00 | MT434003.1 |
| 7 | TRPN-14 | *T. harzianum* clone HC-1 | 99.01 | MK552405.1 |
| 8 | TRPN-15 | *T. lixii* strain F-2 | 99.82 | MT434003.1 |
| 9 | TRPN-17 | *T. harzianum* isolate M3951 | 99.66 | MK738149.1 |
| 10 | TRPN-18 | *T. asperellum* | 99.82 | MH215555.1 |

**Table 4.** Efficacy of different isolates of *Trichoderma* spp. on pre-emergence and post-emergence damping off of tomato

| Sl. No. | Treatment | Pre-emergence damping off | | Post-emergence damping off | |
|---|---|---|---|---|---|
| | | Disease incidence (%)* | Per cent reduction over control | Disease incidence (%)* | Per cent reduction over control |
| 1 | T1- *Trichoderma* isolate TRMW-2 | 27.61 (31.68) | 72.01 | 9.80 (18.24) | 90.20 |
| 2 | T2- *Trichoderma* isolate TRKR-2 | 27.80 (31.83) | 71.82 | 10.00 (18.43) | 90.00 |
| 3 | T3- *Trichoderma* isolate TRPN-3 | 31.45 (34.09) | 68.12 | 10.22 (18.64) | 89.78 |
| 4 | T4- *Trichoderma* isolate TRPN-11 | 28.25 (32.24) | 71.36 | 9.67 (18.11) | 90.33 |
| 5 | T5- *Trichoderma* isolate TRPN-17 | 34.67 (36.06) | 64.85 | 10.12 (18.53) | 89.88 |
| 6 | T6- *Trichoderma* sp. (KAU strain) | 35.14 (36.34) | 64.38 | 11.24 (19.58) | 88.76 |
| 7 | T7- Copper oxychloride (3 $gl^{-1}$) | 40.10 (39.27) | 59.35 | 11.42 (19.74) | 88.58 |
| 8 | T8- Control (Pathogen inoculated) | 98.65 (83.82) | - | 100.00 (90.00) | - |
| | CD (0.05) | 3.28 | - | 0.120 | - |

*Mean ± SD of three replications; Values in parentheses are arcsine transformed

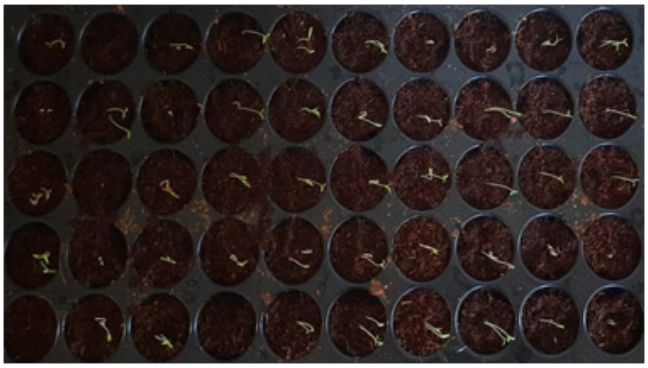
## DISCUSSION

*Trichoderma* species are increasingly being used as biological control agents, and a few isolates are now commercially available Several strains of *Trichoderma* exerted significant reducing effects on plant diseases caused by pathogens such as *R. solani, S. rolfsii, P. aphanidermatium*, *F. oxysporum*, *F. culmorum,* and *Gaeumannomyces graminis* under greenhouse and field conditions, according to the findings of various studies (Sivan and Chet, 1993; Inbar *et al.,* 1994). *Trichoderma* spp. are widely used as biocontrol agents against soil-borne pathogens as it has the ability to multiply and spread fast with ease to isolate and culture (Pandya *et al.,* 2011).

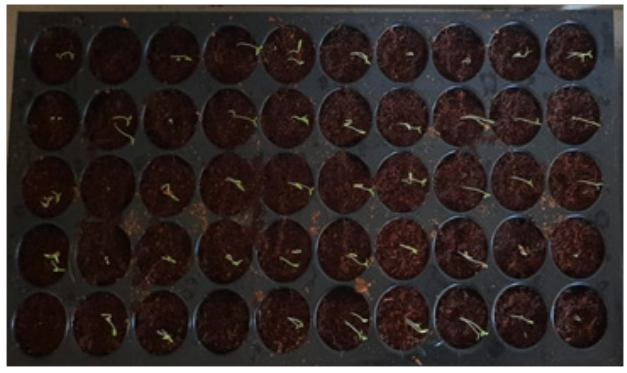
Different *Trichoderma* spp. vary in their growth pattern, sporulation, texture, colony colour, conidiophore branching, phialide shape and size, phialide branching, etc. As a result, in order to identify the various isolates, these characteristics are used as morphological and cultural descriptors. The colour of the mycelium, the texture of the colony, sporulation, and growth pattern varied among the 13 isolates in this study. Similarly, Das *et al.* (2018) found that *T. asperellum*, *T. brevicompactum* and *T. harzianum* varied in the shape and colour of conidia produced. According to Singh *et al.* (2020), the type of growth of *Trichoderma* spp. was either submerged or cottony, conidiation in ring-like formation or no ring formation, phialide shape varying from ampulliform to lageniform and cylindrical, conidia shape varying from ovoid to ellipsoidal or subglobose, and colour varying from hyaline to green. *T. harzianum, T. viride* and *T. aureoviride* isolates had colony colours that ranged from light green to dark green (Shalini and Kotasthane, 2007). In ten isolates of *Trichoderma* sp. isolated from groundnut rhizosphere,

TRMW-2

TRKR-2



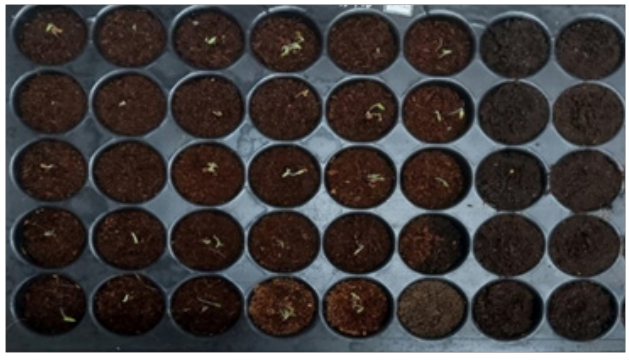
TRPN-3



TRPN-11

TRPN-17

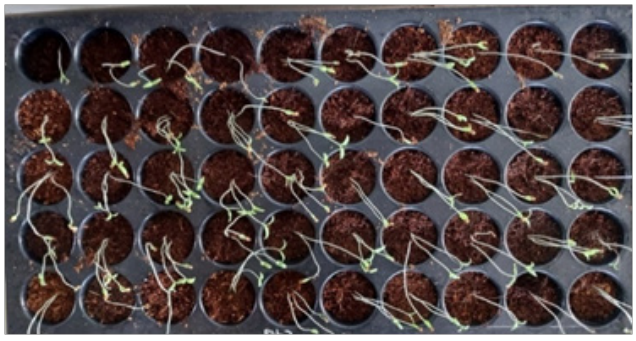
*Trichoderma* sp. (KAU strain)



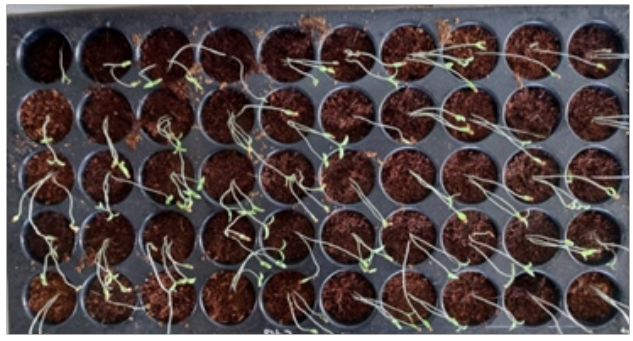
Copper oxychloride (3 g $l^{-1}$)

Control (Pathogen-inoculated)

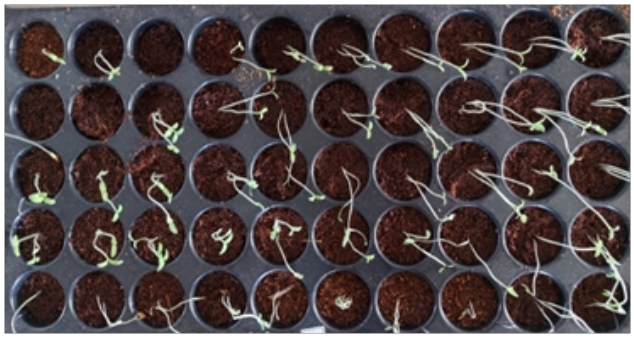
**Plate 3.** Efficacy of different isolates of *Trichoderma* spp. on pre-emergence damping off of tomato.



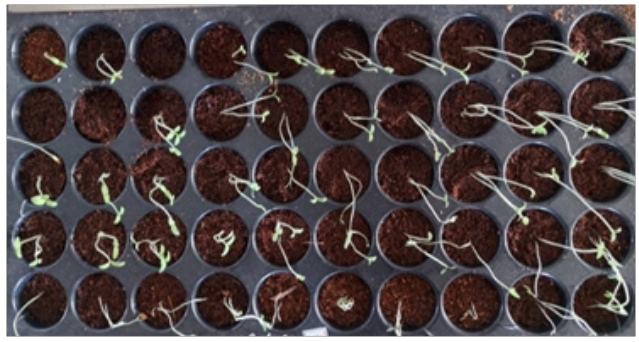
TRMW-2



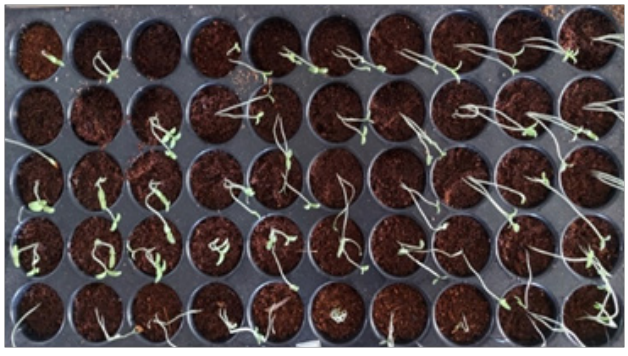
TRKR-2



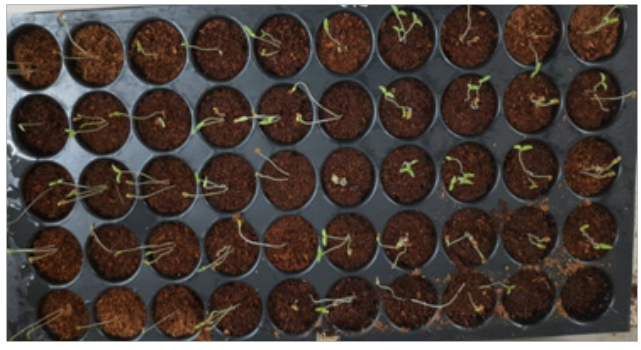
TRPN-3



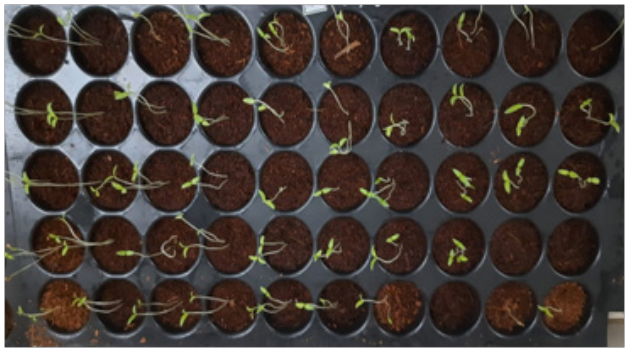
TRPN-11



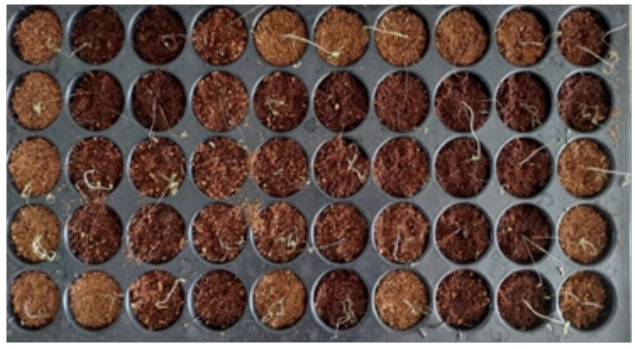
TRPN-17



*Trichoderma* sp. (KAU strain)



Copper oxychloride (3 g $l^{-1}$)



Control (Pathogen-inoculated)

**Plate 4.** Efficacy of different isolates of *Trichoderma* spp. on post-emergence damping off of tomato.

Sekhar *et al.* (2017) reported wide differences in colony colour such as white, pale yellow, blue-green, and dull green. Jaisani and Pandey (2017) in their study on morphological properties of several isolates of *Trichoderma* spp. illustrated that *T. viride* possessed conidiophores that were branching in a regular to the irregular pattern, whereas *T. harzianum* had conidiophores that were compact to loosely organized. The phialides of *T. viride* were hyaline and globose, but those of *T. harzianum* was flask-shaped and formed large globoid masses. Phialospores ranged in colour from light to dark green and were globose, ellipsoidal, or subglobose in shape. *Trichoderma* spp. isolated from sorghum had distinct features such as pale whitish mycelial colour, compact cottony mycelial form, loose tufts of conidiation in concentric rings, repeated paired branched conidiophores, and ampulliform phialides.

In the present study, *in vitro* screening of *Trichoderma* isolates was carried out against *P. aphanidermatum* and *F. oxysporum*. Against *P. aphanidermatum,* the isolates that exhibited highest inhibition were TRPN-7, TRPN-18 and TRML-1. Against *F. oxysporum*, isolates TRSN-1, TRMW-2, TRKR-2, TRPN-3, TRPN-11, TRPN-14, TRPN-17 and TRML-1 displayed high inhibitory activity. Similar results were observed by Cyriac *et al.* (2021). According to Mishra (2010) *T. viride*-1433 inhibited *P. aphanidermatum* the most followed by *T. harzianum*-4572, *T. viride*-793, *T. harzianum*-4532, and *T. virens*-2194. Mazrou *et al.* (2020) found that the 12 *Trichoderma* isolates showed antagonistic ability against the pathogens *viz., R. solani, P. ultimum* and *A. solani* in which *Trichoderma* isolate T6 showed the highest percentage inhibition against all the pathogens. Chao and Zhuang (2019) showed that *T. simonsii* 8702 and *T. pyramidale* 7921 showed high inhibition of *R. solani* infecting cowpea.

Mycoparasitism, antibiosis, competition for nutrients and space, induced resistance, stress tolerance through improved root and plant development, solubilization and sequestration of inorganic nutrients, and inactivation of pathogen enzymes are some of the antagonistic features (Harman, 2000). The current study revealed that antibiosis and overgrowth were the two antagonistic characteristics that were found to be prominent among all the *Trichoderma*-*P. aphanidermatum* interactions in moderate to high levels while overgrowth was found to be the major antagonistic character shown by all the *Trichoderma* isolates against *F. oxysporum* in moderate to high levels. Antibiosis was also documented by Mendoza et al. (2015) after 48 h, with a colour change in the medium due to the synthesis of secondary metabolites. *Trichoderma* spp. is known to produce antibiotics like Trichodermin, Trichodermol, Harzianum A, and Harzianolide (Dennis and Webster, 1971b; Kucuk and Kivanc, 2004), as well as cell wall degrading enzymes like chitinases and glucanases, which break down polysaccharides, chitins, and -glucanase, destroying cell wall integrity (Elad, 2000).

*Trichoderma* spp. produces volatile and non-volatile compounds that inhibit the growth of a variety of pathogenic fungi. The current study revealed that volatile metabolites of all the isolates produced more than 55 per cent inhibition of the pathogen. Against *P. aphanidermatum*, volatile metabolites of isolates TRPN-17 and TRPN-18 brought about the highest inhibition. *Pythium* spp. was controlled using virdin antibiotic generated by *T. viride* colonising pea seeds, according to Bhattacharjee and Dey (2014). Pakora *et al.* (2017) found that crude organic extracts from *T. viride* and *T. harzianum* reduced the mycelial growth and spore germination of the Oomycete pathogens associated with cocoa black pod *viz., Phytophthora palmivora, P. megakarya*, and *P. capsici*. Four *Trichoderma* strains, *T. erinaceum* (IT-58), *T. gamsii* (IT-62), *T. afroharzianum* (P8) and *T. harzianum* (P11) produced non-volatile organic compounds that inhibited *P. myriotylum* mycelial growth *in vitro* (Tchameni *et al.,* 2019). Hajieghrari et al. (2008) found that the volatile and non-volatile inhibitors generated by *T. hamatum* T612 inhibited radial growth of *F. graminearum* to the greatest extent. The appearance of an inhibition zone in dual culture without hyphal contact in *T. virens* T523 and *T. harzianum* T969 treatments suggested that the *Trichoderma* isolates secrete a diffusible non-volatile inhibitory chemical.

Because morphological features are limited due to culturing conditions such as temperature and the quality of the media used, molecular approaches must be used to identify the organisms (Hassan *et al.,* 2014). The Internal Transcribed Spacer (ITS) region is one of the most dependable markers for species-level identification (Kullnig-Gradinger *et al.,* 2002; Hassan *et al.,* 2019). Characterization using ITS region amplification revealed the identity of the ten novel *Trichoderma* isolates to be *T. harzianum* (TRMW-2, TRPN-3, TRPN-14, TRPN-17), *T. koningiopsis* (TRKR-2), *T. lixii* (TRPN-11, TRPN-15) and *T. asperellum* (TRPN-18). Sathiyavathi and Parvatham (2011) used partial 18S rRNA sequencing with the ITS primer set ITS 1/ ITS 4 target to the ITS region of the rDNA complex to characterize *Trichoderma* spp. on a molecular level, and discovered the presence of a novel strain named *Trichoderma* sp. MS 2010, which is an efficient producer of laccase and xylanase for industrial use. ITS PCR was used to detect an amplified rDNA fragment of 500-600 bp in *Trichoderma* sp. by Chakraborty et al. (2010). Shahid *et al.* (2013) used universal primers (ITS-1 and ITS-4) for the amplification of the 28SrRNA gene fragment to characterize the strains of *T. longibrachiatum*.

In the present study, talc-based formulations of all the isolates applied as seed treatment and as an additive in potting medium brought about successful control of pre-emergence and post-emergence damping off, among which TRKR-2, TRMW-2, and TRPN-11 were more effective compared to the treatments with other isolates. Several workers have demonstrated the successful control of seedling diseases by employing *Trichoderma* spp. As evaluated under controlled conditions, a mixture of antagonistic microorganisms, including *Trichoderma* spp. could provide the most effective suppression of soil-borne diseases caused by fungal and Oomycete pathogens (Spadaro and Gullino, 2005). *P. aphanidermatum*, the causal agent of beans damping off was inhibited efficiently by T105 strain of *Trichoderma* spp. (Kamala and Indira, 2011). *Pythium myriotylum* in tomato plants was found to be suppressed by *T. asperellum* (Mbarga *et al.,* 2012). Elshahawy and El-Mohamedy (2019) discovered that soil treatments with *Trichoderma* isolates' conidia, either alone or in combination, reduced the occurrence of tomato damping off and root rot caused by *P. aphanidermatum*. *T. asperellum* T24 was found to be an effective biological alternative to pesticides for the control of *P. capsici* in pepper in a study conducted by Segarra *et al.* (2013). Pythium damping-off has been effectively controlled by treating pea seeds with isolates of *Trichoderma* spp. (Harman *et al.* 1980; Wright, 1956). Earlier investigators have also reported that seed or soil treatment with isolates of *Trichoderma* spp. reduced damping-off induced by *R. solani* (Chet and Baker, 1981; Elad *et al.,* 1981; Karpagavalli and Ramabadran, 2001).

## CONCLUSION

From the present study, it can be concluded that the native isolates of *Trichoderma* spp. from virgin forest soils of Kerala could effectively reduce the pre- and post-emergence damping-off of tomato compared to the reference isolates. The promising isolates were characterized as *T. harzianum*, *T. koningiopsis*, *T. lixii* and *T. asperellum*. *Trichoderma* spp. being a bio-agent is not only an alternative to fungicides but also a safe, eco-friendly and sustainable option for plant disease management. As soil-borne diseases pose a serious threat to crop production all over the world, biocontrol interventions are very much essential. Further understanding of the field efficacy of these isolates, development of formulations, use of consortia, etc. will pave way for successful management of plant disease threats, thus improving overall plant health and productivity.

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