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Endophytic interactions of *Trichoderma harzianum* in a tropical perennial rhizo-ecosystem

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Abstract
This study demonstrates the endophytic interaction of the well-known growth promoting and biocontrol agent in black pepper. Trichoderma harzianum, coupled with its rhizosphere fungal flora was evidenced from metagenomics. We employed short-term and long-term strategies to study the interactions of T. harzianum in black pepper rhizosphere. In short-term strategy, T. harzianum was co-cultivated with axenic plantlets while pot culturing of plants in soil mixed with T. harzianum was performed in the long-term strategy. The colonization was investigated by light microscopy and scanning electron microscopy (SEM).

The co-cultivation of T. harzianum with black pepper showed the intercellular colonization at 24 h and formation of intracellular hyphae with vesicles at 48 h of interaction. The long term strategy inferred that T. harzianum was able to colonize the black pepper roots along with the AMF inter- and intra-cellularly. The whole genome metagenomic sequencing brings out the population abundance of the entire rhizosphere fungal flora.

Keywords: Fungal endophyte, Arbuscular micorhizal fungi, *Trichoderma harzianum* and *Piper nigrum*.

Introduction

Black pepper (*Piper nigrum* L.), popularly known as king of spices or black gold, is a highly valued perennial spice crop grown in tropical world. It is propagated vegetative means through stem cuttings. This export oriented spice climber succumbs to several diseases caused by fungi, bacteria and viruses.[29] Among these, foot-rot caused by the soil-borne oomycetous fungal pathogen, *Phytophtora capsici* is a major constraint for the healthy maintenance of this plant.[5] Crop loss due to this disease alone in Kerala, India (major centre of black pepper production) was estimated to range from 3.4 to 9.4%[3].

Infected plant debris in the soil and dried vines in the gardens are the primary source of inoculum of the pathogen.[2] Some of the black pepper associated bacteria such as *Psuedomonas aeruginosa*, *P. putida* and *Bacillus megaterium* were identified as effective antagonistic endophytes against the foot-rot disease[8]. But the mycelial fungus, *Trichoderma harzianum* is being widely used both in the nursery and field as successful integrated disease management component in India.[7,28,35]

Growth promotion[6,28,32,35] and disease suppression[26,28] activities of *Trichoderma* spp. on black pepper are manifold both in the nursery and field conditions. Despite beneficial claims, studies on the interactions of *T. harzianum* with the rhizosphere of black pepper are less attempted. Therefore, such interactions must be brought to the limelight so as to understand the nature of interaction of *Trichoderma* with the black pepper roots and its impact on other fungal population at the rhizosphere towards the expected beneficial effects.

Thus, the specific objectives of the present study were set as: (a) Examination of colonization behavior and the nature of interaction of *T. harzianum* at the rhizosphere of black pepper using the techniques of microscopy and (b) Correlation studies of rhizosphere soil metagenomics on fungal population pursuant to the inoculation of *T. harzianum*.

Material and Methods

Fungal inoculum: Talc formulation of *T. harzianum* MTCC 5179 obtained from the biocontrol laboratory, ICAR- Indian Institute of Spices Research, Kozhikode, Kerala was used for the pot culture study by mixing 3 g of talc with 3.5 kg of top soil. For co-cultivation study (liquid culture of *Trichoderma*), 72 h old culture on potato-dextrose-agar (PDA) plates was cut into 5 mm^2 discs and one such disc was inoculated in conical flasks containing 50 ml PD medium. After 10 days, 100 ml sterile double distilled water (ddH_2O) was added to the flasks and spore mass was scraped out to be used as inoculum for co-cultivation studies.

Co-cultivation (Short-term colonization)

Plant material: Single node cuttings from black pepper variety namely '*Sreekara*' were washed with tween-20 for 15 min and washed under running tap water. The cuttings were immersed in 0.2% copper oxychloride for 15 min followed by wash in sterile ddH_2O twice. The cuttings were surface sterilized with 0.1% mercuric chloride for 5 min on clean bench and then washed twice with sterile ddH_2O.

The cut ends were quick dipped in 8000 ppm IBA (indole butyric acid) and planted in plantons (7.5 × 7.5 × 10 cm, Himedia) filled with pre-sterilized perlite medium and fortified with sterile Hoagland's solution. The plantons were maintained in tissue culture room at 22 +/- 25°C and 3000 lux for the production of saplings.

Co-cultivation and Microscopy: Under aseptic conditions, the saplings were transferred to sterile petri-dish and roots were cleaned with sterile ddH_2O so as to remove the adhered perlite. Liquid culture of *T. harzianum* (10^9cfu/ml) was added to the *in vitro* saplings (only water added to control). The plantlets were maintained in the incubator shaker (Remi CIS 24 Plus, India) at 25ºC under constant shaking (115 rpm). Root samples (from replicas) were collected after 12, 24 or 48 h incubation and then rinsed in sterile water. They were fixed in 25% ethanol and stored at 4ºC. Toluidine blue and cotton blue staining techniques were performed to observe the extra- and intra-cellular colonization. These samples were subjected to SEM analysis for observing the interactions of *T.harzianum* with black pepper roots during exorhizal colonization. Root clearings were used to verify the endophytic colonization.[27]

Pot culture study (Long-term colonization)

Plant material: Cuttings were prepared as described for the short-term study. The cut ends of the cuttings were quick dipped in 8000 ppm IBA and planted on pre-sterilized perlite medium in protray fortified with sterile Hoagland's solution.[21] The protrays with the preparation as above were maintained in greenhouse with the top portion of protray sealed with aluminum foil. The cuttings were sprayed (foliar) daily with Hoagland's solution thrice. After 2 months of growth, when plants were with 4-5 leaves and 24 - 26 cm height; the perlite adhered to the rhizosphere was collected by gentle tapping and analyzed for the presence or absence of *Trichoderma* by plating (spread/pour plate method).

Subsequently, saplings free of *Trichoderma* were transferred to the pots filled with field collected top soil. The nutrient content of the soil was analyzed[34] as (minerals in ppm): 1.6% organic carbon; 1.64 P, 173 K, 197 Ca, 71 Mg, 11.38 Fe, 18 S, 5.56 Mn, 3.24 Zn, 0.92 Cu, 0.16 B and pH, 4.35. Two treatments (with and without *T. harzianum*) with 4 replicates having 3 plants per replicate were designed for the study. Growth parameters *viz.* height of the plant, stem girth (1 cm above from the soil region), leaf area index (LAI) and number of leaves were recorded.

The LAI was calculated using the formula: length (cm) × width (cm) × 0.6. After 120 days, plants were uprooted, the rhizosphere soil (adhered to the roots of pepper plants) samples were collected from 3 biological replicates of both treatment and control for metagenomics using Illumina hiseq. The weights of shoot and root (fresh and dry) were also recorded.

Root clearing: After 120 days of growth in the pots, the plants were uprooted; root samples were collected by cutting the roots at the collar region of the stem, washed in sterile ddH_2O, fully dried in hot air oven (at 60ºC for 16h) and stored in paper bags at 25ºC. For the analysis of colonization frequency, 25 root bits (~1cm in length) were taken randomly from *T. harzianum* treated and non-treated samples. Dried roots were rehydrated with sterile water for 1 h and then 10% (w/v) of KOH was added to roots and boiled in microwave oven for 10 min followed by rinsing with sterile ddH_2O.

Post clearing was performed with alkaline hydrogen peroxide [0.5% NH_4OH and 0.5% H_2O_2 (v/v) in ddH_2O] by boiling the roots in microwave oven for 5 min followed by rinsing with sterile ddH_2O and acidification using 1% HCl.[27] The roots were stained with 0.05% tryphan blue in lactophenol stain for 15 min, followed by destaining (lactic acid: glycerol: water in the ratio; 40:40:20) for 30 min and examined under microscope (Leica DM 5000 B, USA).

Sample preparation for SEM: Dried root bits were rehydrated with sterile ddH_2O for 1 h and two methods were adopted subsequently:

Method 1: The root bits were fixed with 2.5% glutaraldehyde for 2 h followed by wash with sterile ddH_2O twice for 30 min. Secondary fixation was done in 2 % paraformaldehyde in 1.0 M KH_2PO_4 and Na_2HPO_4 buffer (pH 7.2) and washed with the same buffer twice. Samples were dehydrated using a series of ethanol in ascending concentrations (25, 50, 75 and absolute alcohol for 30 min each).

Method 2*:* Root bits were fixed in 100% methanol for 1 h followed by dehydration using a series of ethanol in ascending concentrations (25, 50, 75 and absolute alcohol for 30 min each).

Processed root samples by either method were cut into thin sections (1 - 2 mm) using a fine scalpel and mounted onto the aluminum specimen stubs using double-adhesive coated carbon tabs and gold sputtering was performed using ion gold sputtering unit (20 sec). The samples were then viewed and the images were micro graphed using Hitachi SU6600 field emission scanning electron microscope (Hitachi, Japan).

Metagenome sequencing, assembly and annotation: The rhizosphere soil DNA was extracted from 100 mg of rhizosphere soil using MoBio kit (MO BIO Laboratories, Inc USA) according to the manufacturer's instruction. DNA was isolated from three biological replicas (from control and *T. harzianum* inoculated plants) pooled for analyses**.** Two µg of DNA from each sample was used for the library preparation using NEB Next ultra DNA library prep kit for Illumina. Sequencing of the paired end library was done in illumina Hiseq platform. The sequences were assembled with RayMeta[10] using a k-mer size of 31 using de-bruijn graph method. Filtered contigs with more than 150 bp length were used with Glimmer-MG v 0.3.2[23] to predict the protein coding regions in the contigs. Functional annotation was done using Diamond v 0.7.9[11] for predicted genes against the protein database using the BLAST version 2.2.29+, with an e value of 1e-5. [4]

Statistical Analysis: For the growth parameters, the experimental design adopted was CRD and the data were analyzed by *t*-test. The differential abundance of fungi (metagenome) between the samples was calculated using G-test (w/Yates') + Fisher's test for two sample analysis in STAMP tool.

Results

Growth Promotion: Growth parameters *viz*. the fresh root, fresh shoot, dry root, dry shoot, LAI and height of the plant were significantly increased upon probiotic application of *Trichoderma harzianum* MTCC 5179 when compared to control (Table 1).

Co-cultivation (Short-term colonization study)

Bright field Microscopy and SEM: The external colonization of *T. harzianum* occurred on the roots of the *in vitro* derived pepper plants as surface adherence at 12 h of co-cultivation. The mycelial spread was increased with increasing time (at 24 h and at 48 h) with profusely growing mycelia bearing chlamydospores as evidenced from toludine blue staining (Figure 1 a, b andc). Upon cotton blue staining, intercellular colonization was found at 24 h incubation (Figure 1 d). *T. harzianum* established endophytic colonization inside the cell at 48 h with luxurious intracellular mycelia (Figure 1 e). No fungal growth was observed (external or internal) on the root tissues of control plant.

Root clearings of plants inoculated with *T. harzianum* showed fungal mycelium in the intercellular spaces at 24 h and hyphal tips as dark blue granules inside the cell (Figure 2 a) and intracellular chlamydospores were observed at 48 h (Figure 2 b). Analysis by SEM showed intact cell structures in control roots (Figure 3a) but *T. harzianum* inoculated samples taken at 24 h and 48 h showed an indication of the interaction with the root cells (Figure 3 b and c) and hyphal growth on the surface at 24 h (Figure 3d). At 12 h, the enlargement of hyphal tip as papillae showed its interaction for intracellular colonization (Figure 3e). Massive colonization was observed on root surface at 48h (Figure 3 f). No fungal growth was observed on the surface of the root tissues of control plant (data not shown).

Pot culture study (Long-term colonization)

Bright field microscopy: Roots of control plants showed no fungal colonization externally but *T. harzianum* treated plants showed invasion of mycelia. Root sections of treated plants stained with cotton blue showed AMF vesicles and arbuscules while the control samples showed a few arbuscules with no vesicles (Figure 4 a and b). The arbuscules in control were localized to the zone of elongation of the root. In general, presence of mycelia was found increasing with maturation of the root tissue.

The root tip meristem showed no colonization; however, the elongation zone showed intercellular colonization with more number of vesicles and the maturation zone was densely colonized by inter- and intra-cellular hyphae with comparatively less number of vesicles and large number of arbuscules.

The AMF mycelia were intercellular i.e. along the tangential plane of the cortex in the elongation zone. The mycelia in this region were larger in size and rarely septate were only of AMF, no *Trichoderma* mycelia were seen in this region but it was observed only in the maturation zone of the root as septate mycelium along with the AMF (Figure 4 b and c). *Trichoderma* in this region was found with conidia (Figure 4 d and e). This zone of maturation of root had structurally differentiated AMF mycelia with prominent septation along with monilioids hyphae (Figure 5c).

Microsclerotia were also observed inside the root cells (Figure 5b). The colonization of AMF was found to be that of Arum type (Figure 5a). This portion of roots had vesicles with round, oval, ellipsoidal and irregular shapes (Figure 5d). Some vesicles were originated from monioloid hyphae. The size of the AMF vesicles was ranged from 40 - 147µm.

Compared to control, the treated plants showed higher mycorhizal frequency (100%) after four months of pot culture. The average number of vesicles was ~ 40 per 1 cm root tissue. Interestingly, the root hairs showed no internal mycelium in both the samples though AMF mycelia were present on the external surfaces (data not shown).

Analyses using SEM: Comparing two sample preparation methods employed for the SEM, the methanol fixation was found better in terms of good cell structure which aided visual observation of bacteria and fungus whereas glutaraldehyde fixation distorted the surface structures (Figure not shown). Hence, methanol fixation was taken for further analysis of black pepper root samples by SEM. The root sample from the control showed weak adherence of organisms on the surface (Figure 6a) while the treated roots showed abundant adherence of organisms on the surface upon imaging with SEM (Fig. 6b).

Population abundance evidenced from metagenomics: From the entire profile of fungal flora of metagenome, only 10 most abundant species were taken for the analysis in this study (Fig. 7). Among them, four were endophytes *viz*. *Pestalotiopsis fici, Oidiodendranmaius, Rhizophagus* sp. and *T. harzianum* in which the *Trichoderma* reads were present only in *Trichoderma* inoculated soil and not in the control.

The population abundance of the fungal species with biocontrol potential showed that *Fusarium oxysporum* ($p = 0.013$), *Pestalotiopsis fici* ($p = 0.443$) and *Talaromyces stipitatus* ($p = 0.219$) were high in *T. harzianum* treated soil metagenome. The metagenome of the control sample was high in *Rhizophagus irregularis* ($p=0.034$), *Pseudogymnoasus pannarum* (human pathogenic fungus) ($p=0.488$) and *Oidiodendran* sp. ($p=0.484$).

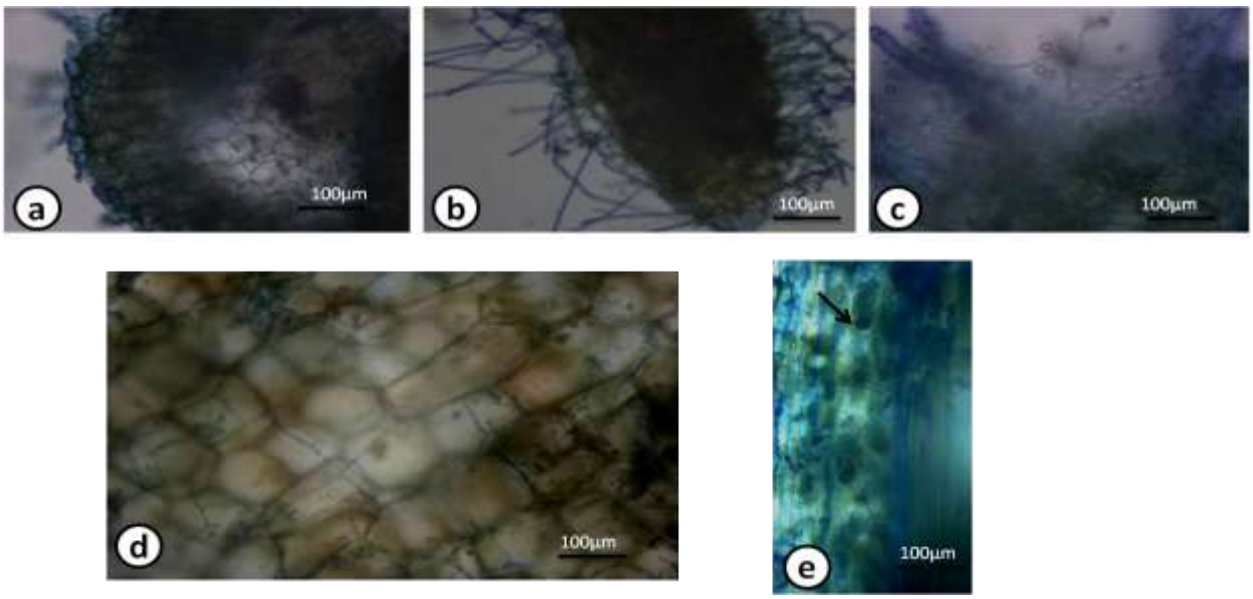

Fig. 1: Extracellular attachment and intracellular interaction of *T. harzianum* during co-cultivation (short-term colonization) with *in vitro* derived black pepper plants. Fig. a to c: toludine blue staining of root tissue - (a) control, (b) after 24 h incoculation and (c) after 48 h inoculation showing hyphae and chlamydospores on the surface of the root section. Fig. d and e: cotton blue staining of root sections - (d) intercellular growth at 24 h and (e) intracellular colonization (arrows) at 48 h.

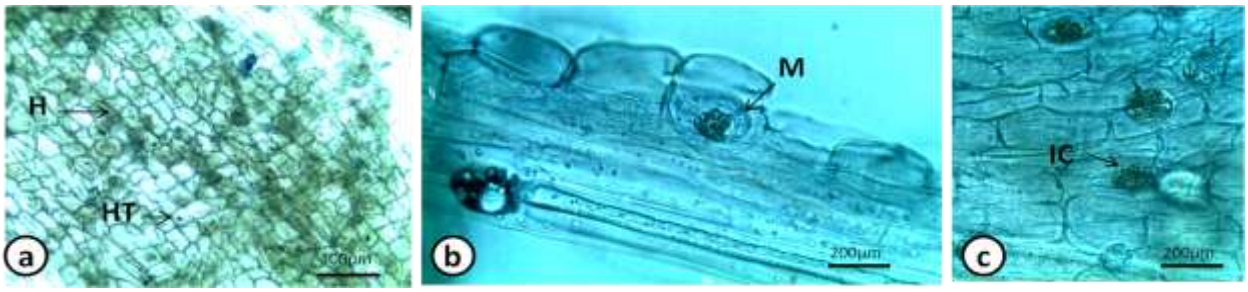

Fig. 2: Bright field micrographs on root clearing of black pepper after co-cultivation with *T.harzianum* showing endophytic interaction. Samples were taken at 12, 24 and 48 h on inoculation, and stained with cotton blue. (a) Inter- and intra-cellular colonization at 24h; (b) and (c) Intracellular colonization at 48h. Hyphae (H), hyphal tips (HT), mycelium (M) and intracellular structure (IC) are marked.

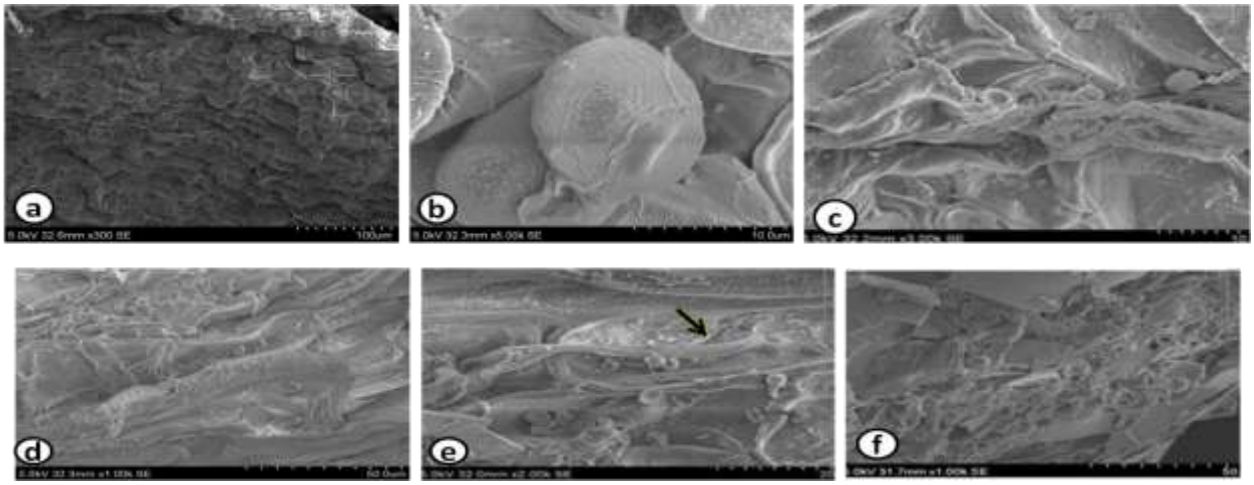

Fig. 3: SEM micrographs taken after co-cultivation of *T. harzianum* 5179 with *in vitro* derived black pepper plant roots; samples taken at 12, 24 and 48 h of inoculation fixed with methanol. (a) control (without *T. harzianum*); (b) spore on cell suface; c) surface attachment at 12 h; (d) hyphal growth at 24 h; (e) enlargement of hyphal tip (arrow-papilla) and (f) dense colonization at 48h.

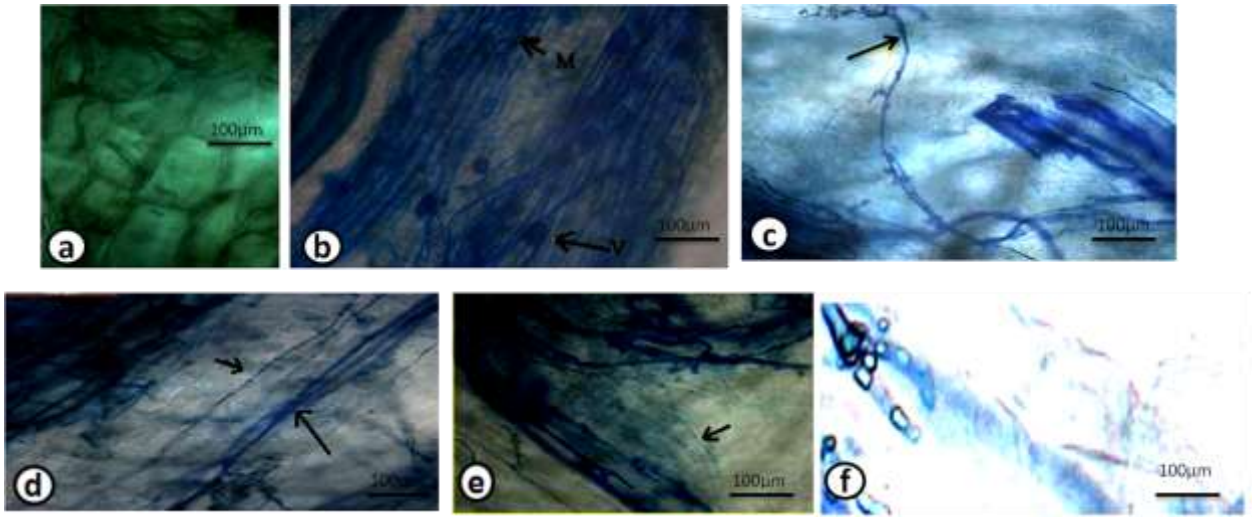

Fig. 4: Images of *T. harzianum* inoculated plant roots with bright field microscopy after 4 months of growth (long-term colonization) in pot culture. The dried roots were cleared and stained with cotton blue. (a) control; (b) *T. harzianum* inoculated; (c) arrow showing extra radicular AMF hyphae; (d) *T. harzianum* (short arrow), AMF (long arrow) hyphae; (e) arrow showing *T. harzianum* mycelium and (f) conidiophore with conidia. Vesicles (V) and mycelium (M) are marked.

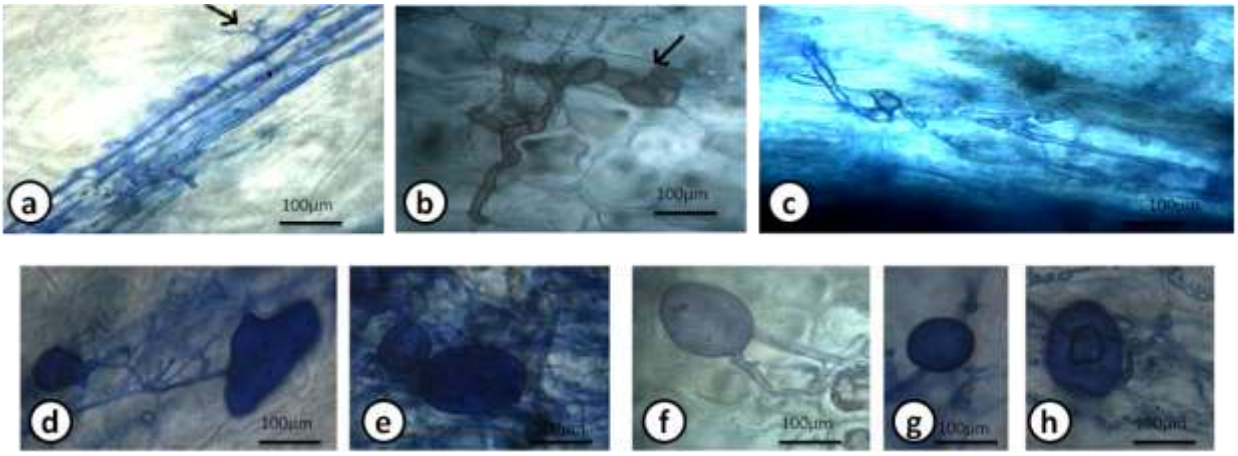

Fig. 5: Maturation zone of black pepper roots showing different AMF structures during long-term colonization (4 months) of *T. harzianum* (a) Tree-like intracellular arbuscules (arrow showing arum type growth of AMF mycelium, (b) intracellular microsclerotia; c) moniliod hyphae of AMF and (d) different shapes of AMF vesicles at the maturation zone of roots.

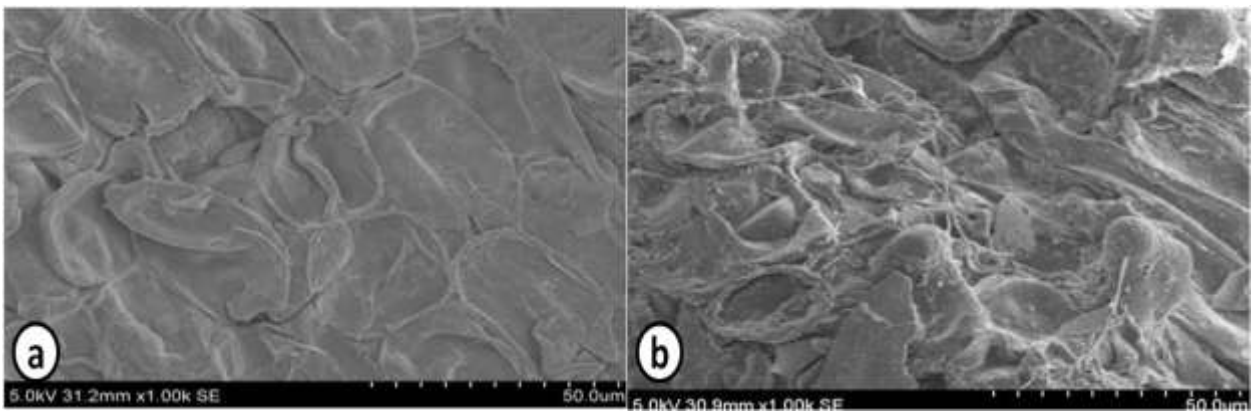

Fig. 6: Scanning electron micrograps of black pepper roots fixed with methanol. (a) Root sample from pots without *T. harzianum* inoculation showing few microbes on the surface and (b) with *T. harzianum* inoculation showing abundant microorganisms on the surface (samples taken after 120 days with or without inoculation of *T. harzianum*).

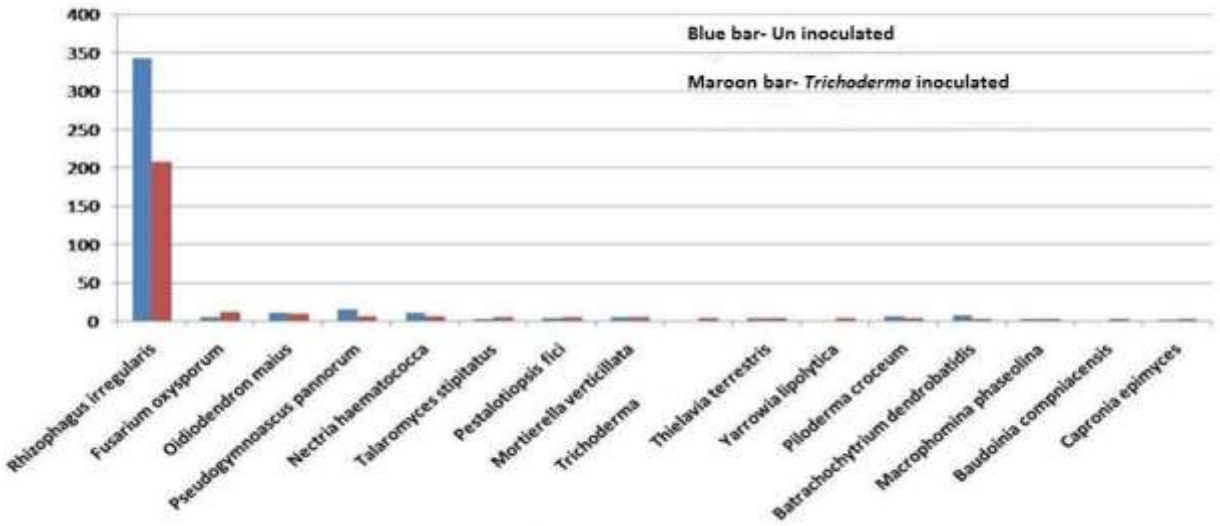

Fig. 7: Abundance of fungal species as observed from the metagenome of black pepper rhizosphere soil using illumina hiseq whole genome metagenomics sequencing.

Table 1
Growth parameters of black pepper with and without *Trichoderma* inoculation

S.N.	Parameters observed	T1 Mean (with *Trichoderma)*	T2 Mean (without *Trichoderma*)	Pr> (*t*)
1	Shoot weight (Fresh)	7.7	3.0	<.0001
2	Root weight (Fresh)	44.5	26.6	0.0050
3	Leaf area index (LAI)	802.5	430.4	0.0028
4	Stem Girth	0.1225	0.1400	0.3896
5	Height of the plant	78.5	44.4	0.0023
6	Root weight (Dry)	1.7	0.7950	0.0018
7	Shoot weight (Dry)	9.9	4.3	0.0003

Discussion

The prime objective of this study was to elucidate the nature of colonization of *T. harzianum* in black pepper both at the anatomical level using microscopic techniques and at molecular level employing metagenomics. When we attempted the study, to our surprise, its endophytic colonization accompanied by AMF was luxuriant upon application of *Trichoderma*.

Co-cultivation study showed that *T. harzianum* is efficiently colonizing the plant roots endophytically. Chacon et al[13] demonstrated intercellular ramification of *T. harzianum* hyphae in the root cells of tomato. They found that some cells were colonized intracellularly only after 48h and occurrence of yeast like structures after 72h treatment. Many *Trichoderma* spp. infecting cocoa plants *viz.T. ovalisporum*[22], *T. paucisporum*[31], *T. evansii*[30] and *T. martiale*[19] were identified as endophytes. TEM[39] showed direct root penetration of *Trichoderma* spp. in cocoa plant. Papillae - the swollen hyphal tips - were also reported in the interactions of *T. harzianum* with tomato roots during adherence[13]. We showed the appearance of papillae on root surfaces at 24 h of inoculation suggesting its quick interaction with the black pepper root system.

The phenomenon of biocontrol and growth promotion does not occur in all *Trichoderma*-host interactions; for instance[16] endophytic colonization of cocoa by *T. stromaticum* was unable to induce plant growth and was resistant to *Magnoporthe perniciosa*. However, some species of *Trichoderma viz. viride, harzianum* and *pseudokoningii* were found promoting growth in cucumber, corn, petunia and pea.[20,37,38] Apart from growth promotion, the endophytic colonization of *T. hamatum* in cocoa was found inducing drought tolerance in the plant[9]. In the present study, the growth promotion was also found to be enhanced by the co-colonization as evidenced from increase in growth parameters (fresh root, fresh shoot, dry root, dry shoot, LAI and height of the plant) in *Trichoderma* treated plants when compared to control which was not showing any co-colonization.

The maturation zone of the black pepper roots showed moniliod hyphae along with structurally differentiated AMF mycelium with prominent septation. Moniliod hyphae of dark-septate fungus in the aquatic angiosperm, *Eorhiza arnoldii* could produce diverse moniliod assemblages[24]. Microsclerotia were also observed inside the cells of pepper and the colonization of AMF was found as Arum type [1]. The region of maturation showed vesicles with different shapes.

We were able to show the *Trichoderma* mycelium and conidia along with the AMF mycelium and vesicles - suggesting co-colonisation inside the roots of black pepper.

The interaction between AMF and *Trichoderma* has been elucidated in many studies: Filion et al[18] reported that *Glomus intraradices* stimulated the conidial germination of *T. harzianum* and Datnoff et al[14] observed a synergistic interaction between them in tomato. Co-inoculation of *T. harzianum* and *T. aureoviride* decreased the time to vegetative sporulation in axenic cultures of these mycorhizal species. Synergistic effect between *G.intraradices* and *T. aureoviridae* in enhancing the growth in citrus in organic substrate has been reported which was higher than the individual effect of *G. intraradices.*[12]

Since the experiment was set up in field soil, the observation is that the roots inoculated with *Trichoderma* had abundant VAM colonization as indicated by the presence of both vesicles and arbuscules. It indicates the native mycorhiza colonizing pepper roots without any hindrance by inoculated *Trichoderma harzianum* MTCC 1579 suggesting the inoculated *Trichoderma* has facilitated mycorhizal colonization where as in the control soil though there was AMF colonization, it was sparse. The native beneficial microbes like mycorhiza had equal opportunity of colonizing black pepper roots the fact that in *Trichoderma harzianum* inoculated roots, the presence of more AMF suggests its active role in helping mycorhizal colonization.

As no choice experiment with insect pests, DeJaeger et al[15] indicated mycoparasitism in one to one interaction. *Trichoderma harzianum* being a saprophyte and opportunistic antagonist [20] in the absence of other nutrient source for its survival perhaps would have colonized AMF. In an experiment by Sibi[32] where selected compatible (*in vitro*) consortia of PGPR (*Pseudomonas mendocina, Bacillus pumilus, Serratia marcescens* and *Rhizbium* sp) inoculated on black pepper rhizosphere, the population of *S. marcescens* was declined to zero when compared to the population in *in vitro* experiments in which the PGPR consortia was compatible suggesting the role of rhizosphere in selecting and maintaining the organisms.

Methanol fixation was found to be good for sample processing for SEM. The attachment of bacterial/fungal population on the surface of *Trichoderma* treated roots showed the rhizosphere competence of soil organisms with *Trichoderma*. Many bacterial cells were found adhering to the surface which was absent in the control roots. With *Arabiodpsis* and barley, Talbot and White[33] also found that methanol-based method was superior to other fixation methods of samples for analysis by SEM.

Reports on growth promotion effect of VAM on black pepper showed that *G. fasciculatum* incorporation as infective propagules (cultured on Rhodes grass) increased the rooting of black pepper at nursery condition.[6] The authors showed more than 80% colonization of *G. fasciculatum* in black pepper roots than control. Detailed study on the effect of biocontrol agent on hardening of tissue cultured black pepper with VAM demonstrated that the treatments wherein *G. fasciculatum* and *T. harzianum* were inoculated showed higher root and shoot mass compared to control.[32]

The growth promotion effect on plantlets inoculated with species of *Pseudomonas, Rhizobium* and *Trichoderma* was checked in the presence or absence of VAM and it was found that the overall growth with VAM was higher than that without VAM.[32] Compared to the effect of *Trichoderma* in black pepper, the treatments inoculated with *Pseudomonas* or *Rhizobium* alone with or without VAM recorded low profile on growth promotion which further indicated the synergistic effect of this fungus.

The comparison of *Trichoderma* treatments with or without VAM recorded higher growth promotion in *Trichoderma* (alone) without VAM suggests the principal action of *T. harzianum* in helping the native VAM fungi present in the soil to colonize the black pepper plants. This was evident from our results with microscopy wherein we showed the endophytic colonization (100% colonization frequency) of AMF along with *Trichoderma* mycelium in *Trichoderma* inoculated soil compared to control.

Metagenomics on fungal population showed population abundance of beneficial fungi *viz*. *Fusarium oxysporum*¸ *Pestalotiosis fici* and *Talaromyces stipitatus* which may impart biocontrol property in *T. harzianum* inoculated plant rhizosphere than in control. Increased population of *Fusarium* spp. showed biocontrol and disease suppression in the rhizosphere of flax.[17] *P. fici*, an endophyte could produce bioactive metabolites and natural products in tea.[36] *Talaromyces* spp. are reported as biocontrol agents against species of *Verticillium and Rhizoctonia* in tomato and potato.[25] Metagenome analysis showed that the AMF *(Rhizophagus irregularis)* was higher in control but less in *Trichoderma* inoculated soil. Microscopic observation on the internal colonization of AMF between these treatments shows increased endophytic colonization of AMF upon *Trichoderma* inoculation.

Although the *Rhizophagus irrregularis* was abundant in rhizosphere soil of control, it had not colonized the tissue to get the benefit of symbiosis from the plant and also the high abundance of this AMF species (*Rhizophagus irregularis)* was ineffective in increasing plant growth in control. The reason for non-colonization in control is not clear where as when *Trichoderma* inoculated root AMF colonization was also facilitated as *Trichoderma harzianum* was known as helper organism for VAM with increase in plant growth. Further targeted studies are needed to understand the time bound interaction of AMF in rhizosphere upon inoculation of *Trichoderma harzianum* MTTC 5179 towards the AMF species abundance.

Conclusion

This study demonstrated the localization and endophytic colonization of *T. harzianum* MTCC 5179 in black pepper. Enhanced AMF root colonization by the *Trichoderma* inoculation in black pepper indicates that *T. harzianum*acts as helper organism in the root ecosystem of black pepper for colonizing AMF on the plant. Moreover, the native microbes that are selectively recruited by black pepper under the *Trichoderma* influenced rhizosphere would have helped to mobilize nutrients and enhanced, the growth. Further, detailed studies on *Trichoderma*, AMF and native microflora with the host in a multipartite interaction would help in developing targeted biocontrol strategy to overcome soil borne pathogens.

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