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Root colonization studies to elucidate the endophytic association of moisture stress tolerant *Trichoderma* isolates in black pepper (*Piper nigrum* L.)

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Abstract

In the present study, endophytic colonization of moisture and temperature tolerant isolates of *Trichoderma viz*., *T. asperellum* (IISR NAIMCC 0049), *T. erinaceum* (IISR APT1), *T. harzianum* (IISR APT2), *T. harzianum* (IISR KL3), *T. lixii* (IISR KA15) and *T. asperellum* (IISR TN3) were studied under *in vitro* conditions. Single node stem cuttings of black pepper (variety IISR Thevam) grown in culture bottles were inoculated with the spore suspensions of *Trichoderma*. Root samples were collected from treated and untreated plants at 24h, 48h, 72h, 3rd, 5th, 7th, 14th, 21st and 28th day after inoculation (DAI). During the early stages of colonization, pre-germinated conidia were abundantly observed on the root surface, subsequently, hyphae penetrated the root system. Among the six tested isolates, *T. harzianum* (APT2) exhibited the earliest intercellular colonization, initially observed in the cortical zone, progressively advancing to the vascular system, followed by intracellular colonization. Whereas the isolates, *T. harzianum* (KL3), *T. erinaceum* (APT1), and *T. asperellum* (TN3) exhibited endophytic colonization only from the 5th DAI. *T. harzianum* (APT2) also demonstrated the highest colonization frequency, while *T. asperellum* (TN3) showed the lowest. Findings of the study highlight the significant variation in root colonization capacities among moisture tolerant *Trichoderma* isolates. The endophytic association of these *Trichoderma* isolates may play a vital role in enhancing resistance to both biotic and abiotic stresses in black pepper.

Keywords: Black pepper, *Trichoderma*, colonization, abiotic stress, biotic stress

Introduction

Trichoderma has developed multiple mechanisms that enhance plant resistance to diseases, promote growth, and improve overall productivity (Valiyambath *et al*. 2024; Enshasy *et al*. 2020; Kapri and Tewari, 2010; Hermosa *et al*. 2012). The ecological adaptability of *Trichoderma* species is evidenced by their prevalent distribution, including under different environmental conditions and on various substrates (Contreras-Cornejo *et al*. 2013). This physiological flexibility together with the antagonistic action of *Trichoderma* spp.

against phytopathogenic fungi facilitated to be used as biological control agent (Druzhinina *et al*. 2011; Kredics *et al*. 2014; Mukherjee *et al*. 2013). Studies indicate that *Trichoderma* spp. could manage abiotic stresses by multiple beneficial effects. Mastouri *et al* (2012) observed enhanced antioxidant defense of tomato seedlings and resistance to water deficit facilitated by *Trichoderma harzianum.*

Black pepper, often referred to as the "King of Spices," is one of the most widely used and valuable spices in the world. Native to the Western Ghats of India, black pepper holds significant medicinal, and economic importance and remains a key ingredient in global cuisines (Vijayan and Thampuran, 2000; Gulcin, 2005; Zou *et al*. 2015). In the current scenario of climate change, abiotic stresses are the major challenges faced by black pepper production systems (Kandiannan *et al*. 2014; Sen *et al*. 2016). Plants under environmental stress suffer losses in their photosynthetic competence through damage to photosystems and other cellular processes triggered by reactive oxygen species (ROS). Valiyambath *et al*. (2024) reported that under moisture stress, black pepper plants inoculated with *Trichoderma* species accumulated higher levels of secondary metabolites, including proline, phenols, malondialdehyde (MDA), and soluble proteins. Studies indicated that plants inoculated with *Trichoderma* respond to salinity stress by modifying physiological and biochemical parameters, which ultimately leads to the restoration of cellular homeostasis, detoxification of toxins and recovery of growth (Brotman *et al*. 2013; Shao *et al*. 2009).

Trichoderma species are primarily regarded as saprophytic soil inhabitants, though some function as opportunistic, avirulent symbionts in plants (Vargas *et al*. 2009, Viterbo and Chet, 2006; Mendoza-Mendoza *et al*. 2018; Morán-Diez *et al*. 2009). Regarding root colonization, *Trichoderma* has been predominantly studied in non-woody plants, such as tomato (Chacón *et al*. 2007; Mastouri *et al*. 2012), cucumber and wheat (Sarrocco *et al*. 2021) and *Arabidopsis thaliana* L. (Alonso-Ramírez *et al*. 2014). Endophytism was also reported from other plants such as olives, cacao etc. (De Souza *et al*. 2008; Ruano-Rosa *et al*. 2016; Zachow *et al*. 2010). Root size and architecture are the factors which determine yield performance, particularly under conditions of limited water availability (Malinowski and Belesky, 2000). The root colonization by *Trichoderma* increases the growth of roots and of the entire plant, thereby increasing plant productivity. *Trichoderma* possesses an endophytic nature though usually limited to superficial layers when colonizing plant roots (Hohmann *et al*. 2011) but no detailed anatomical studies on endophyte–host plant interactions have so far been conducted.

Anatomical studies have proven to be highly effective in understanding the interactions between beneficial microorganisms and their host plants. These studies are particularly valuable for comparing the efficiency of different microorganisms, providing essential insights before developing new strategies for their application in the field. (Massicotte *et al*. 2005); Recent research has identified isolates of many *Trichoderma* spp. that are endophytic on *Theobroma cacao* including aboveground tissues (Bailey *et al*. 2008). Colonization of cacao seedlings by endophytic *Trichoderma* resulted in a delay in many aspects of the drought response.

Studies indicate that *Trichoderma* can colonize plant roots, inducing changes in plant defence systems and plant physiology without destroying plant tissues. The present study aimed to elucidate the endophytic association of moisture-tolerant *Trichoderma* isolates in black pepper, as successful colonization and persistence in the target niche are crucial for their effectiveness against both abiotic and biotic stresses.

Materials and methods

Revival and maintenance of culture

Trichoderma isolates, *viz*., *T. asperellum* (IISR NAIMCC 0049), *T. erinaceum* (IISR APT1), *T. harzianum* (IISR APT2), *T. harzianum* (IISR

KL3), *T. lixii* (IISR KA15) and *T. asperellum* (IISR TN3), shortlisted for temperature and moisture stress tolerance were maintained in Biocontrol laboratory, ICAR-Indian Institute of Spices Research, Kozhikode (Valiyambath *et al*. 2024). The six isolates were revived from stock and maintained on PDA plates and slants for the present study.

Preparation of *Trichoderma* conidia suspensions

Five mm discs of three days old cultures (2nos) were inoculated into 150 ml PDB medium in Roux bottles. The inoculated bottles were incubated at 26 ± 2ºC for 7-10 days. After incubation, the medium was removed by decanting under sterile conditions. Later the mycelial mat was washed with 100 ml sterile distilled water and the washing was repeated. The spore suspension thus obtained was centrifuged at 4400 rpm, 25ºC for 10 min. The concentration was adjusted to 1×10^7 spores/ml with haemocytometer and was used for inoculation.

***In- vitro* inoculation of black pepper plants**

Plug trays were filled with double autoclaved perlite and sand in 2:1 proportion. Individual cuttings of black pepper (variety IISR Thevam) were transplanted at single node stage to plug trays. The plants were watered and maintained for two weeks for proper root establishment. A modified Hoagland solution (0.1%) was prepared using $KHPO_4$, KNO_3, $MgSO_4$, $CuSO_4$, $MnSO_4$, $FeSO_4$, H_3BO_3, and $ZnSO_4$ (Hoagland and Arnon 1950; Sitepu and Mustika, 2000). A volume of 30 ml of the solution was dispensed into culture bottles and sterilized at 121°C for 15 minutes. Stem cuttings from plug trays were uprooted, washed thoroughly to remove soil particles, surface sterilized with 0.1 % sodium hypochlorite solution and rinsed with sterilized water. The sterilized plants were placed in culture bottles, ensuring complete immersion of the root portion in the solution, and maintained under sterile conditions (Fig. 1). Later, 1ml of spore suspension of six test isolates, namely *T. asperellum* (NAIMCC 0049), *T. erinaceum* (APT1), *T. harzianum* (APT 2), *T. harzianum* (KL3), *T. lixii* (KA15) and *T. asperellum* (TN3), was inoculated near the root tip. The upper portion of each bottle was securely sealed with sterile parafilm and a control set of plants was maintained with an equal amount of sterile water. Six replicates were maintained for each treatment and the entire experiment was repeated twice (Rouws *et al*. 2010).

Fig. 1. IISR Thevam grown in culture bottles; Control (1), inoculated with TN3 (2), KL3 (3), APT2 (4), NAIMCC009 (5), KA15 (6), APT1 (7).

Root sampling, fixation, wax embedding and microtome sectioning

Root samples were collected from both treated and control plants at 24h, 48h, 72h, 3rd, 5th, 7th, 14th, 21st and 28th DAI. Root bits of approximately one cm size were pooled from collected samples and made into four sets of root regions (0-2 cm, 2-4 cm, 4-6 cm, 6-8 cm from the tip of root). Then the root bits were surface sterilized with 1% sodium hypochlorite followed by two repeated washes in sterile distilled water.

Root bits were fixed by immersion in a formaldehyde solution (37–41% w/v) for 12 hours at room temperature. The samples were then removed from the formalin and thoroughly washed with water (Slaoui and Fiette, 2011). Wax embedding of root tissues was performed using non-caking paraffin wax, which was melted at 56°C and allowed to solidify in a plastic mould. For cross-sections, paraffin blocks measuring 3.5 cm × 1.5 cm were prepared by positioning root bits (approximately 1 cm in size) perpendicular to the length of the

mould. Similarly, for longitudinal sections, root samples were placed parallel to the length of the block. Once solidified, the blocks were chilled by placing them over melting ice before sectioning. Separate blocks were prepared for each treatment.

Sectioning was performed using a Leica semi-automatic rotary microtome, beginning with the trimming of wax to a thickness of 30 µm. The block was then placed on ice for 1–2 minutes to maintain firmness. Ribbon-like sections were subsequently cut with variable thicknesses ranging from 5 µm to 20 µm, depending on the size of the root bits. The serial sections obtained were carefully transferred to warm water (45–55°C) to separate them from the wax without causing damage to the tissue. Wax debris was regularly removed from the knife using alcohol, and the blade was changed at intervals to ensure precise sectioning.

Root clearing, staining and microscopic observation

The sampled roots were cleared using 10% (w/v) KOH in a water bath at 60°C for 1 hour, followed by an immediate wash with sterile water for 30 minutes (Lux *et al*. 2005; Sonneveld and Voogt, 2009). Uniformly thin sections were then carefully selected from the floating water and placed on a clean glass slide. Similarly, fifty root sections were randomly chosen from each plant at each incubation period and mounted on glass slides for further analysis. A drop of 0.05% lactophenol cotton blue stain was placed on the root bit and kept for 10 minutes. Three types of root tissue samples were observed: longitudinal, transversal and secondary roots without sectioning. The slides were examined under a bright-field microscope (Leica) to observe the hyphal structures of *Trichoderma* in black pepper root tissues. Images were captured at 10X magnification and compared.

Isolation of *Trichoderma* spp. from the roots of *in vitro* inoculated plants

To isolate *Trichoderma* spp., root samples from *in vitro* inoculated plants were carefully collected and washed thoroughly with sterile distilled water to remove any surface contaminants. The roots were then surface sterilized using 1% sodium hypochlorite for a brief period, followed by multiple rinses with sterile water. The washed bits were kept on a sterile filter paper and then transferred aseptically onto solidified *Trichoderma*-selective medium (TSM). The plates were then incubated at 26 ±1º C for 4-7 days and were observed for the presence of *Trichoderma* growth from the inoculated bits on the plates.

Fig. 2. Black pepper (IISR Thevam) plants grown in plug trays.

Effect of shortlisted *Trichoderma* isolates on growth parameters under different moisture levels

Three-week-old black pepper cuttings (var. IISR Thevam) were uprooted, surface-sterilized, and replanted into plug trays with sterile potting mix under greenhouse conditions for two weeks. Each *Trichoderma* isolate was tested with six replicates (Fig. 2). A substrate of farmyard manure and corn flour (7:1) was moistened, sterilized, and inoculated with *Trichoderma* discs, then incubated at 26 ± 2°C for two weeks and shaken daily for uniform fungal growth. Moisture stress was imposed at two levels—field capacity (FC) and 75% FC—by withholding water for three days, then maintaining desired moisture levels. Plants were inoculated with 10 g of *Trichoderma* culture per plant, with uninoculated plants as controls. Growth parameters including shoot/root length, number of leaves, and fresh/dry weights were recorded 3 MAI (months after inoculation).

Isolation of *Trichoderma* spp. from the roots of greenhouse inoculated plants

Roots were initially rinsed with distilled water, small sections were cut, surface-sterilized using 1% sodium hypochlorite, followed by thorough rinsing with sterile distilled water. The sterilized root bits were placed on PDA plates supplemented with streptomycin and incubated at 28 ± 1°C.

Results

Root tissue staining and microscopy

All tested isolates adhered to the root surface and subsequently colonized the root system at varying time intervals, ranging from 24 hours to 5 DAI. During the initial phase, (24 hours after inoculation), pre-germinated conidia of three isolates, *T. asperellum* NAIMCC0049, *T. harzianum* APT2, and *T. lixii* KA15 were abundantly observed on the root surface, with some penetrating the root interior and localizing in the outer cortex (Fig. 3a & 3b).

Three weeks after inoculation with *Trichoderma*, hyphae were abundantly present in the epidermis and outer cortical layer but were less frequent in the inner cortical layer and completely absent in the vascular cylinder. *Trichoderma* spp. was not observed in the roots of uninoculated black pepper plants.

Intercellular and intracellular colonization by *Trichoderma* spp.

(a) T. harzianum IISR APT2

Among the six tested isolates, *T. harzianum* APT2 exhibited the earliest signs of intercellular colonization. This isolate also demonstrated a superior colonization rate throughout the experimental period. At 24 hours after inoculation, pre-germinated conidia of *T. harzianum* APT2 were abundantly observed on the root surface, with some penetrating the root interior and localizing in the outer cortex. At 48h after inoculation hyphal swellings were observed and later hyphae had spread extensively, covering large portions of the root surface. This proliferation indicated an active and sustained interaction between *Trichoderma* and the root tissues. From seventh day after inoculation, a dense mass of hyphae was observed in the cortical region. At 21 DAI, chlamydospores had become the most abundant fungal structure, covering large areas of the black pepper root epidermis (Fig.4a & 4b).

The progressive colonization pattern suggests that *Trichoderma* initially establishes itself externally before systematically infiltrating the root structure, enhancing its symbiotic association with the host plant.

(b) T. asperellum IISR NAIMCC0049 & T. lixii IISR KA15

The colonization pattern of isolates *T. asperellum* IISR NAIMCC0049 & *T. lixii* IISR KA15 were similar and at 24 hours after inoculation, hyphae were prominently observed on the epidermis of the black pepper roots. The first signs of intercellular colonization became evident in the cortical zone, with the hyphae progressively advancing towards the vascular system. However, colonization within the vascular tissue was not observed. By 48 hours after inoculation, intracellular colonization

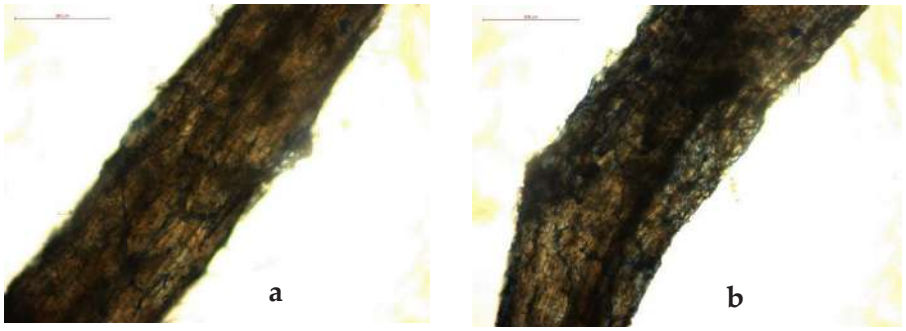

Fig. 3. Root surface colonization by **a)** *T. asperellum* NAIMCC0049 **b)** *T. harzianum* IISR APT2.

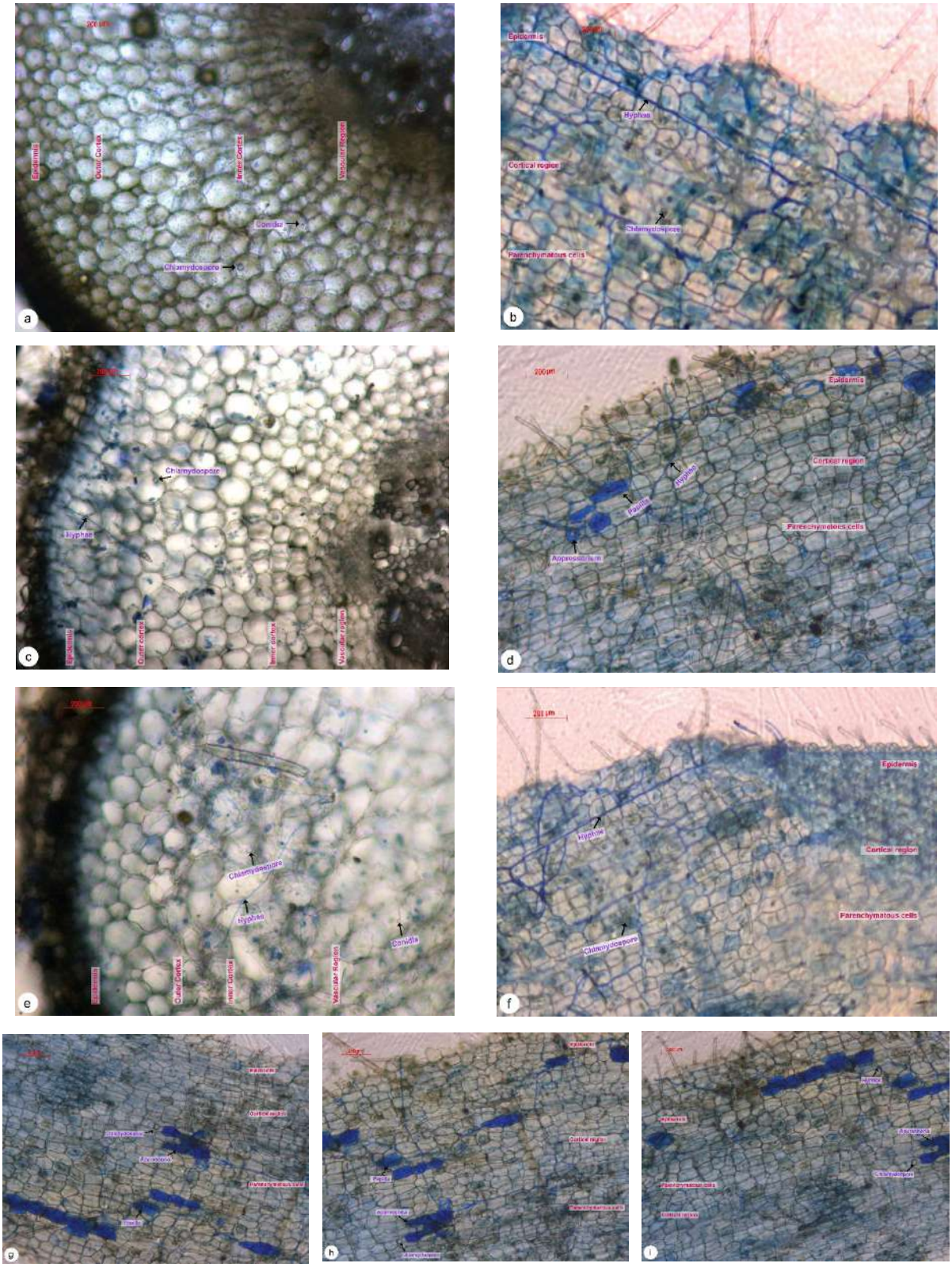

Fig. 4. Cross sections and longitudinal sections of black pepper root showing colonisation of *Trichoderma harzianum* IISR APT2 **(a,b)**, *T. asperellum* NAIMCC0049 **(c,d)**, *T. lixii* IISR KA15 **(e,f)**, longitudinal sections of showing colonisation of *T. harzianum* IISR KL3 **(g)**, *T. erinaceum* IISR APT1**(h)**, *T. asperellum* IISR TN3**(i)**.

had begun, with hyphae penetrating the root cells. At three days after inoculation (DAI), extensive hyphal growth was noted, covering large portions of the root surface (Fig.4c, 4d, 4e & 4f). At 21 DAI, chlamydospores were the most abundant fungal structure, covering large areas of the black pepper root epidermis.

(c) T. harzianum (IISR KL3), T. erinaceum (IISR APT1) and T. asperellum (IISR TN3)

Hyphae and spores of *T. harzianum* KL3, *T. erinaceum* APT1, and *T. asperellum* TN3 were detected in the internal root tissues five days after inoculation (Fig.4g, 4h & 4i). In cross-sections, hyphal structures were observed growing parallel to the longitudinal axis of the root. Additionally, some appressorium-like swollen structures emerged from intracellular hyphae and contacted root cells. However, penetration through the host cell walls was not observed, indicating a surface-level interaction rather than direct intercellular contact.

Comparison of colonization capacities of *Trichoderma* spp.

The colonization frequencies of the test isolates were calculated by counting the number of root segments exhibiting colonization at various root lengths from the root tip (Fig. 5).

The mean values are presented in Table 1. All isolates were successfully isolated on *Trichoderma*-selective agar medium (TSM) from root samples collected across all four root regions (Fig. 6a, 6b, 6c & 6d). Among the tested isolates, *T. harzianum* APT2 showed the highest colonization in the black pepper root system across all samples. In the first set of root samples (root tip region, 0–2 cm), colonization levels varied significantly for four of the isolates, while *T. harzianum* KL3 and *T. asperellum* NAIMCC0049 exhibited relatively consistent colonization. The lowest colonization was observed in *T. asperellum* TN3, whereas *T. harzianum* APT2 had the highest. Colonization rates decreased across all isolates as the distance from the root tip increased, with differences among the isolates becoming less pronounced in the three subsequent regions. However, all test isolates were present in the last root region (6–8 cm from the root tip), indicating the endophytic nature. Notably, *T. harzianum* APT2 maintained significant colonization even in this last region, distinguishing it from the other isolates.

Growth parameters of plants under different moisture levels

The lowest values for all growth parameters

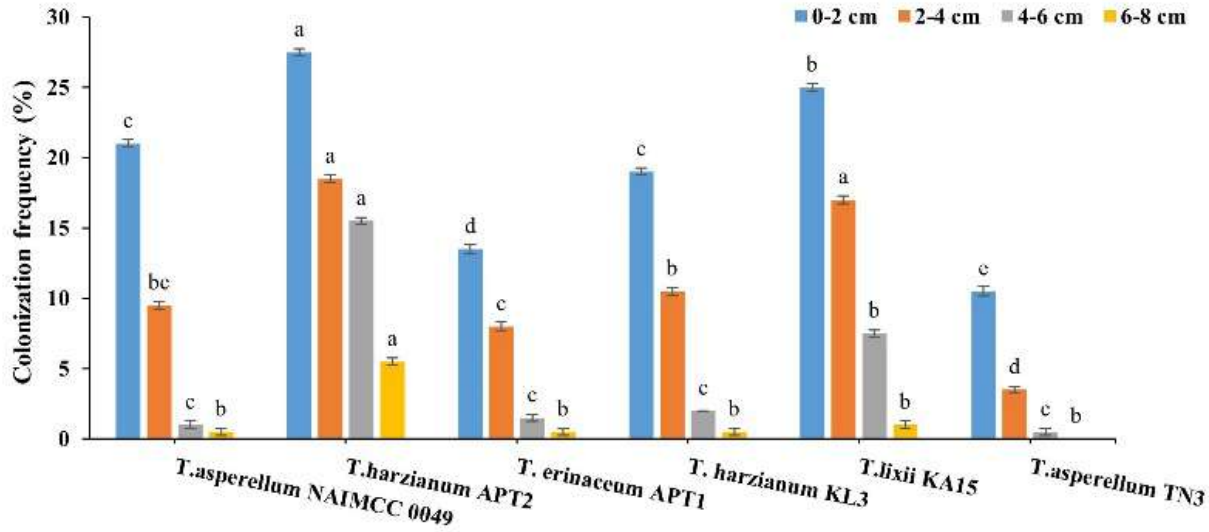

Fig. 5. Comparison of colonization frequencies among *Trichoderma* isolates.

Table 1. Comparison of number of segments colonized by *Trichoderma* isolates

Isolate name	Average number of segments colonized			
	Distance from root tip (cm)			
	0-2	**2-4**	**4-6**	**6-8**
NAIMCC0049	10.5^{c}	4.75^{bc}	0.50^{c}	0.25^{b}
APT1	6.75^{d}	4.00^{c}	0.75^{c}	0.25^{b}
APT2	13.75^{a}	9.25^{a}	7.75^{a}	2.75^{a}
KL3	9.5^{c}	5.25^{b}	1.00^{c}	0.25^{b}
KA15	12.5^{b}	8.50^{a}	3.75^{b}	0.50^{b}
TN3	5.25^{e}	1.75^{d}	0.25^{c}	0.00^{b}

Means followed by the same letter in a column do not different significantly according to Duncan's multiple range test at P=0.05

were observed in the control and IISR TN3 inoculated plants. Similar trend was observed under 75% moisture level and plants inoculated with IISR APT2 recorded significantly higher plant height, root length and number of leaves followed NAIMCC0049 (32.03 ± 0.07 cm) and IISR APT1 (Table 2, Fig 7a, 7a1 & 7b).

Under FC conditions, significant variations in growth of black pepper plants were observed among different isolates. The fresh weight of plants was significantly highest in IISR APT2 (39.14 ± 4.04 g), followed by IISR NAIMCC0049 (30.02 ± 3.01 g) and APT1 (26.11 ± 2.11 g) while the control exhibited the lowest value (15.13 ± 2.02 g). Among the isolates, IISR APT2 recorded the highest plant height (35.03 ± 0.04 cm), root length (18.27 ± 0.07cm), and number of leaves (22.12 ± 0.05) followed by NAIMCC0049 (32.03 ± 0.07 cm) and IISR APT1 (28.04 ± 0.05 cm) for plant height and leaf number (Fig. 8a & 8b).

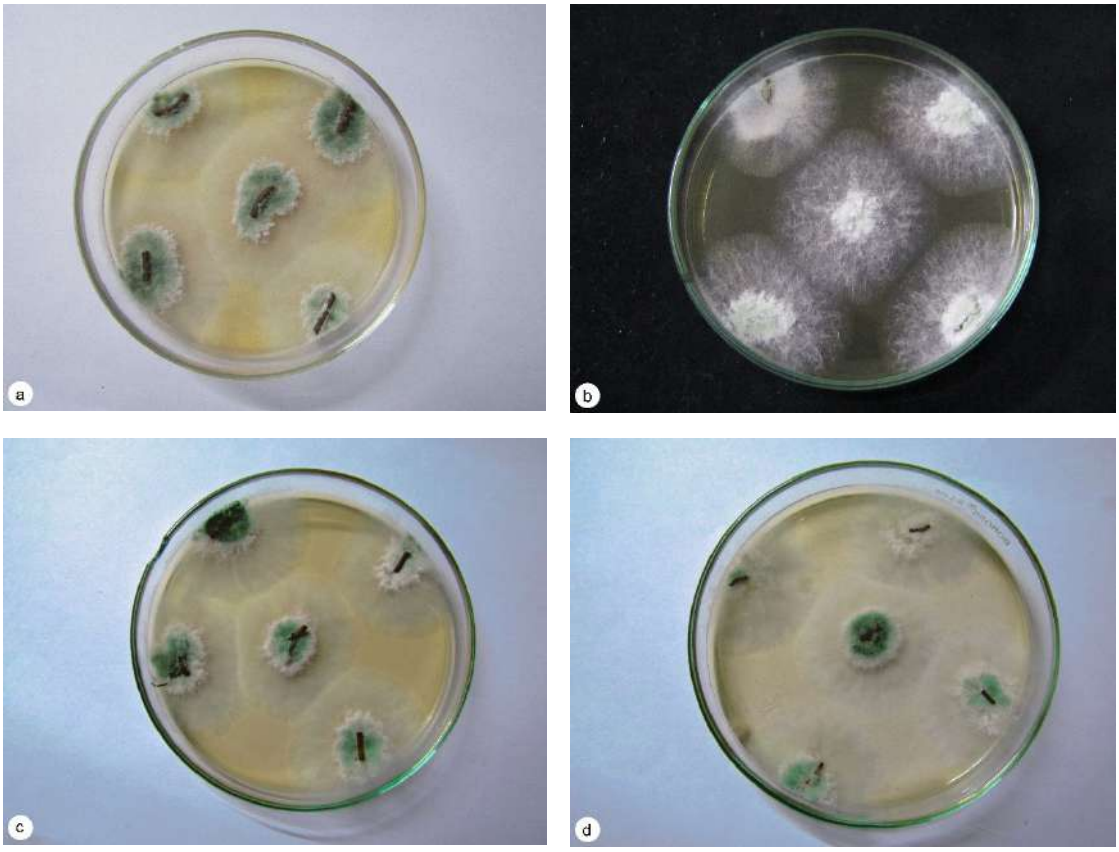

Fig. 6. Re isolation of **a)** *T. asperellum* NAIMCC0049 (100% FC), **b)** *T. asperellum* NAIMCC0049 (75% FC), **c)** *T. harzianum* IISR APT2 (100% FC), **d)** *T. harzianum* IISR APT2 (75% FC).

Table 2. Effect of *Trichoderma* isolates on growth parameters at 100 & 75% FC (30 DAI)

Isolate	Fresh weight (g)	Dry weight (g)	Plant height (cm)	Root length (cm)	Number of leaves
Moisture level- 100% FC					
Control	15.13 ± 2.02 ^{e}	5.24 ± 1.06 ^{f}	20.21 ± 0.04 ^{e}	8.24 ± 0.01^{f}	11.45 ± 0.04 ^{e}
NAIMCC0049	30.02 ± 3.01^{b}	7.14 ± 1.01^{e}	32.03 ± 0.07 ^{b}	16.16 ± 0.06 ^{b}	18.13 ± 0.07 ^{c}
IISR APT2	39.14 ± 4.04^{a}	12.21 ± 2.02 ^{a}	35.03 ± 0.04 ^{a}	18.27 ± 0.07 ^{a}	22.12 ± 0.05 ^{a}
IISR KL3	25.24 ± 3.03^{c}	9.05 ± 1.01 ^{c}	30.23 ± 0.01 ^{c}	12.13 ± 0.06^{d}	17.33 ± 0.03 ^{c}
IISR APT1	26.11 ± 2.11^{c}	11.07 ± 1.35 ^{b}	28.04 ± 0.05 ^{c}	15.14 ± 0.03 ^{c}	16.23 ± 0.01 ^{d}
IISR TN3	24.32 ± 2.07 ^{c}	8.04 ± 1.04^{d}	22.01 ± 0.06 ^{e}	9.11 ± 0.05 ^{f}	12.04 ± 0.07 ^{e}
IISR KA15	22.27 ± 3.04^{d}	7.06 ± 1.05 ^{e}	25.24 ± 0.07^{d}	10.34 ± 0.04 ^{e}	20.16 ± 0.06 ^{b}
Moisture level- 75% FC					
Control	10.21 ± 1.02 ^{e}	3.02 ± 0.04 ^{e}	10.25 ± 0.02 ^{f}	5.33 ± 0.08 ^{e}	6.21 ± 0.01 ^{e}
IISR APT1	18.12 ± 1.22 ^{d}	6.24 ± 0.03 ^{d}	20.04 ± 0.03^{b}	7.14 ± 0.07 ^{c}	10.34 ± 0.05 ^{b}
IISR APT2	30.34 ± 1.52 ^{a}	10.11 ± 0.02 ^{a}	25.03 ± 0.04^{a}	10.33 ± 0.04 ^{a}	14.11 ± 0.07 ^{b}
IISR KL3	21.27 ± 1.12 ^{c}	7.23 ± 0.04 ^{c}	16.67 ± 0.06^{c}	6.71 ± 0.06 ^{d}	9.14 ± 0.03 ^{c}
NAIMCC0049	22.25 ± 1.07 ^{b}	9.13 ± 0.07 ^{b}	15.24 ± 0.01^{c}	8.42 ± 0.01 ^{b}	10.25 ± 0.03 ^{b}
IISR TN3	23.22 ± 0.02 ^{b}	6.26 ± 0.02 ^{d}	14.07 ± 0.07 ^{d}	5.43 ± 0.03^{e}	8.43± 0.02 ^{d}
IISR KA15	20.31 ± 1.05 ^{c}	5.25 ± 0.04 ^{e}	12.33 ± 0.02 ^{e}	8.16 ± 0.05 ^{b}	8.21 ± 0.02 ^{d}

Means followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at P=0.05.

Fig. 7. a & a1 - Black pepper plant inoculated with *T. harzianum* IISR APT2 (Moisture level- 75 % FC), **b -** Control plant (Moisture level- 75 % FC).

Fig. 8. IISR Sreekara plants maintained at **a)** 100%, **b)** 75% FC (3 MAI) and observations were recorded after three months; 1-IISR APT2, 2-NAIMCC0049, 3-uninoculated.

Discussion

The genus *Trichoderma* is cosmopolitan in nature, comprising of widely distributed species that inhabit a variety of environments. According to Hu *et al*. (2020), the diversity and taxonomic structure of *Trichoderma* species are significantly influenced by factors such as the ecosystem, climatic conditions, and biogeographic patterns.

Trichoderma species commonly colonize a wide variety of ecological niches, including soil, plant tissues, dead wood, organic matter in sediments, and other substrates. Additionally, several *Trichoderma* species isolated from plant tissues exhibit endophytic associations with plants, enhancing their tolerance to both biotic and abiotic stress factors. (Nascimento *et al.* 2023; Qi and Zhao, 2013; Harman *et al*. 2005). A major advantage of endophytic *Trichoderma* species is their remarkable ability to efficiently colonize host plants and sustain long-term persistence within plant tissues. The strong colonization ability helps to improve the growth and extension of the root system of crops. Thereby the contact area between root and soil enhances and promotes the secretion of various extracellular enzymes, such as urease, phosphatase, and organic acids, within the rhizosphere. While some *Trichoderma* strains colonize only specific localized areas of the roots, rhizosphere-competent strains can colonize the entire root surface, maintaining their presence for several weeks or even months (Harman *et al*. 2005).

Our results demonstrated the endophytic interaction of the tested *Trichoderma* isolates with black pepper plants. The *Trichoderma* strains were found to colonize the root surface and penetrate the root epidermis later establish itself within the root hairs. Among the six isolates tested, *T. harzianum* APT2 showed the earliest signs of intercellular colonization. Similarly, the colonization patterns of *T. asperellum* IISR NAIMCC0049 and *T. lixii* IISR KA15 were alike, with hyphal growth clearly observed on the root epidermis of black pepper within 24 hours of inoculation.

The colonization of *T. asperellum* NAIMCC0049 was previously attempted in black pepper plants grown under in field conditions (Umadevi *et al*. 2017). Although *Trichoderma* species are widely known for their ability to colonize plant roots (Lopez-Bucio *et al*. 2015; Ruano-Rosa *et al*. 2016), the invasion of hyphae into root cells observed in this study may suggest a closer and more intimate symbiotic relationship compared to other *Trichoderma* strains and species. Interestingly, the reprogramming of root development including inhibition of primary root growth and stimulation of lateral root branching is a well-known characteristic of arbuscular mycorrhizal fungi (Bonfante and Genre, 2010), but has also been reported in *Trichoderma*-colonized *Arabidopsis* roots (Contreras-Cornejo *et al*. 2015). When *Trichoderma* species establish endophytic associations within plant tissues prior to pathogen infection, the likelihood of effective protection increases. This is largely

due to the mutualistic relationship formed between *Trichoderma* and the host plant, which enhances the plant's defense responses against invading pathogens. The primary direct effect of colonization was promotion of root growth and many *Trichoderma* spp. can confer fitness benefits to plants, including increased tolerance to drought stress (Rodriguez and Redman, 2005). In certain plants, strains of *Trichoderma* become systemic, living throughout the plant, while in other plants these same strains are confined to the roots (Bae *et al*. 2011).

In the present study, the colonization frequency of the selected *Trichoderma* isolates was evaluated by assessing the number of colonized root segments at varying distances from the root tip of black pepper. The results indicated that *T. harzianum* APT2 consistently demonstrated the highest colonization in the black pepper root system whereas *T. asperellum* TN3 recorded the lowest colonization frequency. The presence of *Trichoderma* colonies at the root tip region (0–2 cm) supports the endophytic nature of the tested isolates, as this zone is typically protected by rapid cell division and root cap structures that resist surface colonizers. Moreover, its sustained colonization in the distal segment (6–8 cm) further reinforces its endophytic behaviour, suggesting capacity for long-term and systemic root association. Similar root-tip colonization has been observed in other studies on *Trichoderma* endophytes, where early penetration correlates with stable internal colonization and beneficial plant interactions. Contreras Cornejo *et al*. (2024) demonstrated that successful root colonization by *Trichoderma* involves the secretion of effectors that suppress plant defences, enabling penetration at vulnerable zones like root tips. Dutta *et al*. (2023) further emphasize that early root penetration is key to systemic endophytic establishment and plant growth promotion. During colonization, *Trichoderma* species formed papilla-like or appressoria-like structures on host roots, facilitating effective penetration and entry into the root tissues (Lu *et al*. 2004; Chacón *et al*. 2007; Druzhinina *et al*. 2011). The early infection measures of *Trichoderma* involving the formation of appressoria on cell surfaces and the succeeding growth of hyphae within cells of host plant roots, including microsclerotia formation, have been explained by earlier authors (Behie *et al*. 2017). Penetration of the root tissue is usually limited to the first or second layers of cells, however, a strain of *Trichoderma stromaticum* that is endophytic in the vascular system in cocoa has been described (Bailey *et al*. 2008). During the asymptomatic colonization of plant roots by *Trichoderma*, the host plant activates several physical or biochemical responses, which limit the invading fungus to a few roots cortical cell layers in plant roots (Vargas *et al*. 2009).

Under field capacity and 75% moisture level the plants inoculated with IISR APT2, NAIMCC0049 and IISR APT1 recorded enhanced plant height, root length and number of leaves. The application of *Trichoderma* species has shown promising results in enhancing the growth and resilience of black pepper plants under varying moisture conditions. In our study, isolates such as *Trichoderma harzianum* (IISR APT2) and *Trichoderma asperellum* (NAIMCC0049) significantly improved plant growth parameters, particularly under moisture stress suggesting that *Trichoderma* spp. play a significant role in mitigating moisture stress in black pepper.

Several *Trichoderma* species have been reported to stimulate plant growth (Contreras-Cornejo *et al*. 2009) and there are reports that *T. atroviride* and *T. virens* specifically shown to promote root hair development (Contreras-Cornejo *et al*. 2015; González-Pérez *et al*. 2018). Studies in other crops have also elucidated the mechanisms by which *Trichoderma* spp. confer moisture stress tolerance. In rice, endophytic *T. harzianum* isolates delayed wilting and drought responses by enhancing root development and delaying stomatal closure, reducing accumulation of proline, malondialdehyde (MDA), and H_2O_2, while increasing phenolic compounds (Shukla *et al*. 2012). In maize, *T. harzianum* inoculation improved antioxidant enzyme activities (SOD and APX), stimulated accumulation of specific secondary metabolites, and enhanced biomass under salinity and drought stress (Eftekhari *et. al.* 2025). Further

studies in tomato and mustard have shown that *Trichoderma* inoculation improves relative water content, preserves chlorophyll content and photosynthetic efficiency under water deficit, maintains cell membrane integrity, and encourages root architectural changes through secretion of auxin and related compounds (Rawal *et. al*. 2022).

Dhanya *et al*. (2024) reported that beneficial microbes, such as arbuscular mycorrhizal fungi and pink pigmented facultative methylotrophs, play a vital role in enhancing the survival and growth of black pepper under drought conditions. Additionally, research by Verma *et al.* (2024) highlighted that foliar application of *T. viride* and *T. harzianum* not only reduced disease incidence in black pepper but also promoted plant growth by increasing leaf area, shoot length, and root length. Their findings emphasize the dual role of *Trichoderma* spp. in growth promotion and disease suppression, making them beneficial under stress conditions. The findings of this study highlight the potential of microbial inoculants in alleviating water stress, reinforcing the endophytic nature of *Trichoderma* spp. and their role in stress management.

Conclusion

Understanding the behaviour of *Trichoderma* species in their target environment and their interaction with host plants is crucial for determining their survival, persistence, and effectiveness. In the present study, the endophytic association of *Trichoderma* isolates with the root system of black pepper was clearly demonstrated. The application of *Trichoderma* spp. showed promising results in promoting plant growth and enhancing the resilience of black pepper under moisture-deficit conditions. Among the tested isolates, *T. harzianum* (IISR APT2) and *T. asperellum* (NAIMCC0049) effectively colonized the root surface, penetrated the root epidermis, and established themselves within the root tissues. In addition to successful colonization, these isolates significantly improved plant growth parameters, particularly under conditions of moisture stress. The endophytic nature of these *Trichoderma* isolates may also contribute to the activation of the plant's defense mechanisms, offering protection against both biotic and abiotic stresses in black pepper.

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