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ORIGINAL ARTICLE

Dual culture and VOC analysis reveal *Trichoderma harzianum* as an effective biocontrol agent against *Fusarium oxysporum* f.sp. *ciceris* on chickpea

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Abstract

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* (Foc), is a major constraint in chickpea production in Turkey, leading to yield losses of up to 50%. This study aimed to characterize the morphological and molecular traits of Foc isolates and evaluate the antagonistic efficacy of three *Trichoderma* species under in vitro and greenhouse conditions. A total of 20 Foc isolates were obtained from diseased chickpea plants in Tarsus, Mersin, of which 10 were confirmed by PCR amplification of the ITS region. The isolates showed variation in colony morphology, spore type, and pathogenicity, with Foc8, 10, 14, 15 and 20 being the most virulent. Dual culture assays demonstrated that *Trichoderma harzianum* (T3) had the highest antagonistic activity, inhibiting mycelial growth of Foc isolates by up to 68%. Headspace GC–MS analysis indicated that *T. harzianum* VOCs were rich in antifungal ketones, in contrast to the alcohol- and acid-dominated VOCs of Foc. Greenhouse experiments revealed that *T. harzianum* not only suppressed disease development but also significantly enhanced chickpea growth by increasing root biomass, seed weight, and relative water content. These findings highlight *T. harzianum* as a promising biocontrol agent for the sustainable management of Fusarium wilt in chickpeas. Further field validation and formulation development are recommended to support its use in integrated disease management programs.

Keywords: antagonist, chickpea, *Fusarium oxysporum* f. sp. *ciceris* (Foc), PCR, virulence, VOCs

Introduction

Chickpea (*Cicer arietinum L.*) is one of the essential legume crops, cultivated globally for its high nutritional values and agronomic significance. Turkey ranks second in the world after India (13.5 million tons) with production of 0.58 million tons (FAO 2022). However, its productivity is consistently affected by diverse abiotic and biotic stresses, among which Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *ciceris*, is one of the most significant (Mossa *et al.* 2023). This devastating fungal disease significantly impacts the quantity and quality of chickpeas, thus posing significant challenges to agricultural sustainability (Bayraktar and Dolar 2012). *F. oxysporum* f.sp. *ciceris* primarily targets the plant's vascular system, disrupting water and nutrient transport, and subsequently leading to wilting, chlorosis, and necrosis of the leaves and stems. The resulting physiological and metabolic changes severely compromise the plant's ability to produce pods and viable seeds. Consequently, there is a significant reduction in overall yield potential. The extent of yield losses varies between 10 and 90% depending on several factors including disease severity, environmental

conditions, cultivar susceptibility, and prevailing cultural practices (Jiménez-Díaz *et al*. 2006, 2015).

Two different pathotypes and 10 strains are identified based on variations in symptoms, colonization patterns, and virulence on different chickpea cultivars. *F. oxysporum* spreads through various mechanisms that enable it to move from one plant to another and establish new infections (Aydın 2019). Diseases spread primarily through soil, water, infected plant debris, contaminated tools and equipment, root grafting, plant-to-plant contact, natural dispersal, insects, and nematodes. Optimal conditions for *F. oxysporum* growth can vary depending on the forma specialis (f. sp.) and chickpea cultivar (Almourrh *et al*. 2024). Slightly acidic to neutral soil pH (5-6.5), 25°C and low humidity are conducive to the growth of *F. oxysporum*, while adequate nutrient availability, particularly nitrogen, can enhance its growth (Bayraktar and Dolar 2012).

Conventional management strategies for this disease have primarily relied on cultural practices (crop rotation, polyculture farming, adjusting fertilization, irrigation rates, etc.), and chemical treatments. Chemical treatments have demonstrated limitations in terms of environmental and health concerns, and disease resistance development (Almourrh *et al*. 2025). An eco-friendly alternative to synthetic chemical controls lies in the utilization of beneficial microorganisms as biocontrol agents. Specifically, a species of the fungal genus *Trichoderma* is applied in agriculture to control Fusarium fungi responsible for serious diseases in crops, for example, wilt and root rot. It helps by parasitizing *Fusarium*, stealing its food and space, and producing chemical substances that suppress the fungus. *Trichoderma* makes plants stronger by inducing their defense mechanisms. According to research, *Trichoderma* helps plants and is safe for the environment, making it a great choice for improving agriculture and controlling pests (Kayim *et al.* 2018). These filamentous fungi are renowned for their multifaceted growth-promoting attributes and antagonistic potential against various plant pathogens (Kocalar and Bilgili 2020; Jamil 2021). *Trichoderma* species are ubiquitous, fast-growing, cosmopolitan and widely distributed as a dominant microflora in the soil. *Trichoderma* belongs to the phylum Ascomycota, Class Sordariomycetes, order Hypocreales, and family Hypoceaceae (Daguerre *et al.* 2014).

This species is characterized by rapidly developing colonies that are initially transparent but later turn green. But not all produce green colonies, some may be white or yellowish. *Trichoderma* is a diverse genus with numerous species, each having distinct characteristics, and species, which in turn, are further classified into different strains based on genetic and morphological differences (Basim *et al.* 1999; Benítez *et al.* 2004; Harman *et al*. 2004). *Trichoderma* species are versatile and can thrive under a range of environmental conditions (Kayim *et al.* 2018). They act as biocontrol agents by suppressing the growth of phytopathogens through competition inhibition for nutrients, and space (Mohammed and Kayim 2021; Jalali *et al.* 2024).

Trichoderma produces certain antibiotics, e.g., gliotoxin, glivirin, viridin and trichoviridin. *Trichoderma* has also been known for plant growth stimulation through various mechanisms such as plant growth-promoting hormones, bioactive metabolites and plant defiance enzymes, chitinase and β 1,3-glucanase (Lee *et al.* 2016). Some *Trichoderma* species are mycoparasitic, which penetrate the hyphae of pathogenic fungi and disrupt their growth, eventually leading to death. *Trichoderma* species are known to produce volatile organic compounds (VOCs) that play a crucial role in agriculture (Rajani *et al.* 2021). Volatile organic compounds are chemically diverse and include amines, aromatics, hydrocarbons, terpenes, and thiols (Hung *et al*. 2015). However, little is known about their metabolic origin in fungi and their impact on plant growth. This research endeavored to delve into the effectiveness of *T. harzianum, T. atroviде* and *T. virens* against chickpea Fusarium wilt and the efficacy of *Trichoderma* volatile compounds on chickpea biomass.

Materials and Methods

Sample collection and preparation

Twenty isolates of Foc were purified using hyphal tip microdissection from infected chickpea plants which showed typical symptoms of wilting and yellowing of leaves, browning of vascular tissue, and overall stunted growth, from chickpea fields grown in Çukurbağ, Ziftlik, Pınar Oluk, and Emissili of Tarsus town of Mersin province, Turkey. Cultures were maintained on potato dextrose agar (PDA) as described by Kayim *et al.* (2018).

Antifungal strains

Trichoderma strains, *T. atroviде (ACP-Ta5:* MH208260-ITS*), T. harzianum* (ASF-2 ITS:MH208242; tef1a: MH208249) and *T. virens* (ACP-Tv14: ITS:MH208257; tef1a: MH208259) were obtained from fungal stock at the Department of Plant Protection, Çukurova University, Adana, Turkey. These fungal strains were isolated from broad bean leaves and the rhizosphere region of pea plants.

Molecular identification of *Fusarium oxysporum* f.sp. *ciceris* (Foc)

Fusarium oxysporum pathogen isolates, grown on potato dextrose agar (PDA) medium, were incubated in Petri dishes under controlled conditions of 25 ± 2°C with a photoperiod of 16 hours light and 8 hours dark for seven days. Subsequent DNA extraction was executed using a Qiagen Dneasy plant kit following the established instructions for DNA isolation. PCR amplification was conducted using primer sets ITS4-TCC TCC GCT TAT TGA TAT GC, ITS5- GGA AGT AAA GTC GTA ACA AGG (Singh *et al.* 2006). Typical PCR Cycling Parameters: Initial denaturation: 94–95°C for 3–5 minutes, PCR cycles (usually 30–35 cycles): Denaturation: 94–95°C for 30 seconds, Annealing: 55–60°C for 30 seconds (depended on primer Tm), Extension: 72°C for 45–60 seconds (1 min per kb of product), Final extension: 72°C for 5–10 minutes, and Hold: 4°C. To effectively target the desired ITS region a PCR reaction mixture was prepared as follows: 10 x PCR buffer (2.5 µl), 25mM $MgCl_2$ (2.5 µl), 2mM dNTP (2.5 µl), 10 µM forward primer (0.5 µl), 10 µM reverse primer (0.5 µl), Taq polymerase (0.5 µl), Template DNA (2 µl). To maintain 25 µl reaction volume 14.25 µl water was added. PCR amplified products were visualized on 1% agarose gel under UV transilluminator (BioRAD-Versadoc 4000MP) at a wavelength of 302 nm, and digitally photographed.

Sequence profiling and pedigree analysis of Foc isolates

To determine the pathogenic species and investigate the phylogenetic relationship between the Foc isolates, the core sequences of ITS genes were aligned using ClustalW. A comparison of ITS gene sequence with other global Foc isolates was performed on NCBI, employing the Blastn program for species-level identification of isolates. Furthermore, MEGA7 was employed to elucidate the phylogenetic relationships among Foc isolates.

Plant material

The chickpea genotype ILC482, known to be susceptible to *F. oxysporum* f. sp. *ciceris* (Foc), was obtained from the Eastern Mediterranean Agricultural Research Institute, Adana, Turkey. Seeds were surface-sterilized prior to use. This genotype was employed to assess the virulence of Foc isolates and the defensive efficacy of *Trichoderma* species under greenhouse conditions. Additionally, the impact of *Trichoderma* treatments on chickpea plant biomass was evaluated. Chickpea seeds (10 seeds per pot) were sown in sterilized peat moss under five treatment conditions using plastic pots measuring 20 cm in diameter and 25 cm in height. The treatments were as follows: (1) Negative control – clean seeds in sterilized soil; (2) Positive control – untreated seeds in Foc-contaminated soil; (3–5) Treatments with three different *Trichoderma* species.

After 10 days of germination, seedlings were transplanted into treatment pots containing the appropriate inoculate, including Foc (for the positive control and *Trichoderma* treatments) and sterile soil (for the negative control). For the pathogenicity assay, 20 Foc isolates were individually inoculated into chickpea seedlings. Each isolate was tested with three seedlings per pot and three replications per treatment. Disease severity was assessed using a 0–4 disease rating scale, as described by Navas-Cortés *et al.* (2000), and the most virulent isolates were identified based on their ability to cause wilt symptoms. These highly virulent isolates were subsequently used in biocontrol trials involving *Trichoderma* species.

Dual culture technique

To assess the antagonistic activity of *Trichoderma* spp. against highly virulent *F. oxysporum* f. sp. *ciceris* (Foc) isolates, a dual culture technique was employed, following the method described by Bubici *et al.* (2019), with modifications.

A mycelial disc (5 mm in diameter) from an actively growing 7-day-old culture of *Trichoderma* was placed at one end of a Petri dish (90 mm diameter) containing potato dextrose agar (PDA), while a disc of the corresponding Foc isolate was placed at the opposite end, equidistant from the edge. For control treatments, each fungus (*Trichoderma* or Foc) was cultured individually under the same conditions.

The plates were incubated at 25 ± 1°C with a 16-hour light/8-hour dark photoperiod for seven days. The radial growth (colony diameter) of Foc was measured daily using a ruler (in millimeters) for all treatments. The percentage inhibition of radial growth (PIRG) of Foc by *Trichoderma* was calculated on the seventh day using the following formula by Skidmore and Dickinson (1976):

$$PIRG\ (\%) = [(R_1 - R_2) / R_1] \times 100,$$

where:

R_1 – radial growth of Foc in control (alone),

R_2 – radial growth of Foc in dual culture with *Trichoderma*.

All treatments were performed in triplicate, and the experiment was repeated twice for validation. The zone of interaction (Figure 4) between the two fungi was also visually noted, including signs of overgrowth, and contact inhibition.

Control against Foc under greenhouse conditions

To evaluate the biological efficacy of *Trichoderma* spp. as antagonists against three highly virulent *F. oxysporum* f. sp. *ciceris* (Foc) isolates, a quinoa-based solid inoculum method was used for both Foc and *Trichoderma* spp.

Inoculum preparation

For each isolate, 25 g of washed quinoa grains were dispensed into 250 mL Erlenmeyer flasks. Ten milliliters of distilled water were added to each flask. Flasks were sealed with cotton plugs and wrapped in aluminum foil to prevent contamination. A total of 30 flasks was prepared — 15 for Foc isolates and 15 for *Trichoderma* species. All flasks were autoclaved at 121°C for 20 minutes on two consecutive days to ensure complete sterilization.

After cooling, each flask was inoculated with 5 mm mycelial discs taken from the edge of actively growing 7–10-day-old cultures of Foc or *Trichoderma* spp., grown on PDA. Only solid mycelial plugs were used — no liquid cultures were applied. The flasks were incubated at 25 ± 1°C with a 16-hour light/8-hour dark photoperiod and relative humidity at approximately 65–75%, for 10–15 days until full colonization of the quinoa substrate was observed. Fungal growth in the inoculum was visually confirmed, and viability was tested by subculturing small amounts onto fresh PDA plates to ensure active growth before pot application.

Soil infestation and inoculum application

Fifty grams of Foc inoculum (quinoa colonized by each isolate) were thoroughly mixed into the top 10 cm layer of sterilized peat moss in each pot (20 cm diameter × 25 cm height). The mixture was gently pressed down and moistened. Pots were then covered with plastic wrap for 48 hours to allow the pathogen to establish. After this pre-infestation period, 25 g of *Trichoderma* spp. inoculum (solid colonized quinoa) was added to the same pots and mixed thoroughly into the upper substrate layer. No holes were made in the soil; the inoculum was evenly distributed and incorporated into the medium to ensure uniform colonization.

Control treatments included

- Positive control: Foc-infected soil without *Trichoderma,*
- Negative control: sterilized soil without any fungal inoculum.

Each treatment was replicated three times, with three chickpea seedlings per pot. Plants were monitored for disease symptoms, and pathogen suppression by *Trichoderma* spp. was evaluated based on disease severity, plant biomass, and visual health indicators.

Volatile organic compound (VOC) analysis by GC–MS

To investigate VOC profiles of *Trichoderma* spp. and *F. oxysporum* f. sp. *ciceris* (Foc), dual culture assays were established in sealed glass tubes prior to headspace VOC extraction (Li *et al.* 2016). Glass culture tubes (2 cm in diameter × 9 cm in height) were filled with 25 ml of sterilized potato dextrose agar (PDA).

Fungal plug preparation

Instead of using a standard mycelial disc, a sterile inoculating needle was used to transfer a small portion (0.5 mm in diameter) of the actively growing mycelium from 7-day-old fungal cultures grown on PDA. A sterile needle was used to gently pick up a small amount of actively growing mycelium from the edge of a 7-day-old fungal colony. The collected mycelium was then carefully transferred onto the surface of the PDA inside a culture tube for incubation. In dual culture setups, *F. oxysporum* f. sp. *ciceris* (Foc) and *Trichoderma* spp. were inoculated at opposite ends of the same PDA slant. For monocultures, a single inoculum was placed in the center. Tubes were sealed and incubated at 25 ± 1°C with a 16-hour light/8-hour dark photoperiod for seven days before VOC sampling.

VOC headspace sampling via SPME

Headspace VOCs were extracted using a solid-phase microextraction (SPME) fiber coated with 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). Prior to sampling, the fiber was conditioned at 230°C for 1 hour to remove residual volatiles. VOC collection was performed by exposing the fiber to the headspace of each sealed tube at room temperature (22–25°C) for 1 hour. Control samples (PDA only) were also analyzed to subtract media-derived VOCs.

GC–MS conditions

The adsorbed VOCs were thermally desorbed in the injection port of a QP 2010 Ultra GC–MS system (Shimadzu, Japan), equipped with an Rtx-Wax capillary column (60 m × 0.25 mm ID × 0.25 µm film thickness). The injector was operated in splitless mode at 230°C for 10 minutes. Helium was used as the carrier gas at a constant flow rate of 1.0 ml/min. The oven temperature was programmed as follows:

- Initial hold at 35°C for 5 minutes,
- Ramp at 5°C/min to 230°C for *Foc* samples or 2°C/min for *Trichoderma* samples,
- Final hold at 230°C for 15 minutes,

- Mass spectra were collected using electron ionization (EI) at 70 eV, scanning from 35 to 500 m/z.

Compound identification and quantification

To calculate retention indices (RI), a standard alkane mixture (C7–C40 in n-hexane) was injected under the same GC–MS conditions. Tentative identification of VOCs was achieved by comparing mass spectra and RI values against the NIST 11 library using AMDIS 2.7. A match factor of ≥90% and RI deviation of ±4% were required for identification. The identity of major VOCs (e.g., ethanol, 2-phenylethanol, 1-hexanol) was confirmed using authentic standards (Sigma-Aldrich, USA). Peak area integration and linear regression were used for relative quantification.

Replicates

Each treatment (dual and monoculture) was analyzed with two biological replicates.

Statistical analysis

All treatments were analyzed by using statistical tools. All mean data were subjected to PAST software for Cluster and PCA analysis. A level of significance of 0.05% was maintained. Graphs and ANOVA tables were analyzed by Excel, PAST and Statisx 8.1 software.

Results

Collection of *Fusarium oxysporum* f.sp. *ciceris*, (Foc) isolates

Diseased samples were collected from chickpea fields cultivated in the Tarsus district of Mersin, Çukurbağ--Pınaroluk neighborhood in 2022. Ten plants showing symptoms from five different fields, totaling 50 plants (Fig. 1), were collected, and brought to the laboratory.

Morphological features of Foc

Fusarium species were purified based on morphological features. Twenty isolates were subculture, and preserved for future studies. It was observed that some isolates of Foc developed micro, and macroconidia more rapidly on artificial media than other isolates. Morphological structures of Foc4, Foc6, Foc8, Foc12, Foc14, Foc15, and Foc20 isolates were examined according to Huner *et al.* (1998) under the microscope, and are given in Figure 2. Length, width, and number of septations per conidia were measured under the microscope at 40x magnification (Table 1). As given in Table 1, Foc4 isolate forms both micro and macroconidia. The shortest macroconidia were 3 µm long, while the longest microconidia were 17 µm, and the longest macroconidia were 6 µm long and the shortest, 0.5 µm. The widest were 2.5 µm.

As shown in Table 1, and Figure 2, the Foc12 isolate only formed microconidia. This situation continued even in 2–6-month-old Foc12 cultures. Micro- and macroconidia-forming isolates formed chlamydiospores after one month on artificial media, while some isolates also formed fruiting bodies, and abundant macroconidia (Fig. 2).

Molecular identification of Foc isolates

DNA was successfully isolated from 10 out of the 20 *F. oxysporum* f. sp. *ciceris* (Foc) isolates. PCR amplification of the internal transcribed spacer (ITS) region was performed using a standard protocol. All 10 isolates yielded amplicons of approximately 700 base pairs (bp).

To confirm species identity, sequencing was carried out on the PCR products. Sequences were obtained in both the 5'–3' and 3'–5' directions from approximately 100 µl of ITS amplicon per isolate. The resulting sequences were analyzed using the BLAST algorithm against the NCBI GenBank database. The results confirmed that all sequenced isolates were identified as *F. oxysporum* f. sp. *ciceris*, with high similarity to known reference sequences (Fig. 3).

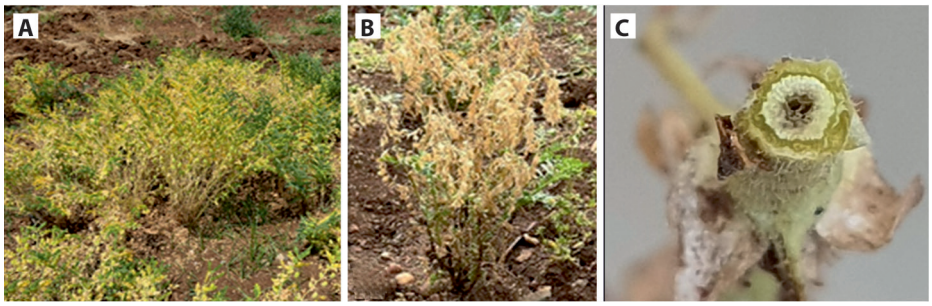

Fig. 1. Chickpea plants infected with Foc (A) yellowing, (B) wilting symptoms (C), and browning of stem on transverse cuts of plants

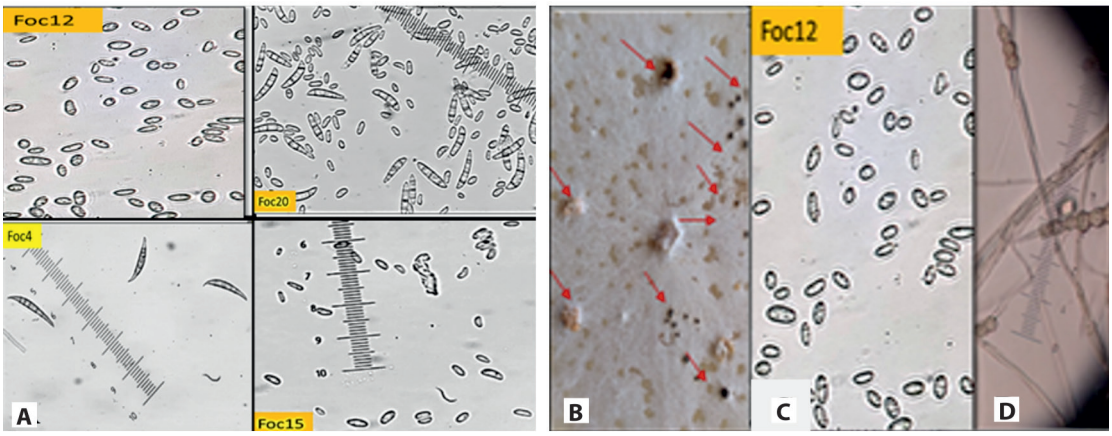

Fig. 2. Macro- and microconidia formed by different *F. oxysporum* f. sp. *ciceris* (Foc) isolates on PDA medium, observed under the microscope. (A) Macroconidia, (B) Fruiting bodies (sporodochia), (C) Microconidia, and (D) Chlamydospores. The image represents the Foc-8 isolate, a yellowing pathotype associated with necrosis. Macroconidia, fruiting bodies, and chlamydospores were formed on PDA after 4–6 weeks of incubation (structures indicated by arrows)

Table 1. Macro, and microconidia measurements of five different Foc isolates under a light microscope (40x magnification)

Foc isolates	Macroconidia length [µm]	Macroconidia width [µm]	Microconidia length [µm]	Microconidia width [µm]
Foc4	7–17	1–2.5	1–2	0.5–1
Foc6	6–15	1–2.5	2–4	0.5–1
Foc8	14–24	3–4	4–6	2.5–3
Foc12	not observed	not observed	3–7	2–3
Foc14	16–21	1.5–3	4–8	2–3
Foc15	17–22	1.5–3	4–7	2.5–3
Foc20	12–14	2–3	3–5	2–3

According to Figure 3 (Table 2), all isolates of fungus were identified as *F. oxysporum* f.sp. *ciceris*. However, there were slight differences between the isolates in terms of some nucleotides, which was also reflected in the family tree. However, it has also been shown by the genetic family tree that it is a different species when compared to other *Fusarium* species. On the other hand, the isolates showed 100% sequence similarity to *F. oxysporum f. sp. ciceris* of the Foc isolates 4, 6, 7, 8, 12, 14, and 15 and were completely homogeneous in the first group, while *F. equiseti* reference isolates were in the second group. *F. brachygibbosum,* and *F. solani* isolates branched in the third and fourth clades with a long distance.

Virulence determination of Foc

The disease severity scale for 20 Foc isolates was determined by treating Foc susceptible chickpea line ILC482. The Foc7, Foc8, and Foc10 isolates, which are yellowing, and necrotic pathotypes, had the highest disease severity value. The disease severity values of other isolates differed, of which the Foc1 isolate gave the lowest disease severity. On the other hand, most of the wilt pathotypes were found to be more virulent pathogens in terms of disease severity, while Foc 14, 15, and 20 caused the disease at the most severe level (4). Foc17 caused the lowest disease severity (1), and the harvest intensities of other isolates ranged from 2 to 3.

The effect of three different *Trichoderma* species against eight selected *F. oxysporum* f. sp. *ciceris* isolates (Foc5, Foc6, Foc7, Foc10, Foc11, Foc12, Foc17, and Foc20), previously identified as highly virulent, was evaluated under in vitro conditions. The suppression of radial mycelial growth by the *Trichoderma* isolates over seven days is presented in Figure 4.

A more detailed inhibition profile for isolate Foc5 is shown in Figure 5, highlighting the progressive suppression of mycelial growth. From the fifth day onward, *Trichoderma harzianum* (T3) demonstrated the most significant suppression of radial mycelial growth, reducing the pathogen colony size to just 11.33 mm.

Among the antagonists, *T. harzianum* (T3) achieved the highest inhibition rate at 68%, followed by *T. virens* (T1) at 55.9%, and *T. atroviride* (T2) at 46%, making it

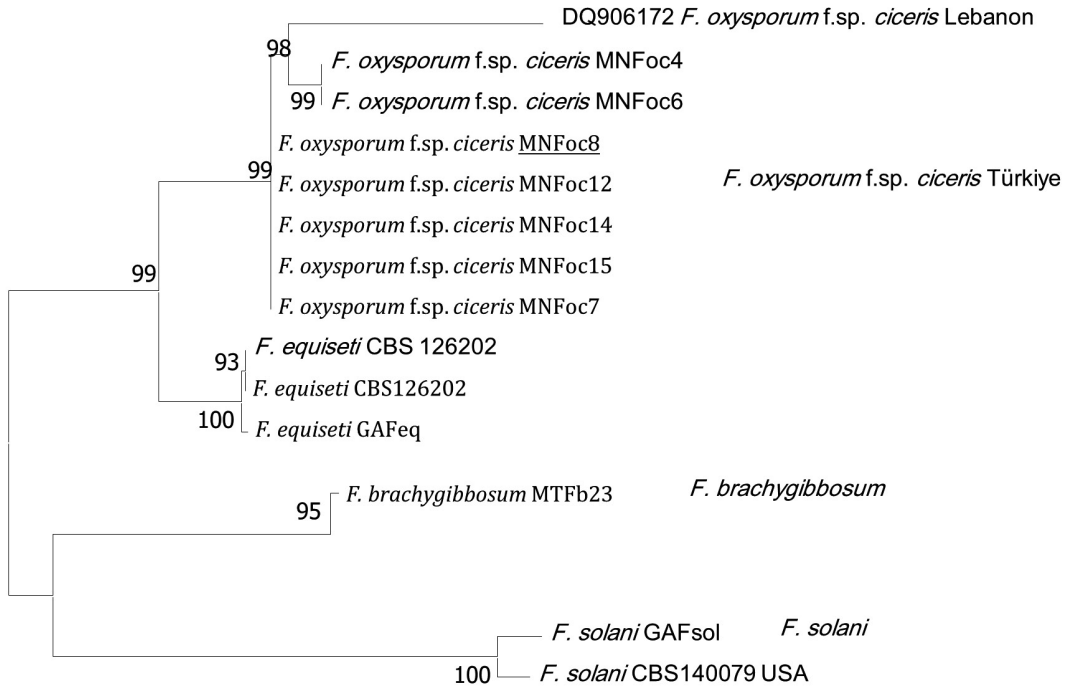

Fig. 3. A maximum parsimony phylogenetic tree based on the ITS gene sequence. of representative Foc isolates. The bootstrap analysis of 1000 replicates was conducted to assess the reliability of the groupings

Table 2. Molecular identity of fungal pathogen and Antagonist *Trichoderma* spp.

Host plant	Location	Pathogen fungal identity	GenBank accession number assigned to species study
Chickpeas	Adana and Mersin, Turkey	*Fusarium oxysporum* f.sp. *ciceris*	OP456678 (MNFoc4) OP456679 (MNFoc6) OP456680 (MNFoc8) OP456681 (MNFoc12) OP456682 (MNFoc14) OP456683 (MNFoc15) OP456684 (MNFoc7)
Antagonist *Trichoderma* spp.			
Patates rizosfer	Çukurova, Adana, Turkey	*Trichoderma virens* (T1)	MH208258
Pisum sativum	Çukurova, Adana, Turkey	*Trichoderma atroviride* (T2)	MH208260
Vicia faba	Seyhan, Adana, Turkey	*Trichoderma harzianum* (T3)	MH208247

the least effective among the tested species. These findings confirmed the superior antagonistic activity of *T. harzianum* against the Foc5 isolate under dual culture conditions.

Statistically significant differences were observed between treatments. T3 treatments generally exhibited the highest inhibition percentages (above 60%) and were significantly more effective than T1 and T2 for most Foc isolates. Notably, Foc-14 was the least inhibited by all *Trichoderma* species, with significantly lower values ($p < 0.05$), represented by red bars. T3 treatment of Foc-11 and Foc-14 showed modest improvement over T1 and T2 but remained significantly lower than other T3 interactions. Dark green bars indicate significantly higher inhibition rates ($p < 0.05$), while orange and red bars represent significantly lower efficacy. These findings confirmed that T3 is statistically superior in controlling most Foc isolates, particularly Foc-8, Foc-10, and Foc-5.

Biplot of physiological parameters are given and it shows that seed weight, no. of pods, no. of flowers, root length, shoot length and relative water content

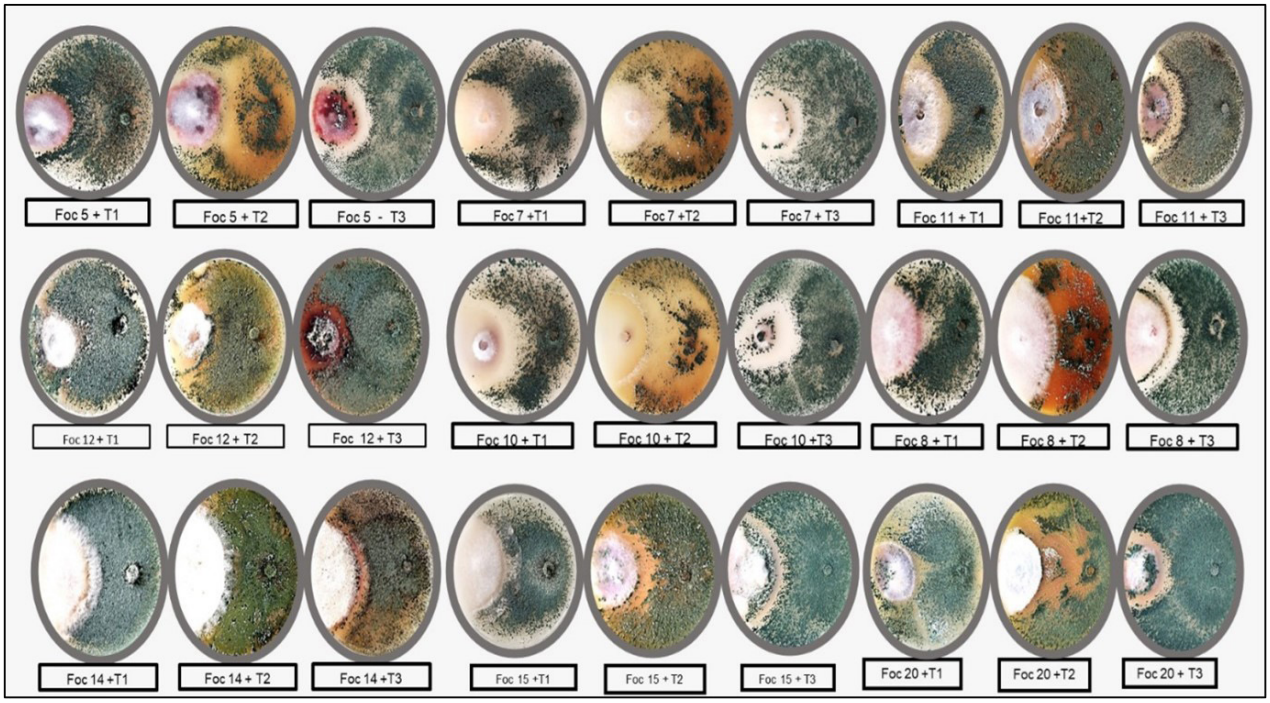

Fig. 4. The 7-day radial mycelial growth suppression, and % inhibition rate of three different *Trichoderma* species on all the selected Foc isolates

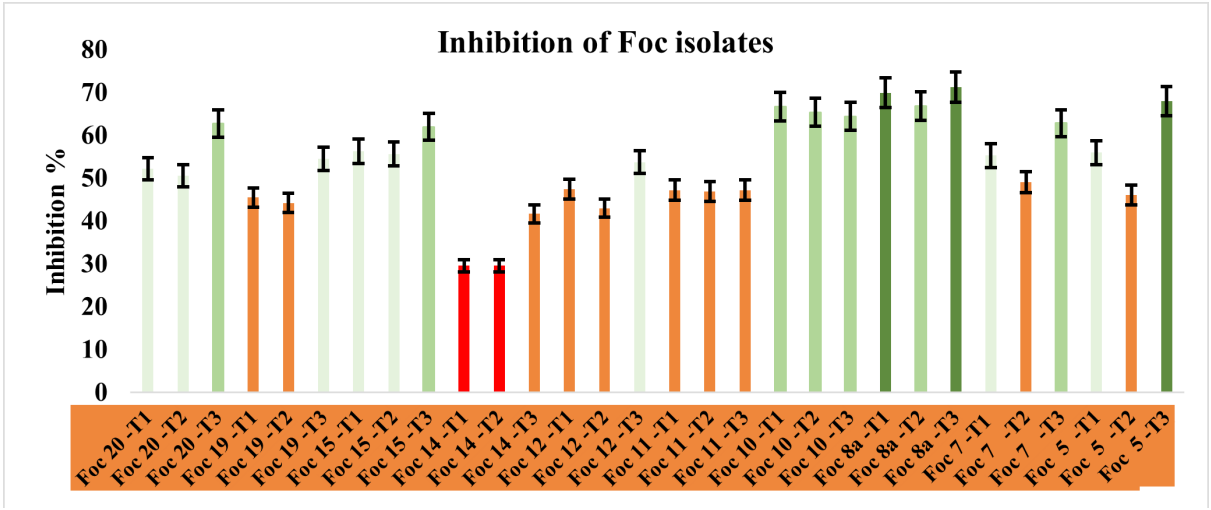

Fig. 5. Bar graphs present the behavior of different isolates and their relationship with *Trichoderma* species

Figure 5. Inhibition of *F. oxysporum* f. sp. *ciceris* (Foc) isolates by three *Trichoderma* species (T1, T2, and T3) in dual culture assays. Bars represent mean inhibition percentages, with error bars indicating standard error (SE). Treatments were statistically analyzed using ANOVA followed by Excel, PAST and Statisx 8.1 software ($p < 0.05$).

in the first and positive quadrant of the biplot while excise leaf water loss is in the negative quadrant. While treatments are mostly in the positive quadrant, which shows their significance on parameters, positive and negative control are presented in the negative quadrant. Excised leaf water loss was minimal in all cases and it supported the research plan, as fusarium affects the water supply chain of plants and when it lessens it will lose less.

This shows two distinct dendrograms of treatment behavior. In the clusters Foc-14+T1 and Foc-8+T3, Foc-14+T1 and Foc-14+TR(T2) are similar in controlling disease. Foc-12+T3, Foc-8+T2, and Foc-12+T2 showed similar results, which is why they are in the separate dendrogram. Table 3 shows PCA analysis for Foc isolates against different *Trichoderma* species concerning positive and negative control.

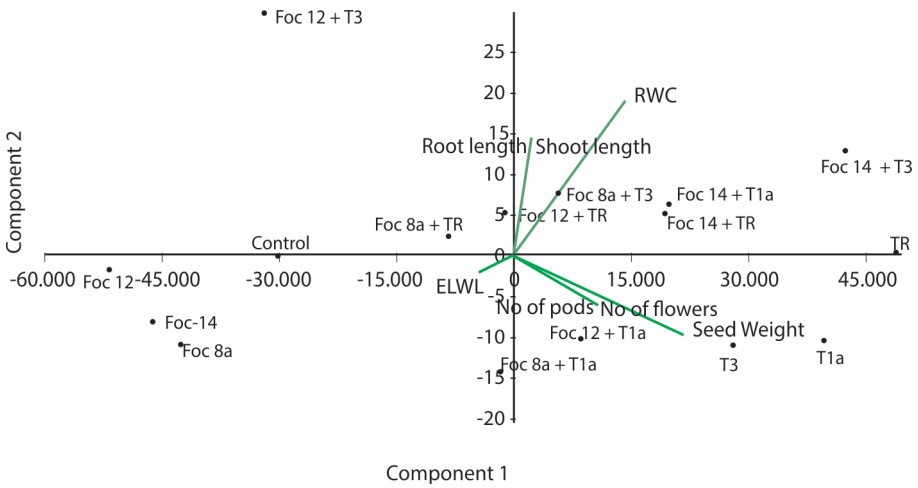

Fig. 6. Biplot presents the interaction between treatments and physiological parameters of the chickpea plant

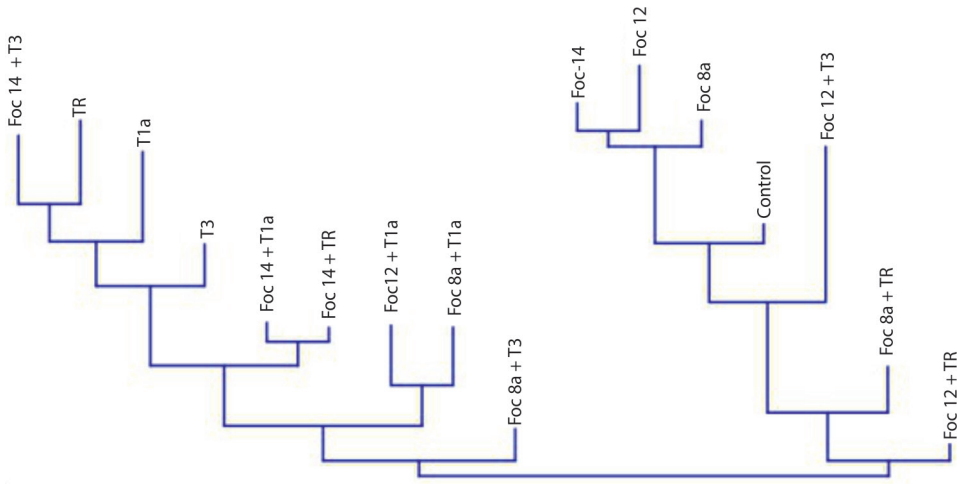

Fig. 7. Cluster analysis of different parameters with different treatments

Table 3. Principle component analysis (PCA)

	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
Foc 12 +T3	–31.668	29.097	0.16425	4.2889	–2.0091	–0.10336	–3.78E–17
Foc 12 +T1a	8.6479	–10.552	12.637	2.4673	–0.97312	–0.18906	–6.87E–15
Foc 12 + TR	–1.0344	4.7217	–2.1347	–3.1193	–0.38261	–0.47217	–5.25E–15
Foc 8a + T3	6.0103	7.32	0.98501	–1.8247	–1.1992	0.21886	–4.39E–15
Foc 8a + T1a	–2.0874	–14.328	6.6644	–3.1471	–1.8535	–0.44986	–9.21E–15
Foc 8a +TR	–8.2687	2.2709	–10.504	–6.4389	–3.2877	–0.5719	–5.51E–15
Foc 14 + T3	42.694	12.515	6.2785	–0.33758	5.3815	–0.56131	–3.52E–15
Foc 14 + T1a	20.138	6.31	3.2176	–0.9063	0.25658	–0.55592	–4.87E–15
Foc14 +TR	19.271	4.8263	3.3296	–4.2622	–0.18327	2.112	–5.33E–15
Foc-14	–45.965	–7.9926	2.8018	1.0647	–0.97429	0.046811	–8.04E–15
Foc 12	–51.412	–1.7242	–3.0859	1.5637	3.2695	0.20263	–5.73E–15
Foc 8a	–42.677	–10.917	0.9972	–2.2442	3.0458	–0.10007	–8.59E–15
T3	27.996	–10.884	–2.765	5.4289	–3.3936	0.19851	–2.91E–15
T1a	39.788	–10.212	–12.965	1.6616	3.3724	0.13194	–5.45E–15
TR	49.121	–0.20839	–1.8571	2.7704	–1.6241	–0.17971	–3.39E–15
Control	–30.555	–0.24206	–3.7635	4.0345	0.55471	0.27266	–3.68E–15

Table 4. Analysis of variance

Source	DF	Seed weight	No. of pods	ELWL	RWC	No of flowers
Replication	2	80.12	20.083	34.3595	34.922	20.083
Treatment	15	1697.74	417.365	90.4789	885.173	417.365
Error	30	3.50	1.194	5.2174	21.930	1.194
Total	47					

ELWL – excised leaf water loss; RWC – relative water content

Analysis of variance for all studied treatments and parameters is presented in Table 3. All parameters showed significant differences among parameters.

In Table 5 regression analysis for all the physiological parameters was discussed. The table contains the results of the trend line and the value of R^2. Results showed that seed weight, no. of pods, ELWL and RWC were significantly affected by fusarium wilt, while, in comparison to others, root length and shoot length were not affected by fusarium.

Table 6 presented the descriptive statistics of relative water content, seed weight, no. of pods, no. of flowers and excise leaf water loss (ELWL). The table consists of maximum, minimum, means, standard error, and standard deviation values against all parameters. All physiological parameters show significant differences which means that plants showed effects of the treatments under study.

After four weeks, the results showed that plants grown with *T. harzianum* had larger leaves. Overall, roots were larger in exposed plants by the end of the sixth week. By the end of the seventh week, the *Trichoderma* -exposed plants were darker green and visibly larger in size. In general, chickpea plants grown with *T. harzianum* grew quickly, produced more biomass, and looked healthier than control plants. The test plants had significantly greater root fresh weight and more lateral root growth than the control and treated plants. The average fresh weight of the roots of chickpeas treated with *T. harzianum* VOCs for four weeks was 4.67 mg compared to control plants of 2.61 mg.

Table 5. Regression analysis of physiological treatments under *Fusarium* treatment

Parameters	Regression	
Seed Weight	y = 0.4886x + 49.622	R^2 = 0.76
No of pods	y = 0.5995x + 17.008	R^2 = 0.686
ELWL	y = 0.3149x + 9.7747	R^2 = 0.745
RWC	y = 0.0058x + 59.091	R^2 = 0.67
Root length	y = 0.6365x + 25.426	R^2 = 0.222
Shoot length	y = 0.6439x + 28.361	R^2 = 0.2133

Results of VOCs by headspace GC-MS

Headspace GC–MS analysis of the VOCs of the endophyte (Figure 8), *T. harzianum*, *T. atroviride, and T. virens* and the 10 pathogens, *F. oxysporum* (Foc) (Table 6) were studied. Based on the total integrated peak areas of the GC–MS elution, the major constituents of VOCs could be classified into aldehydes, acids, alcohols, ketones, esters, phenols, hydrocarbons, and miscellaneous compounds (Li *et al.* 2014). Across replications, the emission profile of *T. harzianum* revealed a high content of ketone (63.55%), followed by miscellaneous (28.26%), hydrocarbons (5.48%), and acids (2.63%). As shown in Table 7, for the pathogenic fungus *F. oxysporum* (Foc), alcohols were the most abundant VOCs (65.54%), followed by acids (22.10%), hydrocarbons (5.31%), aldehydes (3.35%), miscellaneous compounds (1.95%), ketones (1.90%), and esters (0.50%). All Foc isolates showed a similar group-wise chemical composition of volatiles. A major component analysis based on the seven VOC categories indicated a clear separation between the pathogenic fungi, the endophyte (*T. harzianum*), and other Foc isolates.

Table 6. Descriptive studies for physiological parameters

	RWC	Seed weight	No. of pods	No. of flowers	ELWL
Min	30.681	17.004	5	8	5.643
Max	83.67	86.644	41.333	44.333	23.865
Mean	59.042	53.77519	22.10412	25.10412	12.45131
Std. error	4.294292	5.94725	2.948743	2.89743	1.372968
Stand. dev	17.17717	23.789	11.79497	13.77597	5.49187

Table 7. Displays the volatile organic compounds (VOCs) induced following the interaction of the endophytic fungus with individual cultures of *Fusarium oxysporum* f. sp. *ciceris* (Foc) and *Trichoderma* spp (T1, T2, T3)

Retention time	Compounds name	% T3	% Foc 14
	Aldehyde		
14.158	Undec–8–enal <cis–>	–	3.35
9.99	Nona–2(E),4(E)–dienal	0.41	–
	Total Aldehyde	0.41	3.35
	Acid		
16.31	Acetic acid (CAS)	1.97	–
37.19	1,2–Benzenedicarboxylic acid, diethyl ester (CAS)	0.67	–
1.495	2–Acetonyl–3–cyano–2,3–dimethylcyclobutane–1–carboxylic acid	–	22.10
	Total Acid	2.63	22.10
	Alcohol		
9.16	EUCALYPTOL (1,8–CINEOLE)	0.30	–
9.40	1–Butanol, 2–methyl–, (S)–	0.40	–
17.27	2 ETHYL HEXANOL	0.34	
2.86	Ethanol (CAS)	–	59.93
9.64	1–Butanol, 3–methyl– (impure) (CAS)	–	2.20
24.22	Drimenol	–	3.41
	Total Alcohol	1.04	65.54
	Ketone ONE		
2.195	Furan–3–one <2–methyl–, tetrahydro–>	2.80	–
12.158	2–Propanone, 1–hydroxy– (CAS)	0.44	–
19.829	,TRANS–.ALPHA.–BERGAMOTENE	0.43	–
22.112	1H–3A,7–METHANOAZULENE, 2,3,4,7,8,8A–HEXAHYDRO–3,6,8,8–TETRAMETHYL–, [3S–(3.ALPHA.,3A.ALPHA.,7.ALPHA.,8A.BETA.)]–	0.55	–
28.848	Ethanone, 1–(1H–pyrrol–2–yl)– (CAS)	0.53	–
33.088	6 AMYL .ALPHA. PYRONE	57.13	–
19.842	Naphthalene, 1,2,3,4,4a,5,6,8a–octahydro–7–methyl–4–methylene–1–(1–methylethyl)–, (1.alpha.,4a.alpha.,8a.alpha.)– (CAS)	1.68	–
7.037	.beta.–Myrcene	–	0.31
10.773	.ALPHA.–TERPINOLENE	–	0.34
22.008	.beta.–Chamigrene	–	1.25
	Total Keton	63.55	1.90
	Ester		
37.188	1,2–Benzenedicarboxylic acid, diethyl ester (CAS)	–	0.25
23.016	Guaiacwood acetate	–	0.25
	Total Ester	0.00	0.5
	Hydrocarbons		
2.837	Benzene (CAS)	3.60	–
8.561	(+)–LIMONEN	0.98	4.57
8.759	l–Limonene	0.91	–
18.32	Benzene, 1–ethyl–4–methoxy– (CAS)		0.74
	Total hydrocarbons	5.48	5.31
	Miscellaneous compounds		
1.494	oxirane	27.53	–
9.817	Furan, 2–pentyl– (CAS)	0.40	–
23.739	Naphthalene (CAS)	0.29	–
1.485	Nitrogen oxide (N2O) (CAS)	–	1.95
	Total oxirane	28.26	1.95

Fig. 8. Sealed glass vials (2 cm diameter × 9 cm height) containing dual cultures of *F. oxysporum* f. sp. *ciceris* (Foc) and *Trichoderma* spp. grown on PDA slants. Fungi were co-cultured in the same vial for seven days at 25 ± 1°C before headspace volatile organic compounds (VOCs) were extracted for GC–MS analysis

Discussion

Along with fighting off pathogens, *T. harzianum* also helped the chickpea's growth, increased the biomass of its roots and made the seeds bigger. This supports earlier observations by Zou *et al.* (2023), who demonstrated that VOCs from *Trichoderma* species stimulate root development through auxin-related signaling pathways. Enhanced nutrient uptake and improved stress resistance may be attributed to the nearly two-fold increase in root fresh weight, which increased from 2.61 mg in control plants to 4.67 mg in treated plants. The influence of VOCs may enhance phytohormones called indole-3-acetic acid (IAA) and cytokinins which may promote improved root architecture, consistent with findings by Zou *et al.* (2023). The findings agree with earlier research that shows *Trichoderma* controls harmful organisms in the soil, and also boosts the health of plants when they are faced with threatening conditions.

In most cases, direct use of VOCs is avoided due to their volatility which means they need to be handled differently. Therefore, these products are formulated as biological products such as granules or seeds and the VOCs are released directly where they are needed. Protecting fungal particles through microencapsulation or alginate-based carriers can ensure both better field performance and consistency.

More research should focus on the genetics behind *Trichoderma* spp. antagonistic behaviors (Nguyen *et al.* 2023). Comparative transcriptomic or proteomic research may identify the most important genes responsible for producing volatile compounds, attacking other fungi and forming a network in plants' roots. Also, since partnering *Trichoderma* spp. exhibit strong biocontrol properties individually, their effectiveness can be further enhanced through synergistic interactions with other beneficial microbes (e.g., *Bacillus subtilis* or *Pseudomonas fluorescens*) or with organic soil improvers (e.g., vermicompost) which could enhance the prevention of diseases (Bapela *et al.* 2023).

Successful use of *Trichoderma* for biocontrol depends a lot on environmental conditions. It has been reported that the *Trichoderma* strain performs optimally at 25–30°C, soil pH 5.5–7.5, and relative humidity of 60–75% (Nawrot-Chorabik *et al.* 2021). In the future, field trials should consider adjusting these variables and keep in mind the type of soil, what it contains and its communities, so *Trichoderma* strains will thrive in the environment.

Biocontrol potential of *T.* harzianum, *T. virens* and *T. atroviride* was found against *F. oxysporum* f. sp. *ciceris* (Foc) isolates in dual culture assays carried out in the lab. On average, all species reduced the growth of Foc by a minimum of 46%, up to 68%. Among all these fungi, *T. harzianum* (68%) had similar levels of suppression as reported by Altinok and Erdogan (2015), where *T. harzianum* inhibited *F. oxysporum* in tomato by 69–72% in dual culture assays. *T. virens* (55.9%) performed best as antagonists, whereas *T. atroviride* (46%) had the lowest effect.

Differences in inhibition could be due to each strain's specific metabolism, processes of producing antifungal enzymes such as chitinases, glucanases, growth speed and competition for nutrients on the agar surface. How many and what kinds of volatile organic compounds (VOCs) are produced may have been important and this fact, along with its high-quality VOCs, may be why *T. harzianum* did so well.

Analysis of *T. harzianum* VOCs by GC–MS discovered that the main compounds were ketones (63.55%) which were followed by miscellaneous substances (28.26%) and hydrocarbons (5.48%). Most of the VOCs in the present samples were 6-amyl-α-pyrone ketones and they are known to slow down

fungi by puncturing their membranes and interfering with their ability to grow long filaments Li *et al.* (2018). This is consistent with findings by Lochmann *et al.* (2024), who reported that 6-amyl-α-pyrone produced by *T. harzianum* plays a major role in inhibiting the mycelial growth of phytopathogenic fungi. Furan-3-one, 2-propanone, 1-hydroxy- and similar additional ketones are also responsible for the antifungal activity (Pelias *et al.* 2022). Smaller amounts of aldehydes such as nona-2(E),4(E)-dienal, have been observed to cause oxidative stress in fungi. Foc isolates were different since they produced a lot of alcohols, of which ethanol represented 59.93%. While low concentrations of ethanol are known to have antimicrobial effects – disrupting membrane lipids and denaturing fungal proteins – excessive ethanol exposure can be detrimental to plant tissues. High levels of ethanol may weaken cell walls, increase membrane permeability, and suppress plant defense gene expression, thereby compromising structural integrity and making the host more vulnerable to opportunistic pathogens such as *Fusarium* (Yuan *et al.* 2012). Furthermore, ethanol-induced stress may alter root exudate profiles, potentially enhancing fungal chemotaxis and colonization efficiency. These findings underscore the importance of VOC concentration thresholds in biocontrol applications and the need for controlled release formulations that balance efficacy with plant safety.

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