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Real-time PCR early detection of *Trichoderma* treatments efficiency against cotton charcoal rot disease

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ABSTRACT

The charcoal rot (CRD) disease agent, the soil fungus *Macrophomina phaseolina*, survives by developing reproductive units (spores and pycnidia) and infects various host plants. In cotton plants, the disease can lead to dehydration and death of the host plant at late-season stages. Pima cv. cotton plants, the leading cotton crop in Israel, are particularly susceptible to this disease. Developing biological pesticides against soil diseases is at the forefront of scientific research globally and their importance is increasing due to the world's trend towards a reduction in the use of chemical fungicides. In this work, eight *Trichoderma* isolates were tested under laboratory conditions against *M. phaseolina*. Two *T. longibrachiatum* isolates (T7507 and T7407) and a *T. asperellum* isolate (P1) achieved promising results. The bioprotective properties of these isolates' secreted metabolites were evaluated in solid and liquid cultures. The T7407 strain was the most influential in the solid medium with 55% inhibition capacity; the P1 strain excelled in the liquid medium with 62% inhibition capacity. The three *M. phaseolina* isolates were then tested in seedlings (up to 42 days) against controls: non-infected plants, infected unshielded plants, and plants treated with non-influencing *Trichoderma* isolate (O.Y. 14707). The bio-shielding agents were added directly to the seeds with the sowing in this growth room pathogenicity assay. At the experiment's end, the *T. longibrachiatum* (T7407) treatment markedly improved the plants' wet weight (45%), height (32%) and phenological development (56%) compared to the non-influencing *Trichoderma* species control. Since the disease is commonly latent in sprouts, statistical differences in the plants' growth parameters are challenging to reach, as occurred here. Still, the real-time PCR tracking of the pathogen DNA inside the plants' roots revealed dramatic changes. The pathogen DNA dropped to near-zero levels in the *T. longibrachiatum*- and *T. asperellum*-treated plants. Interestingly, the O.Y. 14707 isolate, which showed no bioprotective properties in the lab tests or plants' growth indices, had a similar significant repression impact on the pathogen roots infection. The results of this work demonstrate the importance of the early molecular assessment of preventive treatment effectiveness against *M. phaseolina* before a full growing season evaluation.

1. Introduction

Cotton is one of the most important crops in agriculture. Globally, cotton fibers serve as a raw material for textile industries, having a yearly significant economic impact of at least 600$ billion (Khan et al., 2020). Nearly 25 million tons of total cotton is produced worldwide annually. The top three cotton-producing countries are India, China and the United States. *Macrophomina phaseolina* (Tassi) Goid charcoal rot is considered one of the world's most destructive and widespread diseases of crop plants such as cotton.

M. phaseolina, a member of the *Botryosphaeriaceae* family, is a necrotrophic, soil-borne and seed-borne phytopathogen. It has a vast host range including more than 500 cultivated and wild plant species belonging to more than 75 families (Marquez et al., 2021). Many of the species are economically significant crops, such as corn, sorghum, bean, sesame, sunflower, melon, tobacco and safflower, in addition to cotton (Singh et al., 2008). Even though only one species is documented within the genus *Macrophomina* and sexual reproduction in *M. phaseolina* is absent, results showed a high degree of genetic diversity among isolates of this pathogen. Indeed, significant variability in morphology and pathogenicity was identified among isolates from different hosts (Reyes-Franco et al., 2006).

M. phaseolina infects plants from the seedling stage to maturity (Purkayastha et al., 2006). It causes diseases such as stem and root rot (charcoal rot) and seedling blight. The fungus is a high-temperature pathogen (Lodha and Mawar, 2020). Disease severity rises as air and soil temperatures

Abbreviations: ANOVA, one-way analysis of variance; CRD, charcoal rot disease; DDW, double distilled water; PDA, potato dextrose agar; PDB, potato dextrose broth; qPCR, quantitative real-time PCR; P1, *Trichoderma asperellum*; T14707, *Trichoderma* sp. O.Y. 14707; T1607, *Trichoderma asperelloides*; T203, *Trichoderma asperelloides*; T3807, *Trichoderma atroviride*; T7107, *Trichoderma* sp. O.Y. 7107; T7407, *Trichoderma longibrachiatum*; T7507, *Trichoderma longibrachiatum*

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increase from 30 °C to 35 °C, and when soil moisture is limited, below 60% (Marquez et al., 2021). Under such favorable conditions, the fungus causes damage to cotton plants at various growth stages. Microsclerotia (pycnidia) is the principal infective source of *M. phaseolina*. This asexual fruiting body can survive up to 15 years in soil (Gupta et al., 2012). It can infect the host plant roots at the seedling stage via multiple germinating hyphae. Once in the roots, the fungus disrupts water and nutrient transport to the upper parts of the plants.

The pathogen has a vast repertoire of pathogenicity-associated genes that enables it to adhere to the host tissue (i.e., cellulose-binding elicitor lectin and transglutaminase-like proteins), neutralize the early host defense response (i.e., salicylate-1-monooxygenase), and penetrate and attack the plant epidermis. Once inside the host, the pathogen secretes an array of different toxins and cell wall degrading enzymes (CWDEs), and finally overcome the host resistence, resulting in disease establishment and host cell death (Islam et al., 2012).

When *M. phaseolina* invades the roots or stems of cotton, colonization of internal tissues develops rapidly and eventually kills the host plant. Inspection of affected parts discloses dry rot, with numerous tiny black sclerotia dispersed throughout the wood and softer tissues (Kirkpatrick and Rockroth, 2001). Cotton charcoal rot disease (CRD) is widely distributed throughout Egypt. The causal disease agent is frequently and simply isolated from cotton roots, particularly during the late growing season period. Although initial infection of cotton by *M. phaseolina* occurs at the seedling stage, the disease usually remain latent until plants approach maturity (Dhingra and Sinclair, 1978). *M. phaseolina* appears to affect some cotton cultivars less severely than others, suggesting potential genetic resistance to *M. phaseolina*. Still, resistance to *M. phaseolina* is completely lacking in commercial Egyptian cotton cultivars (Aly et al., 2006). Since Israel's cotton cultivation has shifted to extra long staple (ELS) Pima-type (*G. barbadense*) cultivars that generated higher economic value, CRD damages appear to increase in the Pima-type cultivars' fields (Cohen et al. 2022).

Despite significant research efforts to diminish the disease incidence (with varying degrees of success), the management strategies of *M. phaseolina* remain a challenge (Marquez et al., 2021). Crop rotation is not considered efficient since the fungus has competitive saprophytic ability (Almeida et al., 2001). The broad host range and high persistence of *M. phaseolina* in the soil as microsclerotia make disease control an ongoing mission. In the absence of resistant germplasm against virulent strains, the utilization of systemic fungicides is the only potential approach to diminish pathogen density (Bashir et al., 2017). According to Marquez et al., 2021 (Marquez et al., 2021), no fungicides have been registered to control this pathogen. Yet, various fungicides (i.e., carbendazim, difenoconazole, benomyl, azoxystrobin, dazome) at different concentrations were evaluated in vitro and in vivo against the CRD pathogen. One potential treatment is carbendazim (50 ppm). This systemic pesticide disrupts the mycelial growth and formation of sclerotia by inactivating the tubulin function (the building block of microtubules necessary for fungal growth) (Lokesh et al., 2020).

Current practices for controlling plant diseases aim at developing resistant varieties and applying biocontrol agents (Ons et al., 2020; Ahmed et al., 2021). Chemical antifungal compounds are being replaced with biocontrol agents due to the emergence of fungicide-resistant fungal isolates and public concerns regarding these chemicals' health and environmental impacts (Elbrense et al., 2021). While synthetic pesticides have become an integral part of agriculture to meet the increasing global food demand, their extensive use has encountered two main challenges. First, their residual toxicity effects may affect the environment and human health. Among many examples, one study conducted a meta-analysis comprising 52 studies, concluding that long-term exposure to pesticides (especially insecticides and herbicides) increases the risk of childhood leukemia by approximately 1.5–2 times (Hewedy et al., 2020). Another concern is that the use of agricultural chemical fungicides may evoke resistance to drugs used in human therapy. Such a scenario may happen when intensive azole fungicides crop protection leads to the selection of resistance to clinical azole antifungal treatment against *Aspergillus fumigatus* (de Oliveira et al., 2022). Studies that linked pesticides' exposure and diseases such as cancer and respiratory and hormone imbalance led to regulations restricting the use of synthetic pesticides (Ons et al., 2020). Second, due to the emergence of resistant pathogens, the efficacy of fungicides is constantly decreasing (de Oliveira et al., 2022). At the same time, the discovery of new fungicides is becoming more challenging and costly (Ons et al., 2020).

Biological control agents, plant metabolites and elicitors of plant defenses have received increasing attention in recent decades. Among these, *Trichoderma* spp. fungi are considered pivotal potential biocontrol agents and growth-promoting fungi for many crop plants (Hewedy et al., 2020; de Oliveira et al., 2022). They proved to be effective against several soil-borne fungal plant pathogens, including *M. phaseolina* (Bastakoti et al., 2017) (see Table 2). *Trichoderma* species have evolved many antagonistic mechanisms such as antibiotic production, nutrient competition and mycoparasitism (see for example (Zaki et al., 2021; Khan et al., 2021). Furthermore, some species can positively affect plant health and enhance systemic resistance (Martinez-Medina et al., 2016). They can directly attack the CRD pathogen by overgrowth and degrade *M. phaseolina* mycelia (wrapping around the hyphae with appressoria and a hook-like structure). These interactions are accompanied by enzymes (i.e., chitinase and β-1,3-glucanase) (RP et al., 2012) and other metabolites (i.e., volatile and non-volatile organic compounds) (Sridharan et al., 2020) secretion that alter the pathogen's mycelial structure.

To the best of our knowledge, no attempts have been made to study the effects of *Trichoderma* spp.-based treatments on the incidence of charcoal rot on cotton under Israeli conditions. This is the first work to examine the selected *Trichoderma* strains against the Israeli isolate of *M. phaseolina*. Yet, reviewing the literature can provide vital information regarding the biocontrol potential of some of the *Trichoderma* species against the cotton charcoal rot agent. Indeed most species tested in this work showed an antagonistic ability to inhibit *M. phaseolina* in former works (Table 1). The majority of these micoprasitisem assays were performed in culture plates, so they have a restricted ability to predict the success of the bio-treatments in plants.

Developing early evaluation of biopesticides against the CRD agent at the sprouting phase (ca. 40 days growth) can save time, effort, and investment. Therefore, our main objective in this study was to determine the capability of selected species of *Trichoderma* to control the CRD pathogen under controlled conditions in the lab and in sprouts in a growth room. To this end, we used a dual growth confront assay and plate assays (solid and liquid growth media) to evaluate the *Trichoderma* spp.-secreted metabolites' antifungal properties. Eventually, selected *Trichoderma* species were subjected to an in vivo sprouts assay, accompanied by quantitative real-time PCR (qPCR) tracking of *M. phaseolina* DNA inside the host plants' tissues.

Table 1
Literature review of *Trichoderma* spp. biocontrol ability against *M. phaseolina*.

***Trichoderma* species**	**Designation**	**Assay Type**	**Inhibition**	**Reference**
T. asperelloides	GJS 04–217	Plate assay [a]	69%	(de Oliveira et al., 2022)
T. atroviride	T22	Plate assay	72%	(El-Benawy et al., 2020)
T. atroviride	T2	Greenhouse	62%	(Kia and Rahnama, 2016)
T. longibrachiatum	DAOM 167674	Plate assay	73%	(de Oliveira et al., 2022)
T. longibrachiatum	Tl5	Plate assay	73%	(Hewedy et al., 2020)
T. asperellum	Ta1, Ta2	Plate assay	68, 70%	(Hewedy et al., 2020)
T. asperellum	26	volatile metabolites	44%	(Barari and Foroutan, 2016)

[a] Plate assay - dual culture assay.

2. Materials and methods

2.1. Fungal species and growth conditions

The phytopathogenic fungus, *M. phaseolina* (isolate *Mp-1*), was isolated from infested cotton plants in 2017 (Roni Cohen's lab, Newe Ya'ar Research Center, northern Israel). This isolate was identified using pathogenicity, physiology, colony morphology, microscopic characteristics and molecular traits (Degani et al., 2020a). All fungi species were maintained on potato dextrose agar (200 g potato infusion, 20 g dextrose and 20 g agar, PDA) (Difco, Detroit, MI, USA) medium in the dark at 28 ± 1 °C under high humidity for 4–7 days. To prepare the medium, PDA powder (39 g) was dissolved in 1 L of double distilled water (DDW), according to the manufacturer's recommendations. The fungus was sub-cultured routinely to a new plate (according to (Degani and Dor, 2021) for *Trichoderma* spp.) by transferring a 6-mm (in diameter) colony agar disc cut from the margins of the culture to a new PDA containing a Petri dish. The Petri dishes were kept in a 28 °C incubator (MaxQ™ 6000 Incubated/Refrigerated Stackable Shakers, Thermo Fisher Scientific, Waltham, MA USA) in the dark. Submerged cultures were grown using 10 fungal discs in a 250 mL Erlenmeyer flask with 150 mL potato dextrose broth (200 g/L potato infusion and 20 g/L dextrose, PDB, Difco Laboratories Detroit, MI, USA). The PDB was prepared by dissolving 24 g of powder in 1 L. The bottles were sealed with a breathable stopper and incubated for six days (while shaking at 150 rpm) under the above conditions.

All *Trichoderma* species used in this study are listed in Table 2. Six isolates of *Trichoderma* spp. from a marine source (the sponge *Psammocinia* sp.) were obtained courtesy of Prof. Oded Yarden (Hebrew University of Jerusalem, Israel) and were previously characterized (Gal-Hemed et al., 2011). All marine strains showed an ability to tolerate increasing osmotic pressure (halotolerant), making them more adaptable to soils with some saline conditions (for example, in fields irrigated with some degree of salty water). *In vitro* antibiosis assays revealed strong antagonistic activity of those marine strains towards phytopathogens due to the production of both soluble and volatile metabolites. Specifically, all marine-derived *Trichoderma* isolates were tested as a bio-treatment against *Rhizoctonia solani* damping-off disease on beans, *Pseudomonas syringae pv. Lachrimans* disease in cucumber and *Magnaporthiopsis maydis*, the maize late wilt pathogen, exhibiting high potential in some species (Degani and Dor, 2021; Gal-Hemed et al., 2011; Degani et al., 2021a). The other two *Trichoderma* species also included in this study are the well-established biocontrol strain T203 (*T. asperelloides* (Samuels et al., 2010)) and *T. asperellum* (isolate P1). The latter is a fungal endophyte isolated in our lab from the kernel of a maize cultivar susceptible to late wilt disease (Degani et al., 2021a). Its direct addition or the metabolites secreted to seeds, especially the 6-Pentyl-α-Pyrone active ingredient, proved to provide solid protection to maize cultivars against *Magnaporthiopsis maydis* in the lab in sprouts and full-season greenhouse plants (Degani and Gordani, 2022; Degani et al., 2021b). The growth conditions of the *Trichoderma* isolates were similar to those of *M. phaseolina* described above (Degani and Dor, 2021)."

2.2. Plate antagonism assay

The *Trichoderma* isolates were screened individually against *M. phaseolina* by employing the dual culture technique described by Degani et al. (2020a). This technique is also referred to as antagonism, confrontation, or mycoparasitism test. It aims to inspect the *Trichoderma* soluble and volatile metabolites as well as the physical contact impact on the pathogen. *M. phaseolina* and the *Trichoderma* spp. isolates were cultured separately on a solid PDA medium (preparation is detailed in Section 2.1) at 28 ± 1 °C for five days. Each 90-mm-diameter Petri dish was inoculated with a *Trichoderma* spp. colony agar disc (6 mm in diameter) on one side and a *M. phaseolina* colony agar disc on the opposite side. The distance between the colony agar discs was approximately 5 cm. In the control treatment, *M. phaseolina* culture discs were placed in the two poles of the plate. Dishes were labeled and maintained at 28 ± 1 °C in the dark. After three days, the interactions between *M. phaseolina* and the *Trichoderma* isolates were recorded and photographed. *Trichoderma* isolates limiting the pathogen's growth or growing above the pathogen mycelium were marked as having biocontrol potential. There were five independent replicates for each treatment in two duplicate experiments, and similar results were obtained. One representative plate was selected, photographed and presented in the Results section.

2.3. Cotton cultivar selected for this study

Pima cotton, Goliath cv. (extra-long-staple [ELS] cotton), is cultivated routinely in different regions of Israel (supplied by Israel Seeds, Kibbutz Shefaim). In a survey conducted by Roni Cohen across Israel (Newe Ya'ar Research Center, northern Israel), Pima cotton, Goliath cv., was classified as *M. phaseolina* charcoal rot disease-sensitive (Cohen, 2018).

2.4. Solid media assay for the Trichoderma-secreted metabolites

All *Trichoderma* were evaluated for the possibility of producing inhibitory soluble substances in vitro following the methods of Degani et al., 2021 (Degani and Dor, 2021). The preparation of PDA and PDB growth media is detailed in Section 2.1. Briefly, five 6-mm mycelial discs were removed from the edges of 2–3-day-old colonies of selected *Trichoderma* species (Table 2) and incubated in 150 mL sterilized rich liquid (PDB) in 250 mL conical flasks and incubated at 28 ± 1 °C on a rotatory shaker set at 150 rpm for six days. At the growth period end, the cultures' growth medium was filtered through a Buchner funnel with Whatman filter paper no. 3 to remove mycelial mats. The growth medium pH was adjusted to 5.1 ± 0.2 (the pH of PDB medium) with NaOH. Each culture's filtrate was then sterilized using 0.22 μm pore biofilter bottles (BIOFIL 500 mL vacuum bottle filter, Indore, India). The above liquid was used (instead of DDW) to prepare the PDA plates. PDA powder was added to the *Trichoderma* spp. growth medium filtrate as recommended by the manufacturer (39 g in 1 L). After sterilization by autoclave, the mixture was poured into Petri plates and the plates were inoculated with mycelial plugs (6 mm diameter) of *M. phaseolina* at the centers (as described in Section 4.1.).

Table 2
List of *Trichoderma* spp. isolates used in this study.

Species	Designation	Origin	Reference	Confrontation Assay Winner [2]	Tested in Sprouts
Trichoderma asperelloides	T203	ATCC 36042, CBS 396.92	(Degani and Dor, 2021; Samuels et al., 2010)	Antagonism	No
Trichoderma asperelloides	T1607	*Psammocinia* sp. [1]	(Gal-Hemed et al., 2011)	Antagonism	No
Trichoderma sp. O.Y. 7107	T7107	*Psammocinia* sp. [1]	(Gal-Hemed et al., 2011)	T7107	No
Trichoderma atroviride	T3807	*Psammocinia* sp. [1]	(Gal-Hemed et al., 2011)	Antagonism	No
Trichoderma sp. O.Y. 14707	T14707	*Psammocinia* sp. [1]	(Gal-Hemed et al., 2011)	Antagonism [3]	Yes
Trichoderma longibrachiatum	T7507	*Psammocinia* sp. [1]	(Gal-Hemed et al., 2011)	T7507	Yes
Trichoderma longibrachiatum	T7407	*Psammocinia* sp. [1]	(Degani and Dor, 2021; Gal-Hemed et al., 2011)	T7407	Yes
Trichoderma asperellum	P1	*Zea mays*, Prelude cv.	(Degani et al., 2021a)	P1	Yes

[1]Mediterranean sponge *Psammocinia* sp. [2] Mycoparasitism assay outcomes, including the following options: *M. phaseolina* or *Trichoderma* sp. covers the colony surface of the other, and antagonism or confrontation occurs where none of the two species can extend above the other, and at the meeting point their development stopped. [3] The *Trichoderma* strain T14707 is a weak antagonist to *M. phaseolina.*

The colony growth rate was measured after three days compared to the growth rate of the fungus grown on a standard PDA substrate. Each treatment was performed in five repetitions. The experiment was repeated twice, obtaining similar results.

2.5. Liquid media assay for the Trichoderma-secreted metabolites

The effect of the *Trichoderma* isolate-secreted products was also examined on liquid media flasks, according to Degani et al. (2021b). To this end, 100 mL was moved from the *Trichoderma* cultures' filtrate into a sterile 250 mL Erlenmeyer flask. Sterilized glucose solution (6% in the stock) was added to a final 2% concentration in the flask, identical to the amount of glucose in a standard PDB substrate. The control was PDB medium *M. phaseolina* cultures maintained under the same conditions. The solutions were poured into a 96-well plate and a 6-mm colony mycelia disc was added to each well as described in Section 4.1. After three days of incubation without shaking, the fungus' area coverage of the well surface was measured. Each treatment was performed in five repetitions, the whole experiment was repeated twice, and similar results were obtained.

2.6. Preparation of infected sterilized wheat kernel

For the seedlings experiment, infected sterilized wheat kernels were used to spread the fungi in the soil, as previously described (Degani et al., 2020b). Wheat kernels were soaked in tap water overnight. The seeds were then dried for about four hours in a fume hood on towel paper and autoclave sterilized for 30 min at a temperature of 120 °C. Plastic 0.5 l boxes were disinfected and used for inoculating 100 g sterilized wheat kernel with 10 mycelial discs (see Section 4.1) of *M. phaseolina*. The boxes were sealed with a lid (tightened to the box using Saran wrap), covered with aluminum foil (to ensure dark conditions), and incubated at 28 ± 1 °C in the dark for 10 days.

2.7. Seedlings experiment in a growth chamber under controlled conditions

Four *Trichoderma* isolates were selected for the pot experiment according to their performance in the in vitro assays (dual culture confront test and the secreted metabolites inhibitory on solid or liquid medium). Three of them, T7507, T7407 (*T. longibrachiatum*) and P1 (*T. asperellum*), excelled in these tests, exhibiting high biocontrol behavior against the CRD pathogen. The fourth, T14707, lacked any clear antagonistic activity against *M. phaseolina* and was chosen as a reference strain and a control. The *Trichoderma*-based protection treatments were conducted by adding three colony agar discs (see Section 4.1) of the selected *Trichoderma* species to each seed with the sowing.

The soil was inoculated one week before the experiment by adding eight gr' sterilized wheat kernels infected by *M. phaseolina* to the top layer of the pot's soil up to a depth of 5 cm. Complementary infection was done on the sowing day by adding three *M. phaseolina* colony agar discs (see Section 4.1) to each cotton seed. The non-infected control pots were treated with sterilized wheat kernels, and sterilized PDA agar discs were added to each cottonseed with the sowing.

Two-liter pots were filled with 70% commercial, non-sterilized garden soil and 30% Perlite No. 4 (for aeration). The commercial garden soil mixture (Garden Mix, Deshanit, Be'er Yaakov, Israel) comprised fibers, coconut peat, a relatively low tuff concentration, and Osmocote (ScottsMiracle-Gro, Marysville, Ohio, USA), a 3–4 month slow-release fertilizer. The Pima cotton cv. seeds were soaked for about 15 min in distilled water and sown in pots to a depth of 5 cm (five seeds per pot). The pots were kept in a growth room with a photoperiod of 16 h of light and 8 h of darkness. All the plants were grown under the same conditions of constant humidity of 45% and a temperature of 27 ± 2 °C. Immediately after sowing, the pots were irrigated to initiate germination. Irrigation was carried out 60 mL once a day using a computerized irrigation system. Throughout the experiment, treatments against various pests and fertilization treatments were performed according to the Israel Ministry of Agriculture Consultation Service (SAHAM). The experiment was conducted in six repetitions per treatment (each repetition is a pot containing five sprouts).

An emergence above-ground test was performed three days after sowing. At the end of the experiment (day 42), all seedlings were gently removed from the ground, thoroughly rinsed with running tap water and dried using paper towels. All plants were subjected to a phenological stage (number of leaves), shoot length and wet biomass estimation. In addition, a 0.7-gram root sample was taken from each pot for DNA extraction.

2.8. DNA purification

For DNA purification, the plants' roots were washed twice in sterile DDW for 30 s. Tissues were sampled by removing a cross-section of approximately 2 cm in length from each of the plant's roots and near-surface hypocotyl under a sterile biosafety hood. Samples from the five plants of each plant pot were combined, and the total weight was adjusted to 0.7 g and considered one repeat. All tissue samples were inserted into universal extraction BioMed bags (Bioreba, Switzerland) with 4 mL CTAB solution per bag. The samples were ground with a tissue homogenizer (Bioreba, Switzerland) for five minutes until the tissues were completely homogenous. The homogenized samples were treated for DNA purification, as previously described (Degani et al., 2019a). The DNA samples were suspended in 100 microliters of ultra-pure quality water and stored in a freezer at −20 °C until used in the qPCR reaction.

2.9. Real-time PCR-based molecular diagnosis

The qPCR reactions were performed as previously described (Degani et al., 2019a) using the ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) for 384-well plates. The MpK FI/RI primers were used for *M. phaseolina* detection (300–400 bp species-specific fragment) (Degani et al., 2020a; Babu et al., 2007). The housekeeping COX gene encoding the enzyme cytochrome c oxidase – the mitochondria's last enzyme in the cellular respiratory electron transport chain – aimed at normalizing the *M. phaseolina* pathogen DNA (Weller et al., 2000). The COX amplification was done using the COX F/R primer set (Weller et al., 2000; Li et al., 2006). Calculation of relative gene abundance was made according to the ΔCt model (Yuan et al., 2006). Similar efficacy was assumed for all samples. All amplifications were performed in triplicate.

To perform the qPCR, each well contained 2 microliters of the sample DNA, 2.5 μL of Universal SYBR Green Supermix iTaq™ (Bio-Rad Laboratories Ltd., Hercules, CA, USA), and 0.25 μL of the forward and reverse primers (10 μM from each primer per well). The qPCR cycle program was as follows: pre-cycle activation stage (1 min at 95 °C); denaturation stage (15 s at 95 °C) for 40 cycles; annealing and extension (30 s at 60 °C); and finalizing by melting curve analysis.

2.10. Statistical analysis

Data statistical analysis was carried out by one-way analysis of variance (ANOVA) using JMP software, version 15 (SAS Institute Inc., Cary, NC, USA). The means' differences were identified using a t-test for each pair (without correction based on multiple tests). The significance threshold is $p \leq 0.05$. In the analysis of the results of the potted plant experiment and the qPCR analysis, no statistical significance was found due to relatively high natural variations in the fungi pathogenesis. Still, some differences can be recognized as being statistically significant ($p \leq 0.05$) after a one-tailed student t-test (a more powerful test) that compares each treatment separately to the infected control. Those cases are indicated by an asterisk (*) above the bar graphs.

3. Results

The current study investigated the biological control capability of eight *Trichoderma* isolates (listed in Table 2) against the cotton charcoal rot disease agent, the fungus *M. phaseolina,* using screening tests and a sprouts assay in a growth chamber. An antagonism test that examined the inhibitory activity of the *Trichoderma* strains (Fig. 1) showed that at least three isolates, T.7407, T.7507 and P1, have a bioprotective potential, expressed as the ability to grow

on top of the *M. phaseolina* colonies and prevent its spread.

Next, we examined the inhibitory effect of the secretory products of the *Trichoderma* isolates in solid substrate plates (Figs. 2, 3). The growth fluids filtered from *Trichoderma* isolates cultures were used to make rich PDA media plates. They were seeded by *M. phaseolina* and photographed three days later (Fig. 2). Also the radius of the colonies was measured (Fig. 3). Soluble metabolites secreted from isolates T7407 (*T. longibrachiatum*) and T7107 (*Trichoderma* sp. O.Y. 7107) were found to have a significant inhibitory effect ($p \leq 0.05$) on the growth of the pathogen in this test. The inhibitory effect of the *Trichoderma* isolates on the pathogen's development was 55% for the T7407 isolate and 26% for the T7107.

The latest test under laboratory conditions (in vitro) examined the development of the pathogen in a liquid substrate (shallow stationary cultures) with the substrate containing the growth fluid of the *Trichoderma* isolates (after pH correction) and the addition of PDB. After three days of incubation, it could be seen that the isolates P1 (*T. asperellum*), T1607 (*T. asperelloides*) and T7107 (*Trichoderma* sp. O.Y. 7107) significantly inhibited ($p \leq 0.05$) the pathogen growth (by 62%, 16% and 16%, respectively, Fig. 4).

To conclude, the in vitro experiment series (dual culture confrontation and secreted soluble metabolites tests) revealed that three isolates, *T. longibrachiatum* (isolates T7507, T7407) and P1 (*T. asperellum*), best inhibited *M. phaseolina* growth in the mycoparasitism assay, while two of them, T7407 and P1, also excelled in the secreted metabolites tests. Hence, these isolates were selected for biocontrol of *M. phaseolina* in vivo in a sprouts pathogenicity assay. A fourth isolate, T14707, did not exhibit any potent visible antagonistic behavior against *M. phaseolina* and was chosen as a reference/control strain.

In the pot experiment, a bio-pesticide treatment based on selected *Trichoderma* isolates was applied to protect the CRD susceptible Pima cv. cotton plants, in pots, up to the age of 42 days. At the sprout stage, when the disease symptoms are expected to be minor (commonly the disease is latent), the development measures are restricted in their ability to reflect and predict the biocontrol treatment potential. Still, this stage is crucial to rapidly eliminate ineffective treatments and identify possible influences on this delicate developmental stage. Indeed, no statistically significant differences were found between the treatments or the treatments and control (Fig. 5). However, measurable differences can be seen. The above-ground emergence percentages evaluated three days from sowing were high in the non-infected and infected controls (53% and 60%, respectively) and relatively low in the *Trichoderma*-based treatments (30–43%), except for the T7507 treatment, which achieved an emergence percentage similar to the controls (57%). At the end of the experiment, the survival rate and growth indices estimated 42 days from sowing showed a clear advantage for plants treated with *T. longibrachiatum* (T7407) (Fig. 5). This treatment, relative to T71470, the non-influencing isolate treatment, resulted in a 45% improvement in the wet weight of the plants (the positive control was high in this index), 32% in plant height, and 56% in phenological development (expressed as the number of leaves). Most interestingly, the qPCR analysis conducted on the plants' roots revealed the efficiency of the bio-shield treatments (Fig. 6). The DNA amount of the cotton pathogen decreased dramatically ($p \leq 0.05$) in the treatments with T7407, T71470 and P1 isolates.

4. Discussion

Biopesticides have earned substantial scientific attention in recent years because they are a vital alternative replacement for the much debated traditional chemical pesticides used to protect field crops. The extent of *M. phaseolina* infection in cotton plants may result in both pre- and post-emergence plant mortalities. Still, the symptoms of the disease in a natural infection usually appear near the flowering stage (Cohen, 2018). At first, the cotton plant's leaves turn yellow, a phenomenon probably caused by toxins secreted by the fungus, and move to the plant's upper organs by water transport in the vascular system. Later, the wilting of the whole plants that remain standing can be observed. Another typical symptom is the browning of the vascular bundles resulting from the toxins' activity and the plant's counter-reaction. Later, when the thin roots begin to rot, many sclerotia can be seen under the root bark.

In the current study, the biocontrol capability of various isolates of *Trichoderma* spp. against *M. phaseolina*, the causal agent of cotton charcoal rot (CRD), was investigated using in vitro and in vivo assays. To the best of our knowledge, this screening was conducted here for the first time against the Israeli *M. phaseolina* strain with this collection of *Trichoderma* isolates. This preliminary work is essential for revealing the bioprotective potential and pointing out specific *Trichoderma* isolates that are good candidates for follow-up work. Such work should include whole growth period experiments (as demonstrated in other plant diseases (Degani and Dor, 2021; Degani et al., 2021a) and various application methods of the biocontrol fungi or their secreted metabolites. Another future research direction is to identify the active ingredient/s in the *Trichoderma* spp.-secreted metabolites, as recently demonstrated (Degani et al., 2021b).

Following their success in the dual culture confronts assay, three isolates, T7507, T7407 (*T. longibrachiatum*) and P1 (*T. asperellum*), were tested against other *Trichoderma* isolates to study their secreted metabolites' bioprotective potential. T7407 was the most influential species in the solid medium test with 55% inhibition capacity, while P1 excelled in the liquid medium assay with 62% inhibition capacity. These differences may hint at the possibility that different action mechanisms or metabolites are involved in *M. phaseolina* growth suppression in those dissimilar media (Bastakoti et al., 2017). Finally, a biocontrol assay in sprouts under controlled conditions was conducted to complete this series of rapid evaluations of *Trichoderma* spp. candidates (Fig. 5). At the experiment's end of this test (42-day-old seedlings), the soil treatment with *T. longibrachiatum* (T7407) resulted in plant growth promotion in all parameters compared to the non-influencing T71470 isolate (a weak-antagonistic reference strain).

Interestingly, in the sprout pathogenicity assay, both controls with or without *M. phaseolina* inoculation had relatively high growth values. This

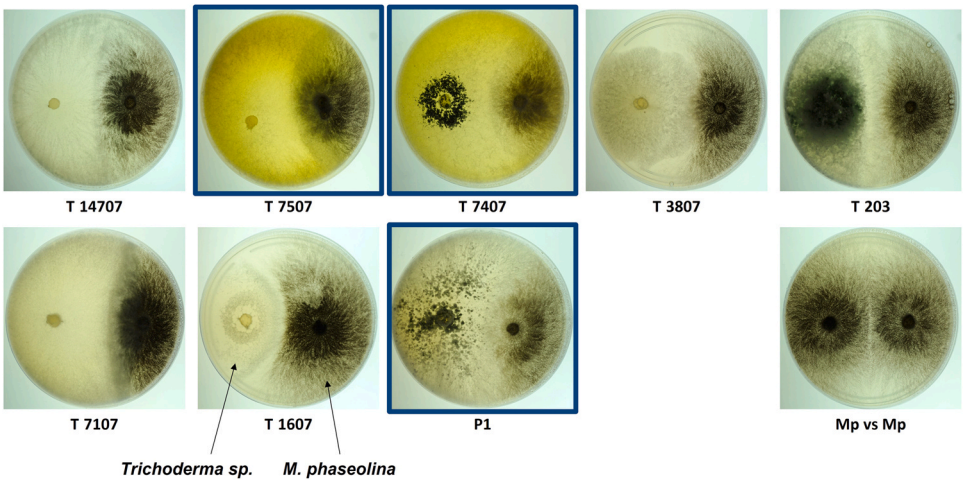

Fig. 1. Mycoparasitism of *Trichoderma* spp. against the cotton charcoal rot pathogen *M. phaseolina*. *Trichoderma* isolates (shown in Table 2) were seeded on the left and *M. phaseolina* (Mp) on the right. The fungi were sown on rich solid substrate (PDA) plates and incubated for three days at 28 ± 1 °C in the dark. The control is a growth plate where *M. phaseolina* was seeded in both poles (Mp vs Mp). Each photo is representative of five independent replicates for each treatment. Three *Trichoderma* isolates that have shown the ability to grow on the colony surface of the cotton pathogen *M. phaseolina* are marked by a blue frame.

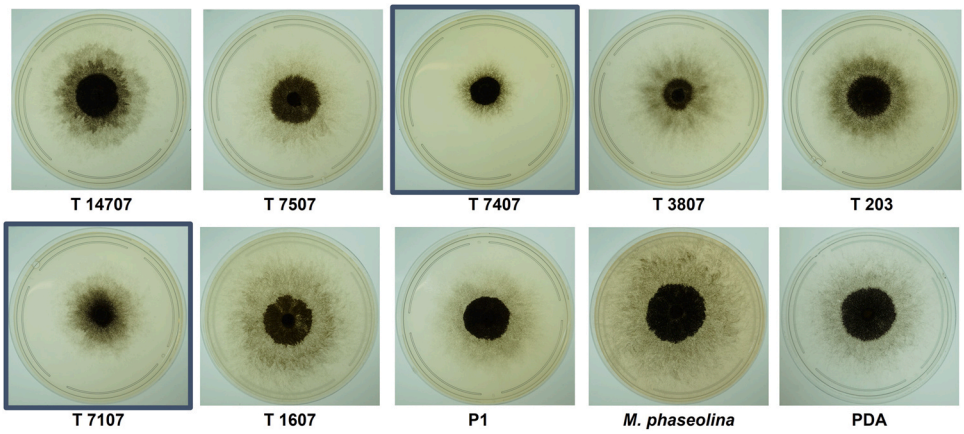

Fig. 2. Effect of *Trichoderma* spp. growth fluid on the development of the cotton pathogen *M. phaseolina* in solid media plates. The fungus *M. phaseolina* was seeded on rich solid substrate (PDA) plates containing the excreted metabolites of *Trichoderma* isolates (shown in Table 2). Plates were incubated for three days at 28 ± 1 °C in the dark. The controls are *M. phaseolina* grown on its own liquid growth medium and *M. phaseolina* grown on regular PDA. Each photo is representative of five independent replicates for each treatment. Two *Trichoderma* isolates whose secretion products inhibited the growth of the cotton pathogen are marked by a blue frame.

may be the consequence of the methodology used. Instead of *Trichoderma* culture discs, we added sterilized PDA agar discs in these controls (see Section 4.1). This procedure may unintentionally supply nutrients to the germinating seeds and enhance plant growth. It is likely that when the PDA agar disc contained the fungus, it consumed its nutrients. Hence, those excessive components will not affect plant growth. It should also be pointed out that such influence was not observed when the same method was applied in maize (*Zea mays* L.) (Degani et al., 2020b; Degani and Cernica, 2014) or in onion (*Allium cepa* L.) (Degani and Kalman, 2021). Thus, the outcome of adding agar discs to the controls may be associate directly with cotton plants cultivation.

For this reason, we added control treatment with the non-influencing T71470 isolate and compared the results to this treatment. This reference isolate had a similar influence to the control in the in vitro experiments and caused the lowest growth values in the seedling assay. Still, isolate T71470 unexpectedly reduced the pathogen DNA in the sprouts' roots comparable to the two successful treatments, T7407 and P1. This result implies a more complex picture and that *Trichoderma* isolates may perform distinctively in different tests.

Although CRD may cause damage to sprouts' survival and development, its major impact is revealed in the late growth phase of cotton plants (Dhingra and Sinclair, 1978; Degani et al., 2020a). In field trials conducted by Dr. Roni Cohen (Institute of Crop Protection, A.R.O., The Volcani Center, Israel) in the Hefer Valley on Israel's coastal plain and the Jezreel Valley (lower Galilee, northern Israel), cotton seeds or young plants were inoculated by *M. phaseolina* hyphae fragments (Cohen, 2018). While the fungus was identified in a significant proportion in the plant roots, no disease symptoms could be observed. The fungal infection appears to be successful, but the onset of symptoms occurs only in some cases and largely depends on environmental conditions.

Indeed, in our potted sprouts experiment, significant statistical differences could not be identified. Still, the molecular base qPCR tracking of the pathogen DNA inside the plants' roots had proved the importance of the biological treatments applied. Such a sensitive experimental technique is essential for the early detection and evaluation of preventing treatment impact, especially at the latent phase of the disease, as previously shown (Degani et al., 2019b). A scientific program to develop disease biocontrol cannot solely rely on a full growth period under field conditions. A complete growing season of cotton can last nearly five months. Such a growing season involves great efforts and being exposed to changes in environmental conditions that cause inconsistent results. So, to focus the challenge of field trials on the most promising bio-treatments, rapid screening should be implied first, as was done in this work.

Similar *Trichoderma*-based control studies were conducted in cotton and other crops against *M. phaseolina*, as illustrated by the following few examples. Aly et al. (2007) screened five *Trichoderma* sp. under greenhouse conditions against 14 isolates of *M. phaseolina* in cotton plants. They conclude that specific *Trichoderma* species (*T. harzianum,* Bi isolate) can be highly effective against one isolate of *M. phaseolina*. The Bi isolate treatment resulted in a 96.7% plant survival rate compared to the unprotected control plants with only a 46.7% survival rate. But they also showed that this *Trichoderma* isolate might have minimal effects on other isolates of *M. phaseolina*. In line with this finding, Cardona and Rodriguez (2006) showed that there was no effect of *T. harzianum* on the incidence of the charcoal rot disease in sesame (Cardona and Rodriguez, 2006).

In another study conducted on soybean, the biological control potential of 11 *Trichoderma* spp. isolates against *M. phaseolina* was explored using screening tests (Khaledi and Taheri, 2016). Among all *Trichoderma* isolates tested, inhibition varied from 20.22% to 58.67% in dual culture tests. Two *T. harzianum* isolates best inhibited the growth of *M. phaseolina* in vitro. In cowpea, unidentified soil *Trichoderma* sp., *Trichoderma koningii* Oudem (IMI 361600) and *T. harzianum* Rifai

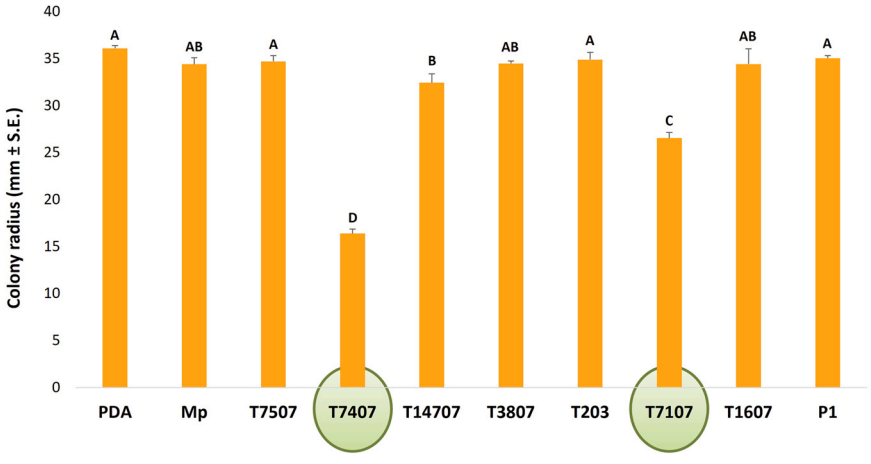

Fig. 3. Measurement of the pathogen *M. phaseolina* radius colonies grown on a solid substrate containing secretory products of *Trichoderma* isolates. The experimental conditions are as described in Fig. 2. Measurement of the radius of the colonies was performed on day 3 of the growth. Averages represent five replicates. Error lines represent a standard error. Different letters above the columns represent a statistically significant difference ($p \leq 0.05$, columns highlighted by a green circle).

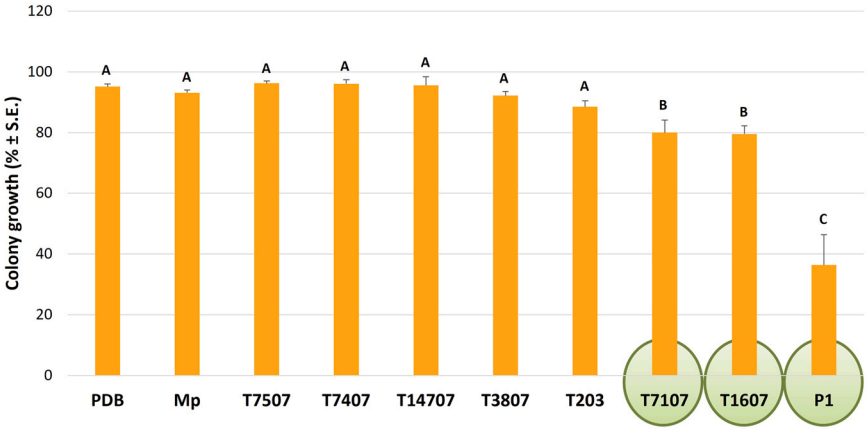

Fig. 4. Effect of the extracellular growth fluid of the fungus *Trichoderma* spp. against the cotton pathogen, *M. phaseolina*, in shallow liquid medium cultures. The fungus *M. phaseolina* was sown on plates containing the excreted metabolites of *Trichoderma* isolates (shown in Table 2). The controls are *M. phaseolina* grown on its own liquid growth medium and *M. phaseolina* grown on regular PDB. Measurements of the colonies' percent coverage in each well were performed on day 3 of the growth. Averages represent five replicates. Error lines represent a standard error. Different letters above the columns represent a statistically significant difference ($p \leq 0.05$, columns highlighted by a green circle).

(IMI 361601) were tested in vitro for their antagonistic behavior against *M. phaseolina* before being applied in the field (Adekunle et al., 2006). In dual dixenic cultures, all three *Trichoderma* species had superior growth and stopped the pathogen's development by the third day. In a groundnut study, *T. viride* (Tv1) and *T. harzianum* effectively reduced the mycelial growth and sclerotial formation of *M. phaseolina* (Karthikeyan et al., 2006). The culture filtrates of *T. viride* (Tv1) excel in the inhibition of the pathogen growth and sclerotial germination even more than the fungus itself. In the same work, the application of *T. viride* with pungam (*Pongamia pinnata*) cake in greenhouse plants markedly reduced root rot incidence, recording 3% compared to 44% in control (Karthikeyan et al., 2006).

Future efforts should be dedicated to screening more *Trichoderma* isolates against the CRD pathogen, identifying the most promising candidates, and testing them against various *M. phaseolina* isolates (obtained from charcoal rot diseased cotton plants in different areas). Such rapid in vitro assays can be accompanied by cotton sprouts' bio-protection if molecular or more traditional phytopathological methods are used to evaluate the pathogen's establishment in the host plants. Eventually, a field assay over an entire growing season is crucial to develop a commercial, validated bio-based product. Future cellular and molecular investigations on the responses activated by *Trichoderma* spp. or other plant growth-promoting fungi against *M. phaseolina* are also critical. Such work will improve our knowledge of the mechanisms involved and help develop novel and environmentally safe strategies to control this economically important cotton disease.

5. Conclusions

Charcoal root rot of cotton caused by the fungus *Macrophomina phaseolina* is a severe disease having few management options. While effective fungicides are available, it is becoming increasingly evident that their widespread use is associated with some problems, such as the potential harmful effect on non-target organisms, the development of resistance races of the pathogen, and public health concerns. *Trichoderma* spp. are considered potential eco-friendly biocontrol agents and growth-promoting fungi for many crop plants. The current study offers such a safe and green approach based on selected *Trichoderma* species to eradicate the cause of the cotton charcoal rot disease, *M. phaseolina*. In a series of laboratory experiments, we examined the

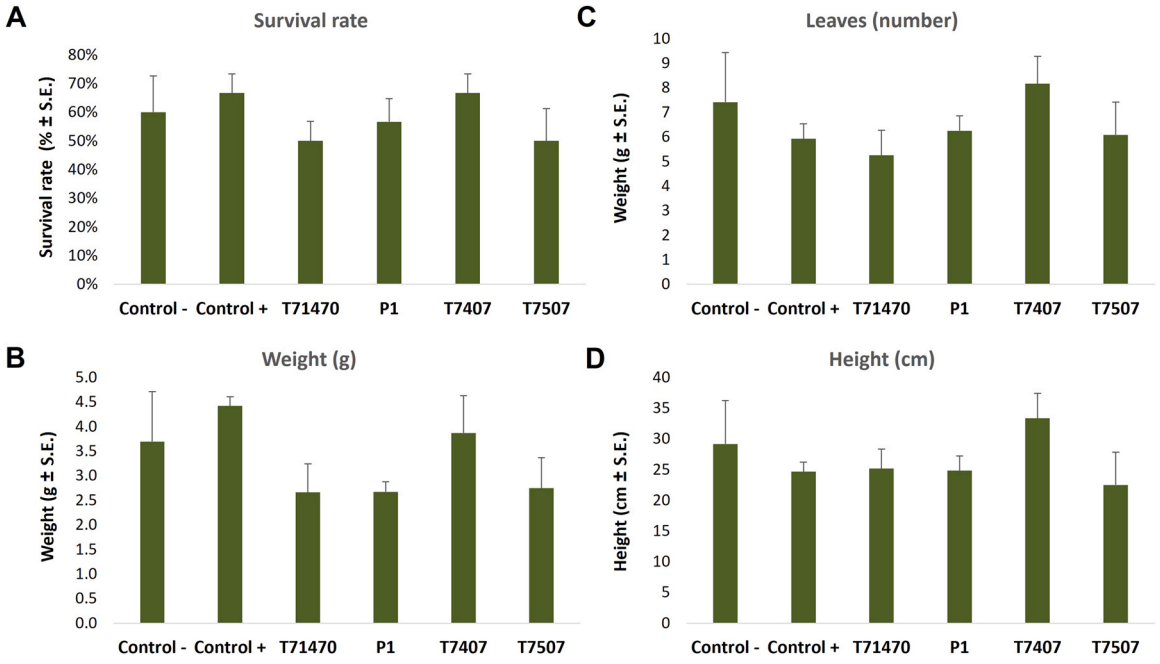

Fig. 5. Bioprotection of cotton sprouts against charcoal rot disease using *Trichoderma* spp. The charcoal rot disease susceptibale Pima cv. cotton buds were treated with the *M. phaseolina* fungus and were grown in a growing room for 42 days. Pesticide treatments were done by adding three mycelial discs (6 mm in diameter) of the *Trichoderma* isolates (shown in Table 2) to each seed at sowing. The seedlings' survival rate (A), fresh weight (B), number of leaves (C) and height (D) were measured at the experiment's end. The controls included healthy untreated plants (control -) and infected plants without *Trichoderma* treatment (control +). Averages represent six replicates. Error lines represent a standard error. No statistically significant difference was found between treatments or treatments and control.

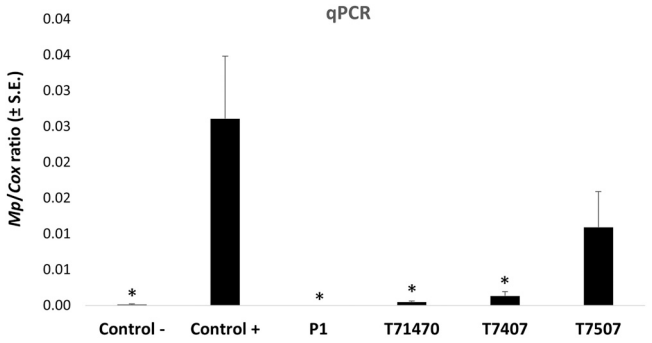

Fig. 6. *M. phaseolina* DNA evaluation in the cotton plants' roots in the experiment is described in Fig. 5. The Y axis shows the amount of the *M. phaseolina* specific DNA segment relative to the housekeeping gene encoding cytochrome C oxidase (COX), the last enzyme in the cellular respiratory system. Values represent an average of six repetitions. Error lines represent a standard error. An asterisk above the columns denotes a statistically significant difference ($p \leq 0.05$ in the T-test versus the infected control (control +). No significance was found in the weaker ANOVA test.

pesticidal potential of the *Trichoderma* species. Two *T. longibrachiatum* isolates (T7507 and T7407) and a *T. asperellum* isolate (P1) efficiently restricted the pathogen growth in a dual culture confrontation assay, and their secreted metabolites strongly inhibited the pathogen growth in solid and liquid media cultures.

In cotton sprouts at the age of 42 days, the *T. longibrachiatum* (T7407) bio-shield treatment recovered the plants' growth indexes to the level of the uninfected control and reduced the pathogen DNA in the plants' roots to the threshold levels. The results presented in this study should encourage a follow-up examination of *T. longibrachiatum* (T7407) and perhaps other *Trichoderma* isolates over an entire growth period under field conditions. A combination of different biological pesticides should be considered to enhance the treatments' effectiveness. In addition, examining the biological application in combination with a chemical pesticide may produce an effective control strategy that could be applied in severe cases. The importance of bio-chemical combined pesticides is that they disciplinarily reduce the use of pesticides while maintaining high efficacy and stability to the bio-shielding.

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CRediT authorship contribution statement

Conceptualization, O.D., P.B. and A.G.; data curation, O.D., P.B. and A.G.; formal analysis, O.D., P.B. and A.G.; funding acquisition, O.D.; investigation, O.D., P.B. and A.G.; methodology, O.D., P.B. and A.G.; project administration, O.D.; resources, O.D.; supervision, O.D.; validation, O.D.; visualization, O.D.; writing (original draft), O.D.; writing (review and editing), O.D., P.B. and A.G. All authors have read and agreed to the published version of the manuscript.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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