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RESEARCH

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Exploring the efficacy of a *Trichoderma asperellum*-based seed treatment for controlling *Fusarium equiseti* in chickpea

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Abstract

Background Chickpea plant (*Cicer arietinum* L.) is an important legume crop that is vulnerable to various fungal pathogens causing significant yield losses. Among them, *Fusarium equiseti* is a pathogen that has started to raise concern. In contrast, *Trichoderma* species have been explored for their ability to control such pathogens. In this study, the efficacy of a novel seed treatment formulation was explored for controlling *F. equiseti* in chickpea plants. The formulation was designated to enhance growth in chickpea plants as well as the ability to protect plants from infection. In addition, this formulation was tested for its effectiveness in maintaining the conidia of the antagonist in the soil after sowing.

Results Applying the *Trichoderma asperellum*-based formulation promoted growth, as well as root and aerial biomass. In seedlings derived from treated seeds, the shoot length increased by 36.8%, and the average number of leaves also increased than the control. Following evaluation of disease severity and the foliar alteration index (FAI), a protective effect was noted, as the symptoms of *Fusarium* were significantly reduced in treated plants than the infected control. Re-isolation from plants infected with *F. equiseti* was successful in the roots (72.7%), root crown (84.5%), stem (64.4%), and even in petioles (36.1%).

Conclusions Due to both direct antagonist activity and indirect growth promotion ability, the findings suggested that tested formulation can be a sustainable and eco-friendly alternative to chemical fungicides for managing *F. equiseti* in chickpea seeds.

Keywords Chickpea, Formulation, *Fusarium equiseti*, Growth, *Trichoderma asperellum*

Background

Pulses play an important role in the food system as a source of vegetable protein and are increasingly present in the Mediterranean diet, especially among people with limited income (Ali et al. 2017). Chickpea (*Cicer arietinum*) is one of the most important food legumes in the world according to the Food and Agriculture Organization of the United Nations (FAOSTAT 2020). In Morocco, chickpea cultivation accounts for approximately 40% of the national production of consumable legumes, second to faba beans. The crop can resist drought (Jerotich and Mugendi 2013), but is susceptible to fungal

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diseases, which are considered a major limitation of its development. The impact of diseases induced in chickpeas by *Ascochyta rabei*, *Rhizoctonia solani*, *Fusarium oxysporum*, and *Morrophomina phaseolia* (Mabsoute and Saadaoui 1996) was more prominent under abiotic stress conditions (Sinha et al. 2019).

Fusarium wilt is a destructive disease caused by fungal soilborne pathogens such as *F. oxysporum* or *F. solani* that causes significant losses for many important vegetable and crop plants, including Chickpea, all over the world (Knights and Hobson 2015) causing significant economic losses up to a 100% yield loss. Despite the progress made in recent investigations on pathogen resistance in germplasms, breeding lines, and local chickpea varieties (Sharma and Ghosh 2016), the risk of developing this disease is high (Singh et al. 2022). *Fusarium equiseti* has received more attention recently about its pathogenicity and its impact on different crops. In Morocco, *F. equiseti* was identified as the causal agent of rot symptoms and root necrosis in chickpea plants, leading to stunted growth and reduced plant vigor (El Hazzat et al. 2023).

There are many methods to control fungal diseases, such as conventional fungicides, which are often used, but their excessive use causes serious damage to the environment (Pal et al. 2013). Thus, fungicides harm the mycoflora of the soil (Ratna et al. 2017) and human health (Nicolopoulou-Stamati et al. 2016). Pathogens also develop resistance to the continued use of fungicides (Yin et al. 2023). Pathogen populations that develop resistance to one fungicide can sometimes simultaneously resist one or several other fungicides; this phenomenon is known as cross-resistance (Yang et al. 2019). Another limitation is the relatively high costs of these fungicides. Therefore, it is wise to look for other alternative means, such as biological ones, which are less expensive, can coexist with the soil microflora, and have long shelf life.

Species of the genus *Trichoderma*, particularly *T. harzianum* and *T. asperellum*, have been studied extensively for their ability to promote plant growth and enhance soil health as well as their potential to control plant pathogens, such as *Verticillium dahliae* and *F. oxysporum* on tomato (Sghir et al. 2016), *Curvularia spicifera*, *Bipolaris sorokiniana*, and *F. roseum*e on wheat and barley (Qostal et al. 2020b), and *Pyricularia oryzae* and *Helminthosporium oryzae* on rice (Ratna et al. 2017). Based on the study by Sanjeev et al. (2015), *Trichoderma* strains, as conidial suspensions, cannot be used directly as a plant treatment under field conditions. They must be used in the form of preparations and transported via specific carriers. These formulations facilitate storage, commercialization, and application to plants.

Although many techniques are available for seed treatment with antagonists (Berg 2009), it is still difficult to standardize the formulations of products based on these microorganisms, their distribution, and the methods of their application in the field (Herrmann and Lesueur 2013). In most cases, seed treatment is performed in the field before sowing (O´Callaghan et al. 2012). Nevertheless, sowing is usually a busy time for farmers, and the task of seed handling is an extra task. Before sowing, pretreatment of seeds with a stable and viable inocula of antagonistic microorganisms is preferable (Oukara et al. 2017). However, the survival of microorganisms in the seeds is low (Swaminathan et al. 2016). Therefore, an ideal formulation should satisfy a set of criteria. For example, it should ensure the survival of conidia for a long storage period, not have a toxic effect on crop plants, and tolerate harsh environmental conditions (Sanjeev et al. 2015).

The objective of the present study was to verify the efficacy of a chickpea seed treatment formulation, which also ensures the viability of *Trichoderma* spores on the seed and in the soil, and test the stimulating and protective effects of *T. asperellum*, delivered through this formulation, against *F. equiseti*-induced chickpea wilt and root rot.

Methods

Fungal material

The microorganisms were cultured on potato dextrose agar (PDA, Biokar diagnostics) in Petri plates and incubated at 28 °C. The strain TH2 of *T. asperellum* accession number MW074122 was used in the study. The biocontrol agent was isolated from banana soil in a previous work, and it was selected out of several microbial candidates according to their performance (Kribel et al. 2019). The *T. asperellum* solution was prepared from a 7-day-old culture by immersing each dish in 10 ml of sterile distilled water. The cultures were scraped, and the resulting spore suspension was filtered through a muslin cloth. The concentration was adjusted to 10^7 spores/ml on a Malassez slide. Ten percent of gelatin was added to the solution as an adhesive substance. This preparation was used as a formulation combined with talcum powder. For every 100 g of seeds, 10 ml of the formulation was used. *F. equiseti* N3 accession number MT111122, isolated from a chickpea plant showing symptoms of desiccation and root rot (El Hazzat et al. 2023), was used in the present study. Soil inoculum was prepared from 7-day-old *F. equiseti* N3 cultures. The cultures were immersed in 10 ml of sterile distilled water in each dish, and the spore suspension was filtered through a muslin cloth and adjusted to a concentration of 10^6 spores/ml using a Malassez slide.

Plant material

Seed of chickpea (*C. arietinum* L.) RIZKI (FLIP 83–48 C) was obtained from the National Institute for Agronomic Research INRA, Rabat, Morocco.

Glasshouse experiment

The pot experiment was conducted with four treatments in five replications from October 2018 to March 2019 under a glasshouse (at 20–25 °C) in the Department of Biology, Faculty of Science, Ibn Tofail University. Mamora soil (Table 1) was sieved and sterilized at 200 °C for 2 h. Plastique pots were filled with 2 kg of sterilized soil. Chickpea seeds were surface-sterilized with 90% ethanol solution for 30 s, rinsed three times with sterile distilled water, and dried between two layers of sterile filter paper. The surface-sterilized chickpea seeds were inoculated with *T. asperellum* formulation. (L_{Ta}), three inoculated seeds of each treatment were sown in each pot. After one week of germination, thinning was done to retain one seedling per pot. The same procedure was repeated, but this time with soil inoculated with 10 ml of the pathogenic N3 strain of *F. equiseti* (10^6 spores/ml) 24 h before sowing ($L_{Ta/Fe}$). The control was treated with only sterile distilled water, and another group was inoculated with *F. equiseti* (L_{Fe}). Pots were arranged in a randomized block design, and each treatment was replicated five times. Pots were irrigated with tap water as needed.

Assessment of plant growth

The seedlings of chickpea emergence were observed, and the germination percentage was assessed during the first week after sowing. For measuring growth parameters, three plants were randomly uprooted by digging from each pot, 60 days after sowing. Plant roots were washed in running water to remove the adhering soil particles without any teasing of the roots. Shoot length, root length, fresh weight, and number of leaves and twigs were recorded, and the dry weight was assessed after drying at 70 C for 72 h in a hot air oven.

Estimation of FAI

The disease was estimated by calculating the foliar alteration index, noted according to the following scale (Douira and Lahlou 1989):

Score	Appearance of leaves
0	Healthy appearance
1	Cotyledonary leaf: wilting or yellowing
2	Cotyledonary leaf: fall
3	True leaf: wilting or yellowing
4	True leaf: necrosis
5	True leaf: fall

The scores related to the number of leaves constitute the foliar alteration index, which was calculated according to the following formula (Douira and Lahlou 1989):

$$\mathrm{FAI} = [\Sigma(\mathrm{i} \times \mathrm{Xi})]/6 \times \mathrm{NtF}$$

Here, FAI stands for foliar alteration index; i represents the leaf appearance notes 0–5; Xi represents the number of leaves with note i; and NtF represents the total number of leaves. The average index was calculated for each batch of plants.

Assessment of disease severity

In the laboratory, the roots of the plants were examined visually, and the disease was assessed according to Greaney et al. (1938), who distinguished six classes of severity depending on the type of symptoms observed: S0, no infection; S1, small necrotic lesions scattered in the roots; S2, necrotic lesions in the basal part of the plant, especially under the crown and roots; S3, large necrotic lesions in the crown and roots with reduction in the vigor of the plant; S4, rotting of the root part, chlorosis of the plant; and S5, death of the plant.

Pathogen re-isolation

After evaluating the disease, 30 roots, crown, and leaf segments were randomly selected from the plants grown on the inoculated substrate. These segments were then washed with water, sterilized with 90% alcohol for 1 min, rinsed thoroughly with sterile water, dried on sterile filter paper, and placed in Petri dishes containing PSA medium. This study aimed to isolate this pathogenic strain and verify Koch's postulates. The results were read after seven days of incubation. The re-isolation percentage (RP) was calculated.

Table 1 Physicochemical characteristics of the Mamora forest soil (Talbi et al. 2016)

Physicochemical characteristics	pH	Organic matter (%)	Nitrogen (%)	Phosphorus P_2O_5 (%)	Potassium K_2O (meq/100 g)	Magnesium Mg (meq/100 g)	Calcium Ca (meq/100 g)
Mamora soil	7.53	0.7	0.05	0.239	0.15	0.2	7251.5

Root colonization and maintenance in the substrate

To assess the colonization of *Trichoderma* in the root system of chickpea plants, the roots of the plants were washed and superficially disinfected with 90% alcohol for 1 min. The roots were then rinsed thoroughly with sterile distilled water, dried, cut into small pieces, and placed in Petri dishes containing water agar. The percentage of root colonization was calculated from the number of root fragments surrounded by *Trichoderma* mycelium. Ten root fragments were deposited in each Petri dish, and three replicates were obtained from each plant. In addition, *Trichoderma* population estimation in the growing medium was conducted on two types of soil samples with three replicates for each sample: soil of the rhizosphere in direct contact with the roots and soil at a distance from the roots, taken 2 cm from the edge of the pots and 5 cm deep. Using the suspension dilution technique, each dilution (0.1 mL) was spread on the PDA medium. Colony counts were determined after 64 h of incubation at 25 °C. The number of propagules per gram of soil was calculated using the following equation:

$$\text{Nombre CFU g}^{-1}\text{de sol} = \text{CN } 0.1\,\text{mL} * \text{DF}$$

Here, CFU stands for colony-forming units; CN stands for colony number; and DF stands for dilution factor.

Statistical analysis

The collected findings were analyzed by analysis of variance (ANOVA) using the SPSS software package version 22.0. Fisher's least significant difference (LSD) test was used to do post hoc comparisons of the mean values of measured parameters between treatments at the $p < 0.05$ level.

Results

Effect of treatment formulations on plant growth

According to these findings, the application of *T. asperellum* on chickpea seeds did not significantly affect the germination percentage of the seeds for both L_{Ta} and $L_{Ta/Fe}$ groups, with values of 80.7 and 77.9%, respectively. Conversely, the germination rate of seeds infected solely with *F. equiseti* was reduced to 25.9%. The seed treated with *T. asperellum* (L_{Ta}) showed 36.8% augmentation in shoot length compared with the control. The $L_{Ta/Fe}$ lot, plant treated with *T. asperellum* and infected with *F. equiseti* ($L_{Ta/Fe}$), had similar shoot length results as the control, whereas the L_{Fe} lot, inoculated only with *F. equiseti*, showed a reduced shoot length. Plants of $L_{Ta/Fe}$ also had a well-developed root length, with a 7.9% increase compared with the control, whereas L_{Fe} plants had an average root length of 22.23 cm. The *F. equiseti* inoculation reduced the root length of the plants (Table 2).

Table 2 Effect of treatment with the Trichoderma harzianum Th2 strain on different agronomic parameters of 2-month-old chickpea plants

	Control	L_{Fe}	L_{Ta}	$L_{Ta/Fe}$
Shoot length (cm)	31.66^{b}	26^{c}	43.33^{a}	35.83^{b}
Root length (cm)	28^{b}	22.23^{c}	30.22^{a}	29.75^{b}
Root fresh weight (g)	2^{b}	1.07^{c}	4.9^{a}	2.8^{b}
Root dry weight (g)	0.977^{b}	0.166^{c}	2.4^{a}	0.23^{bc}
Shoot fresh weight (g)	4.65^{b}	3.8^{c}	8.96^{a}	4.65^{b}
Shoot dry weight (g)	0.61^{b}	0.2^{c}	1.46^{a}	0.71^{b}
Leaves mean	22.11^{b}	16.66^{c}	37.11^{a}	22^{b}
Twigs mean	1.33^{b}	2^{b}	2.22^{a}	2^{a}

Each two values on the same line followed by the same letter are not significantly different at the 5% level, according to Fisher's least significant difference (LSD) test

Treatment with *Trichoderma* resulted in increased plant mass, L_{Ta} and $L_{Ta/Fe}$ showed fresh and dry root masses of 4.9 g/2.4 g and 2.8 g/0.23 g, respectively. The control was 2 g/0.97 g. $L_{Ta/Fe}$ plants showed reduced fresh and dry root masses compared with the other groups, at 1.07 g/0.16 g. Similar improvements were observed in the fresh and dry aerial part masses, with values of 8.96 g/1.46 g in L_{Ta}, 4.65 g/0.71 g in $L_{Ta/Fe}$, 3.8 g/0.2 g in L_{Fe}, and 4.65 g/0.61 g in the control.

The number of leaves produced by chickpea plants was affected by *T. asperellum* seed treatment. On average, there were 37.3 leaves per plant in the L_{Ta} group, 22 leaves per plant in the $L_{Ta/Fe}$ group, 22.1 leaves per plant in the control group, and 16.6 leaves per plant in the L_{Fe} group. These findings demonstrated that treating seeds with *T. asperellum*-based formulations affected the growth parameters of chickpea plants derived from these seeds, and enhanced plant vigor (Fig. 1).

Estimation of the foliar alteration index

Symptoms of *Fusarium* wilt were observed in chickpea plants during the fourth week of cultivation. The disease, as estimated by the symptoms, was significantly reduced in chickpea plants treated with *T. asperellum*. Leaf chlorosis was observed in plants inoculated with *Fusarium*, and the leaves started to form necrotic lesions on the extremities. In plants from lot L_{Fe} inoculated only with *F. equiseti*, the FAI recorded seven weeks after inoculation was approximately 0.44 (Fig. 2). In contrast, this index was very low in chickpea plants grown from seeds treated with *T. asperellum* and inoculated with *F. equiseti* (approximately 0.28). In plants treated only with *T. asperellum*, the FAI did not exceed 0.18.

Fig. 1 Chickpea plants from *T. asperellum*-treated seeds after two months. L_{Fe}: Plants of a soil pretreated with F. equiseti; L_{Ta}: plants from TH2-treated seeds; $L_{Ta/Fe}$: plants from TH2-treated seeds grown in soil pretreated with F. equiseti

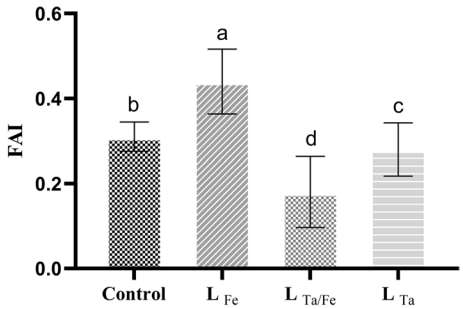

Fig. 2 Evolution of the foliar alteration index, 3 weeks following the appearance of the symptoms. Each two values followed by the same letter are not significantly different at the 5% level, according to Fisher's least significant difference (LSD)

Assessment of disease severity

Treatment of chickpea seeds with *T. asperellum* slowed the development of the disease caused by *F. equiseti* during the culture period in the greenhouse. After two months of cultivation, symptoms of root rot were observed on the majority of the plants whose soil was inoculated with a conidial suspension of *Fusarium equiseti*, and brownish necrotic lesions were visible on the crown and roots (Fig. 3). In lot L_{Fe}, the severity class S4, whose the most accentuated symptom was root rot, was the dominant one (73%), followed by class S3 (17%), and the death rate of plants in this lot was 3%. In the $L_{Ta/Fe}$ lot, the dominant class was S3, with 59% of plants dying.

Pathogen re-isolation

Re-isolation from plants inoculated with *F. equiseti* was positive for the presence of the fungus in different parts of the plant (Fig. 4). The percentages of re-isolation from the roots, crown, and leaf petioles were 72.7, 84.1%, and 36.1%, respectively.

Root colonization and maintenance in the substrate

Table 3 presents the number of colony-forming units per gram of soil CFU.g-1 of *T. asperellum* at the end of the trial in the chickpea plant growing medium, both in the rhizosphere and at the root distance. The conidia of *T. asperellum* were maintained at the rhizosphere level at higher concentrations than those observed at the root distance. The root colonization with *T. asperellum* was 100% in samples extracted from treated plants (Fig. 5).

Discussion

In this study, the treatment of chickpea seeds with the *T. asperellum* formulation did not significantly affect seed germination. Similarly, it was reported that *Trichoderma* sp. treatment of tomato seeds had no impact on either the germination delay or the percentage of germination of seeds, according to Azarmi et al. (2011). In contrast, Ali et al. (2014) reported that the treatment of chickpea seeds by soaking them for 30 min in a conidial suspension of *Trichoderma* sp. increased their germination percentage. The germination rate decreased after transplanting untreated chickpea seeds into substrates contaminated with *F. equiseti*. The effect on germination was lessened when seeds were treated with *T. asperellum* and transplanted onto an infected substrate. Compared with untreated seedlings, chickpea seeds treated with the *T. asperellum* formulation produced plants with better growth characteristics. Similar observations were reported for barley and wheat seeds treated with *Trichoderma* spp.

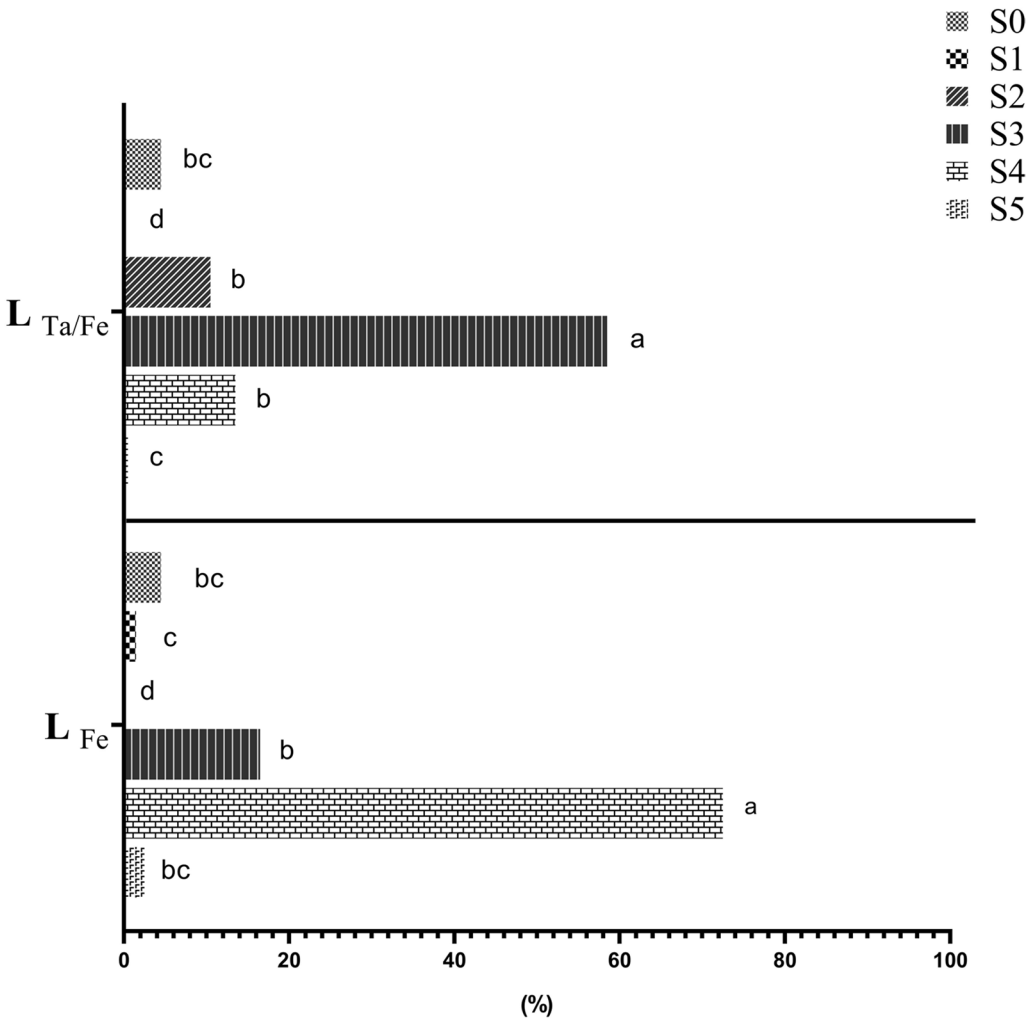

Fig. 3 Root rot severity classes (in %) in chickpea plants after 2 months of culturing. Each two values followed by the same letter are not significantly different at the 5% level, according to Fisher's least significant difference (LSD)

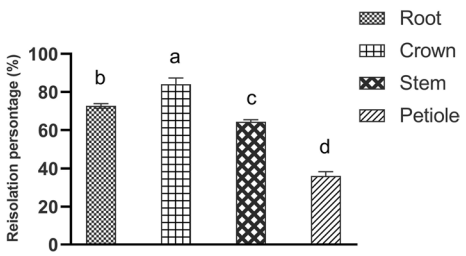

Fig. 4 Re-isolation of *F. equiseti* from different parts of inoculated chickpea plants (expressed as percentages). Each two values followed by the same letter are not significantly different at the 5% level, according to Fisher's least significant difference (LSD)

Table 3 Substrate and rhizosphere colonization, estimated in CFU.g-1 of soil, of chickpea plants from *T. asperellum*-treated seeds after 2 months of culturing

CFU.g^{-1} du sol	CFU (rhizosphere)	CFU (substrate)	Root colonization (%)
L_{Ta}	18,000[a]	38,000[a]	100
$L_{Ta/Fe}$	15,000[a]	40,000[a]	100

CFU: colony-forming units

Each Two values in the same column followed by the same letter are not significantly different at the 5% level, according to Fisher's least significant difference (LSD)

(Qostal et al. 2020a). This improvement was mainly due to better root and aerial growth, higher root and vegetative biomass, and higher numbers of leaves and twigs, with gain percentages varying from 13 to 36.8% (shoot length) and 6.2 to 7.9% (root length). For biomass, the treated plants gained approximately 108% fresh weight

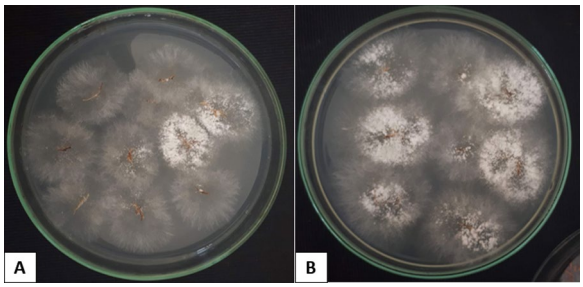

Fig. 5 Colony of *T. asperellum* growing around the roots of chickpea plants from batches L_{Ta} (A) and $L_{Ta/Fe}$ (B), after culturing the roots on culture medium, after 8 weeks of culturing

and 143% dry weight compared with the control. In this context, Mouria et al. (2012) revealed that tomato characteristics were improved after inoculating the roots with *Trichoderma* suspensions. The results of Amna et al. (2014) on the treatment of chickpea seeds with three species of *Trichoderma* revealed that *T. harzianum*, *T. viride*, and *T. koningii* enhanced plant growth.

Zheng and Shetty (2000) reported that *Trichoderma* employs several mechanisms that influence seed germination and plant vigor. Mastouri et al. (2010) tested the hypothesis that T22, a *strain of T. harzianum* reduced damage resulting from the accumulation of ROS in stressed plants. These authors suggested that *Trichoderma* used a mechanism similar to that of the antioxidant glutathione to increase seedling vigor and reduce stress by inducing physiological protection against oxidative damage.

The infected plants with *F. equiseti* developed symptoms of *Fusarium* wilt identical to those described by El Hazzat et al. (2023): leaf necrosis, wilting of plants, browning of plant collars, and root rot. The FAI in inoculated plants was approximately 0.395. Plants from *T. asperellum*-treated seeds grown on a substrate inoculated with *F. equiseti* developed fewer symptoms. The FAI was on the order of 0,28. Mukherjee and Mukhopadhyay (1995) tested the ability of *T. harzianum*, *T. virens*, and *T. viride* isolates to protect seeds against *Pythium* spp. and *R. solani*. When the antagonist was in direct contact with the seeds, its propagules germinated on the surface and colonized the roots of the plants and their rhizospheres.

Compared with the control plants, chickpea plants developed from seeds treated with *T. asperellum* on soil contaminated with *F. equiseti* showed fewer symptoms of root rot, and produced severity class S3, whereas those inoculated with the pathogen alone acquired severity class S4. Dubey et al. (2007) studied the effect of *Trichoderma* on the development of wilt in chickpea plants inoculated with *F. oxysporum* f. sp. *ciceris* and noted a significant reduction in the incidence of the disease as well as a decrease in the symptoms characteristic of the disease, such as falls of leaves and petioles and black internal discoloration of the cortex, including the xylem. Variable *T. harzianum* formulations were able to decrease the percentage of plant mortality against a wilt pathogen complex in chickpea plants, according to Anand et al. (2007).

Present results showed an obvious protective effect in chickpea plants treated with the TH2 strain against the pathogenic N3 strain of *F. equiseti*. The main three mechanisms of the biocontrol of *Trichoderma* spp. are mycoparasitism, antibiosis, and competition for nutrients or space among others which may operate independently or together to suppress plant pathogens (Mukhopadhyay and Kumar 2020).

The role of *Trichoderma* in seed protection is achieved by covering and colonizing the surface of the seeds. A spore suspension formulation effectively prevents infection by plant pathogens. *Trichoderma* controls seed diseases through various mechanisms: inhibiting pathogen growth, inducing seed production of antibiotics, and competing for nutrients. *Trichoderma* also competes for iron through siderophore production, which reduces growth and pathogenicity (Al-Ani and Mohammed 2020).

The production of particular substances and metabolites, such as growth factors, hydrolytic enzymes, siderophores, antibiotics, and permeases, is required for the activation of each mechanism (Benítez et al. 2004). Present results showed that *T. asperellum* conidia could maintain themselves in the soil, especially in the rhizosphere. In addition, *Trichoderma* can colonize the roots of plants from treated seeds. Kribel et al. (2020) reported that the examined isolates of *Trichoderma* can maintain and multiply over time in the diverse substrates under study as well as colonize the roots of wheat (soft and hard) and barley plants.

Conclusion

The formulation used to treat chickpea seeds ensured good installation of *Trichoderma* conidia, which allowed this antagonist to stimulate different agronomic parameters of chickpea plants. This indicated that the maintenance of the viability and persistence of the antagonist conidia around the seeds was achieved. Obtained results suggested that seed treatment with *T. asperellum,* as a formulation based on adhesive substances combined with talcum powder, was an effective way to control *Fusarium* wilt and root rot.

Abbreviations

FAI Foliar alteration index

FAO	Food and Agriculture Organization of the United Nations
MAPMDREF	Ministry of Agriculture, Marine Fisheries, Rural Development, and Water and Forests
PDA	Potato dextrose agar

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Author contributions
AM performed the experiments, wrote the manuscript, and assisted with the software. EN revised the data. MS and MN validated the manuscript. EMA interpreted the data. SK, BR, and OTA supervised the study. DA revised the manuscript, supervised the study, and edited the manuscript.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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