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RESEARCH

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In vitro evaluation of antagonistic potentiality of *Trichoderma harzianum* against *Diplodia* spp. phytopathogenics fungi

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Abstract

Background *Trichoderma* species are able to cause significant changes in the metabolism of host plants, by that means it trigger the plant growth and increasing plant defense to diverse fungal diseases. *Trichoderma harzianum* has been identified as potential biocontrol agent of many phytopathogenic fungi. Therefore, the aim of this study was to assess in vitro antagonistic activity against three *Diplodia* isolates utilizing direct and remote confrontations methods.

Results The results revealed that *T. harzianum* inhibited mycelial growth of the three phytopathogenic fungi compared to the untreated control. The percentage of growth inhibition of *T. harzianum* against *Diplodia* isolates ranged between 58 and 79% for direct confrontation and between 31 and 46% remote confrontation. Considerable antagonistic abilities of *T. harzianum* were exhibited against all tested *Diplodia* isolates. The results of both confrontations showed that the radial growth of the fungal pathogens was statistically significant ($P < 0.001$) and influenced by the antagonist on 6 days following incubation.

Conclusion The findings may reveal a valuable knowledge that may be further used to find a suitable biological control contrariwise Botryosphaeria dieback caused by *Diplodia* species.

Keywords Botryosphaeria dieback, *Diplodia* species, *Trichoderma harzianum*, Dual culture, Direct and remote confrontations

Background

Botryosphaeriaceae family is distributed worldwide on a wide range of different plant hosts (Slippers et al. 2014). In fact, Botryosphaeria species are thought to be present in an endophytic phase before transmuting to a pathogenic phase under abiotic and/or biotic stress factors (Hrycan et al. 2020). In the Mediterranean area, these fungal species have been associated with diseases symptoms including leaf spots, necrosis of the wood, fruit rot, root rot, shoot dieback, and gummosis and branch cankers on agricultural crops, urban and natural forest trees (Phillips et al. 2013). Diverse researches have described Botryosphaeriaceae species as main pathogens associated with dieback on grapevine in California (Urbez-Torres 2011), on Eucalyptus in Portugal (Barradas et al. 2017) and on loquat in Spain (González-Domínguez et al. 2017). Indeed, including Botryosphaeriaceae family, *Diplodia* species are the most aggressive pathogens causing dieback, withering and cankers on ecologically and economically plant (Alves et al. 2014). In Europe, *Diplodia pinea* causes dieback and crown wilt on pines (Luchi et al. 2014). In Italy, *Diplodia olivarum* has been revealed to be

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associated with carob tree canker diseases (Granata et al. 2011). In Tunisia, *Botryosphaeria obtusa* has been described as a causal agent of olive tree branch dieback (Chattaoui et al. 2012). Furthermore, *Diplodia seriata* has been recognized causing branch canker on *Pinus pinea* trees (Hlaiem et al. 2021) and on *Q. coccifera* (Hlaiem et al. 2020). Recently, *Diplodia scrobiculata* has been reported as the causal agent of stem canker of *Tetraclinis articulata* (Hlaiem et al. 2022). Furthermore, *Diplodia gallae* has been characterized as the causal agent of *Quercus suber* dieback (Yangui et al. 2022).

Considering that forest ecosystems are vulnerable to fungal pathogens, especially members of Botryosphaericeae family which can give rise to rapid decline in many regions of the world (Slippers and Wingfield 2007), it is imperative to respond immediately to keep forest robust and healthy under climate change employing appropriate silviculture. Furthermore, few approaches have been utilized for assessing antagonism ability, namely agglutination lectin test (Yang et al. 2009), degradation of mycelium of phytopathogenic fungi after treatment of *Trichoderma* secretions (Xiong et al. 2014), biological control functional genes (Tijerino et al. 2011) and hydrolytic enzymes activities (Qualhato et al. 2013). Nevertheless, no restorative strategies are currently available to effectively control forest disease in Tunisia. *Trichoderma* species have been well known to promote growth and induce resistance against various disease caused by fungal pathogens (Britto and Jogaiah 2022) also as a potential biofungicide since 1932 including members of this genus which have been recognized as among the most potential biocontrol agents of many phytopathogenic fungi (Herrera-Parra et al. 2017). Moreover, Jogaiah et al. (2017) confirmed that *Trichoderma* spp. can facilitate increased availability and efficient uptake of soil nutrients, thereby improving yield of a ratoon crop. Hence, this work was performed in order to evaluate in vitro antagonistic potentiality of *Trichoderma harzianum* against *Diplodia* species by means of direct confrontation (dual culture) and remote confrontation techniques.

Methods

Fungal isolates

The present study evaluated the antagonistic activity of *T. harzianum* TN.112 (GenBank MK123932), isolated from healthy *P. pinea* branches. Three phytopathogenic fungi were used, namely *Diplodia scrobiculata* TN.44 (Hlaiem et al. 2019), *D. pseudoseriata* TN.80 (Hlaiem et al. 2019) and *D. africana* TN.102 (Hlaiem et al. 2020) isolated from branch canker disease of declining *Pinus halepensis*, *Retama raetam* and *Pistacia lentiscus* trees, respectively (Table 1), and were collected from investigated Bizerte forest (37°17′48″N; 10°0′2″E; alt. 41 m) in the Northern Tunisia. All fungal isolates were propagated on potato dextrose agar (PDA) and subcultured into fresh medium as needed.

Direct and remote confrontation

The susceptibility of the three *Diplodia* isolates to *T. harzianum* was estimated in vitro applying direct confrontation (dual cultures) and remote confrontation (distant inhibition method). Dual culture method consisted in placing, a mycelial plug of 5 mm in diameter of *T. harzianum* isolate TN.112 on PDA, about 1 cm from the edge of each Petri dish (9 cm in diameter). Concurrently, a mycelial plug (5 mm diameter) of each *Diplodia* isolate (TN.44, TN.80 and TN.102) was taken from the margin of a 5-day-old colony growing on PDA and placed 6 cm away from the plug of the *T. harzianum* isolate on the opposite side of the same Petri dish (Hibar et al. 2005). Petri dishes inoculated with each *Diplodia* isolate alone placed at the center were used as controls. Each experiment was repeated three times. Incubation of all dishes was performed at 25 °C for 6 days.

The remote confrontation method consists of planting *T. harzianum* (TN.112) and each *Diplodia* isolate (TN.44, TN.80 and TN.102) alone at the center in two separated Petri dishes. Afterward, an assembly was performed by super-positioning the two dishes (*Trichoderma* downside and *Diplodia* isolate upside). The junction between the two dishes was insured by a Parafilm in order to avoid any loss of volatile substances (Daami-Remadi and El Mahjoub 2001). Incubation conditions were similar to those of dual

Table 1 Identity of the three fungal pathogens used in this study and GenBank accession numbers

Host	Isolate	GenBank accession numbers		
		ITS	*EF1-α*	TUB
Pinus halepensis	*Diplodia scrobiculata* TN.44	MK170175	OM428167	MT164530
Retama raetam	*D. pseudoseriata* TN.80	MN123532	MN125371	MN125372
Pistacia lentiscus	*D. africana* TN.102	MK230889	MK746133	MK746134

cultures. The control was carried out by stacking dishes, the upper one contained a mycelial plug (5 mm diameter) of each *Diplodia* isolate and the bottom one contained only PDA. The average diameter of treated colonies was noted when *Diplodia* isolates mycelium in control dishes reached the periphery.

Measurement of radial mycelial growth

Measurement of mycelia radial growth of *Diplodia* colonies in direct and remote confrontations and in control dishes was realized daily. Ratings on the inhibition of the growth and invasion of *Diplodia* colonies by *Trichoderma* mycelium were examined. The percentage of inhibition of radial mycelial growth (IR) was calculated, using the following formula: IR (%) $= (1 - R_T/R_C) \times 100$ according to Hmouni et al. (1996), where R_T is the radial growth measurement of *Diplodia* colonies in the presence of *Trichoderma* and R_C is the radial growth of *Diplodia* colonies in the control dishes. Inhibitory activity of the antagonist was appraised using a scale reported by Sangoyomi (2004), with (S1) 0% inhibition (not effective); (S2) > 0 to 20% inhibition (slightly effective); (S3) > 20 to 50% inhibition (moderately effective); (S4) > 50 to < 100% inhibition (effective); and S5: = 100% inhibition (highly effective).

Data analysis

To evaluate the antagonistic potentiality of *T. harzianum* against *Diplodia* isolates (TN.44, TN.80 and TN.102), one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, using SPSS version 20, was conducted for growth inhibition (%).

Results

Direct confrontation

Trichoderma harzianum reveled antagonistic potentiality against all *Diplodia* isolates tested. The simultaneous subculturing of *T. harzianum* and *Diplodia* isolates showed faster growth of the antagonist than the other pathogens tested. After 6 days of incubation, the radial growth of *Diplodia* isolates (*D. scrobiculata* TN.44, *D. pseudoseriata* TN.80 and *D. africana* TN.102) was obviously inhibited and the Petri dishes were invaded by *T. harzianum* TN.112 isolate (Figs. 1, 2). The radial growth of *Diplodia* isolates was statistically significant ($P < 0.001$), influenced by TN.112 isolate on 6 days, following incubation. Growth inhibition percentage of TN.112 against TN.44, TN.80 and TN.102 were 79, 58 and 69%, respectively (Fig. 2). The level of effectiveness of *T. harzianum* toward all *Diplodia* isolates was effective (S4). The phytopathogenic fungus TN.44 was the most responsive isolate to *T. harzianum*, which have a strong

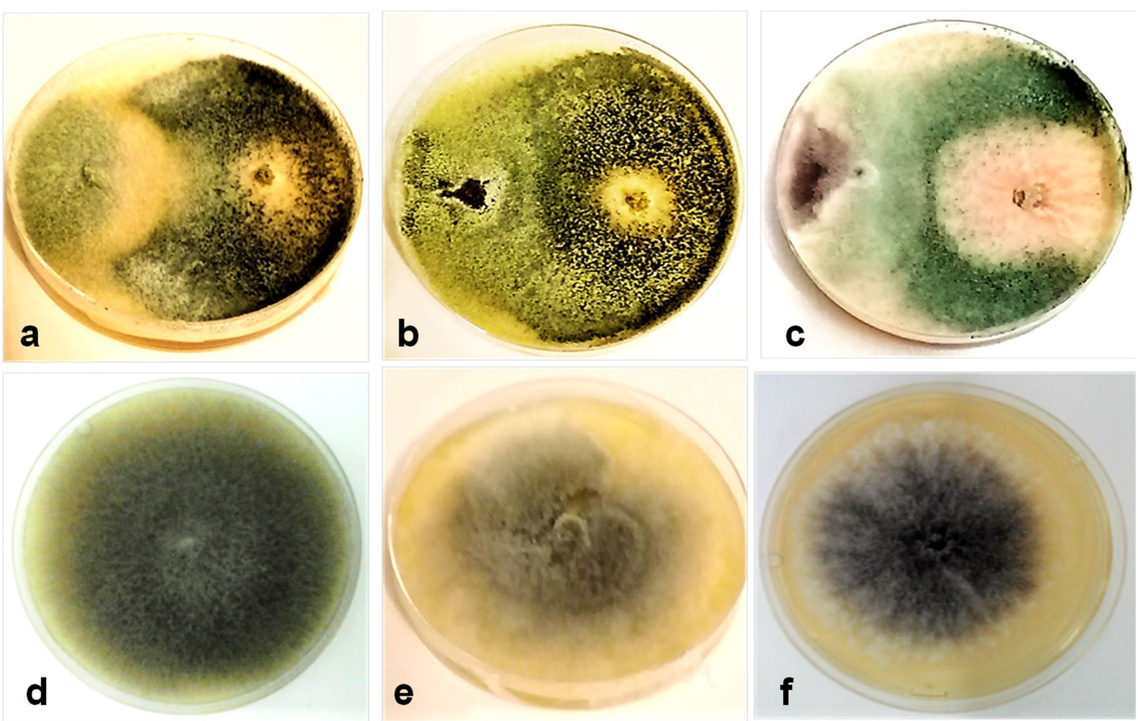

Fig. 1 Direct confrontation (dual culture method) of *Trichoderma harzianum* against *Diplodia* isolates: *Trichoderma harzianum* TN.112 + *D. pseudoseriata* TN.80 (**a**), TN.112 + *D. scrobiculata* TN.44 (**b**), TN.112 + *D. africana* TN.102 (**c**), phytopathogenic fungi (left), antagonist (right); control (**d**–**f**)

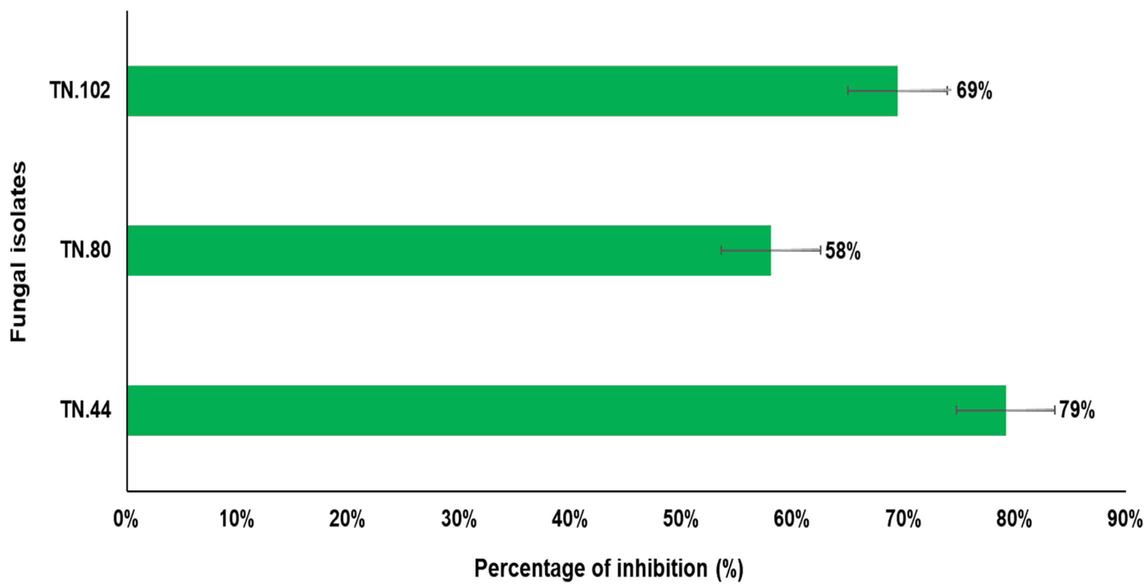

Fig. 2 Antifungal activity (% inhibition percentage) on *Diplodia* isolates (TN.44: *Diplodia scrobiculata*, TN.80: *D. pseudoseriata*, TN.102: *D. africana*) using the dual culture method

antagonism growing faster, covering it completely and sporulated abundantly on colonies of TN.44 in 6 days (Fig. 1b, e).

Remote confrontation

A reduction in the radial growth of *Diplodia* isolates (*D. scrobiculata* TN.44, *D. pseudoseriata* TN.80 and *D. africana* TN.102) in the presence of *T. harzianum* TN.112 was noticed compared to the controls (Figs. 3, 4). The antagonist (TN.112) seemed to have an inhibitory activity on the growth of phytopathogenic fungi in the absence of direct contact. The percentages of growth inhibition recorded were 31, 46 and 40% for TN.44, TN.80 and TN.102, respectively (Fig. 4). TN.80 isolate was the most sensitive isolate toward *T. harzianum* (Fig. 3a, d). The results obtained showed a change in the color of mycelium in TN.44 colonies than the untreated control (Fig. 3b, e). The analysis of the variance exhibited a significant difference ($P < 0.001$) in the radial growth among *Diplodia* isolates after 6 days of incubation. The level of effectiveness of *T. harzianum* against the three *Diplodia* isolates was moderately effective (S3).

Discussion

This the first attempt to evaluate in vitro the antagonistic effect of *T. harzianum* naturally occurring on branches of *P. pinea* trees toward *D. scrobiculata*, *D. pseudoseriata* and *D. africana*, fungal species involved in Botryosphaeria dieback of *Pinus halepensis*, *Retama raetam* and *Pistacia lentiscus* trees, respectively in Tunisian forest. In this study, the simultaneous incubating of *T. harzianum* with each *Diplodia* isolate exhibited that the antagonist inhibited the mycelial growth of the three *Diplodia* species. Moreover, it invaded the colonies of the pathogens and sporulated on them, revealing its hyper-parasitic activity (Dubey et al. 2007). The antagonist *T. harzianum* seemed to be able to achieve more than 50 to ~ 80% of growth inhibition of the fungal pathogens. Furthermore, estimation of antagonistic activity of *Trichoderma* species has been frequently assessed by percentage of growth inhibition in vitro, which is on the basis of ratio between decreased radius of plant pathogenic fungi growing in direct confrontation and radius of the pathogen colonies alone (Zhang and Zhuang 2017). Accordingly, previous studies have shown isolates of *T. asperelloides*, *T. atroviride*, *T. harzianum* and *T. koningii* to be highly effective in inhibiting the growth of Botryosphaeria fungi, including *D. seriata* in vitro (Urbez-Torres et al. 2020). In accordance, Daami-Remadi and El Mahjoub (2001) reported that *T. harzianum* inhibited the radial growth of *Fusarium oxysporum* f. sp. *Radicis-lycopersici* associated with *Solanum tuberosum* rot and also sporulated on them, thus revealing its highly mycoparasitic effect. Recently, Yangui et al. (2020) reported that *T. harzianum* was highly effective against *Biscogniauxia mediterranea*, the causal agent of cork oak charcoal canker disease, which is in agreement with our findings using *Diplodia* isolates. Additionally, Pollard-Flamand et al. (2022) approved the antagonistic activity of *Trichoderma* species isolated from grapevine trees in British Columbia toward Botryosphaeria dieback

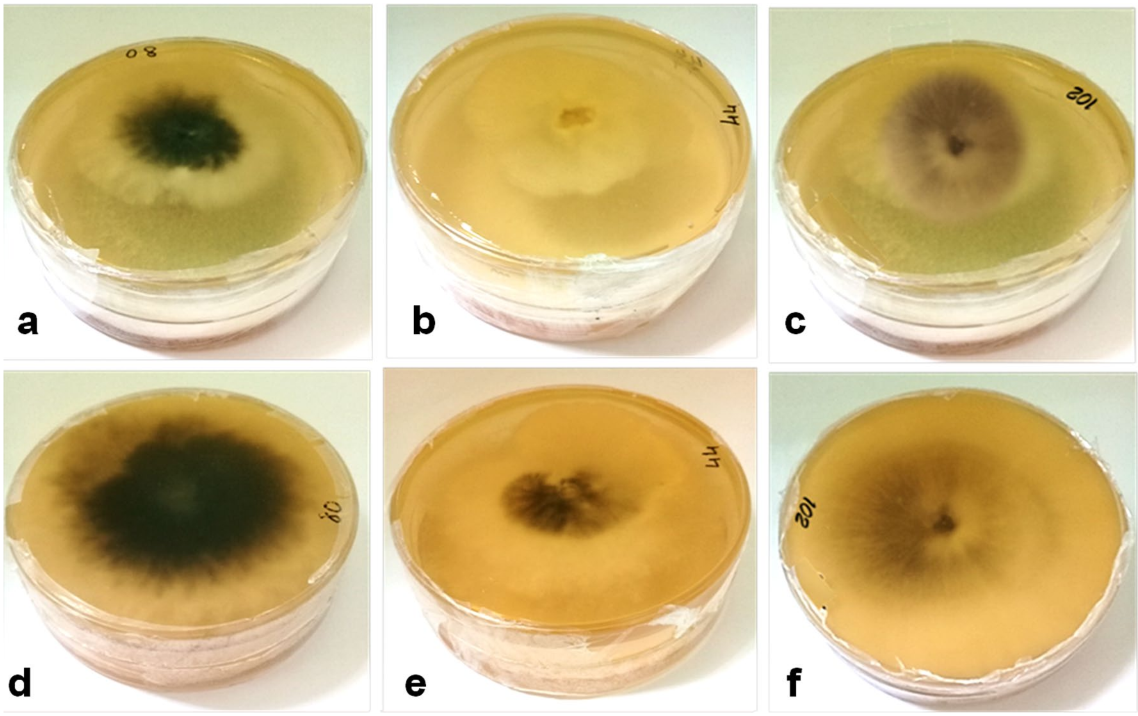

Fig. 3 Remote confrontation of *Trichoderma harzianum* TN.112 against *Diplodia* isolates: *D. pseudoseriata* TN.80 (**a**), *D. scrobiculata* TN.44 (**b**), *D. africana* TN.102 (**c**); the upper one: pathogen, the bottom one: antagonist; control (**d**–**f**)

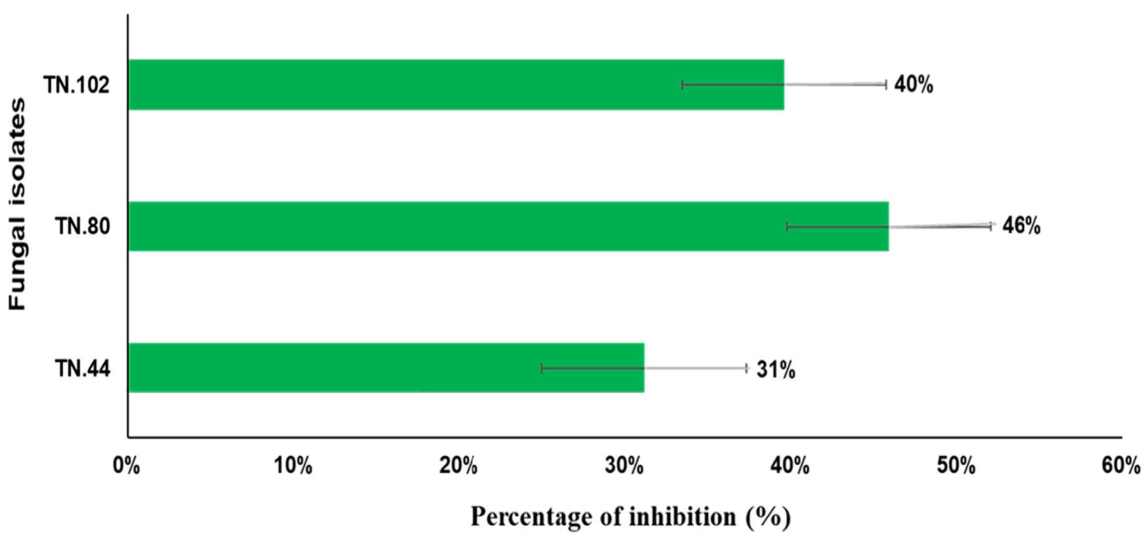

Fig. 4 Antifungal activity (% inhibition percentage) of *Diplodia* isolates (TN.44: *Diplodia scrobiculata*, TN.80: *D. pseudoseriata*, TN.102: *D. africana*) using distant inhibition method

fungal pathogens. Hoitink et al. (2006) revealed that this inhibitory action was due to chemical substances released by *Trichoderma* species, leading to competition, antibiosis and parasitism where the production of specific enzymes (chitinases or proteases) was for cell wall degradation.

On the other hand, present results of the remote confrontation showed clearly the ability of *T. harzianum* to exert an inhibitory activity on the mycelial growth (31 to 46%) of phytopathogenic fungi in the absence of direct contact. This technique enabled us to highlight the inhibiting effect even remotely of the *T. harzianum* on *Diplodia* isolates. This antagonist seemed to secrete volatile substances able to reduce, even remotely, the radial growth of the three phytopathogenic fungi. In accordance, Wheatley (2002) found that these volatile substances could easily diffuse and inhibit the mycelial growth of the fungal pathogens. Moreover, studies of M'zahem and Mihoubi (2017) reported that *T. harzianum* had an antagonistic effect against *Fusarium* sp., *Botrytis* sp., *Alternaria* sp. and *Penicillium* sp. Likewise, a change in the color of the mycelium was observed in the colonies of *D. scrobiculata* tested than in the untreated control. This corroborates the study of Boukarchaoui (2017) reported that the growth inhibition of *Botryosphaeria dothidea* by *Trichoderma* sp. was accompanied by a change in the color of the mycelium of this pathogen. However, the effectiveness of *T. harzianum* in direct confrontation appeared to be greater than the remote confrontation. Furthermore, *Trichoderma* isolates have strong antagonistic and mycoparasitic effects against phytopathogens and therefore are able to reduce disease severity in plants (Viterbo and Horwitz 2010). *Trichoderma* has been recognized as an aggressive mycoparasite that cabled of competing with fungal pathogens at the site of infection (Djonovic et al. 2007).

Conclusion

The fact that forest ecosystems are vulnerable to fungal pathogens, which can cause rapid decline, is imperative to respond quickly to improve forest sustainability under predicted global warming scenarios by developing control measures by providing an efficient biological control method. Subsequently, aiming to estimate the effectiveness of *T. harzianum* under natural environmental conditions, it is suggested to fully carry out biocontrol trials in the nursery and in the field. Fundamentally, it is appropriate to investigated in vivo the role of *Trichoderma* spores in regulating growth and activation of the defense responses of plants against fungi.

Abbreviations

%	Percentage
SPSS	Statistical Package for the Social Sciences
IR	Inhibition of radial mycelial growth
TN	Tunisia
p value	The probability of obtaining an F statistic at least as extreme as that observed when the null hypothesis is true
F-statistic	The ratio of two mean squares that forms the basis of a hypothesis test
Alt	Altitude
N	Latitude
E	Longitude

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Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors. It is original and has not been submitted or published elsewhere.

Consent for publication
It was obtained from all individual participants included in the study. All the authors have seen and approved the manuscript, and all have taken a valid role through either study design, data generation or manuscript preparation.

Competing interests
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