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Antifungal effect of three *Trichoderma* species against the citrus black spot caused by *Phyllosticta citricarpa* in Tunisia

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Abstract

Background Citrus black spot (CBS), even recently reported in Tunisia, could be responsible for considerable losses in lemon growing areas. The present study evaluated different *Trichoderma* species against *Phyllosticta citricarpa* causative agent of CBS using two assays.

Results The obtained in vitro results exhibited the efficacy of these antagonists to reduce *P. citricarpa* mycelial growth and the inhibition rate values varied. *Trichoderma* species showed the highest mycelial growth inhibition with 54.33%, noted for *P. citricarpa* isolate CBS1 treated with *Trichoderma atroviride* (A3), and 51.76 and 53.51% for *P. citricarpa* isolate CBS4 confronted with *T. atroviride* and *Trichoderma asperellum*, respectively. The in vivo assay on Citrus lemon fruits confirmed the decrease of the lesions development caused by four *P. citricarpa* isolates. In fact, the decrease varied from 28.28 to 72.83% for the *P. citricarpa* CBS3 isolate treated with *Trichoderma kunmingense* and *Trichoderma atroviride*, respectively.

Conclusions Based on the current results, the used *Trichoderma* species reduced *P. citricarpa* mycelial growth in vitro and the lesions appearance due to *P. citricarpa* on *Citrus limon* variety Eureka. The efficacy varied upon on the antagonist species and pathogen isolates. Thus, they showed a promising potential to be used to control CBS.

Keywords Biological control, Citrus black spot, *Phyllosticta citricarpa*, *Trichoderma* species

Background

The citrus sector is the first fruit producer worldwide and is dedicated to the juices extraction or direct consumption as fresh fruit (Palou et al. 2015). In Tunisia, citrus is economically important. However, its production is threatened by many diseases, which cause significant losses and social impacts (Khanchouch et al. 2017).

Citrus black spot (CBS), which is caused by the fungus *Phyllosticta citricarpa*, is responsible for considerable losses in various citrus producing region in Australia, Asia, Africa, and America (Boughalleb-M'Hamdi et al. 2020). The symptoms of this disease include false melanose, hard spot, virulent spot and freckle spot on fruits, as well as necrotic lesions on leaves and twigs (Guarnaccia et al. 2019). The symptoms are limited to the flavedo, not changing the quality of the fruits, making it possible to use it in the juice production (Aguilar-Vildoso et al. 2002). However, this disease makes citrus fruits look bad and harder to sell. It also creates problems with exporting to European countries where the disease doesn't exist (Graham et al. 2014).

Current practices for controlling CBS disease are represented in the cultivation management in fields like the litter collection and the pruning and the protective

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fungicides application or their mixture, associated with mineral or vegetable oils (Lanza et al. 2018). Nevertheless, intensive use of chemical pesticides has many problems such as the toxins accumulation in the environment and their effects on human health and the appearance of resistant pathogen isolates (da Cruz Cabral et al. 2013).

Because of these positive effects, certain strains, like *Trichoderma harzianum*, *T. atroviride* and *T. asperellum*, are used to protect plants from diseases and to stimulate plants growth (Verma et al. 2007). The *Trichoderma* species have different biological control mechanisms in plant fungal disease such as the competitive role by fast mycelial growth and strong adaptability to the environment, antibiosis by secreting antagonistic substances and an important number of secondary metabolites like pyrones, sesquiterpenes, peptaibols and mycoparasitism by invading or wound the mycelium, causing the expansion and deformation of pathogen cells. Besides, *Trichoderma* can induce host plants to produce defense responses (Yao et al. 2023). Despite the recognized potential of this genus to control various plant pathogens (Martinez-Medina et al. 2014), there is a deficit of researches about the mechanisms of *Trichoderma* used to control *P. citricarpa*. Pandolfo (2011) revealed the antagonistic capabilities of *T. koningii* against *G. citricarpa* in vitro and suggested this species as an efficient biocontrol agent for citrus black spot (CBS). Lima et al. (2017) showed the ability of *T. harzianum* to produce proteins associated with the management of *G. citricarpa*, as well as the enhancement of plant resistance. Although CBS diseases are starting to be significant in Tunisia (Boughalleb-M'Hamdi et al. 2020), little attention has been made to control this disease. Whereas, without control strategy, losses of marketable fruit would be severe in many citrus orchards. In addition, a study of chemical control has been accomplished (unpublished data). The objective of the present study was to assess (1) the in vitro antifungal activity of three *Trichoderma* species against five *P. citricarpa* isolates associated with

CBS in Tunisia and (2) these antagonists's abilities to reduce CBS disease severity on *Citrus limon* fruits.

Methods

Isolation and characterization of CBS isolates

Four isolates of *Phyllosticta citricarpa*, collected from citrus orchards, showing CBS symptoms, in Tunisia, were confirmed as the causative agent of this disease (Benfradj et al. 2024). One isolate from Argentina was also included in this study (Table 1). The morphological and molecular methods were used to identify these isolates. In fact, colonies with the characteristic of the genus *Phyllosticta* were transferred to PDA, malt-extract-Agar (MEA) and oatmeal-agar, and incubated in the dark at 25 °C for one month. Then, colony characteristics and pigmentation were determined. Conidia from pycnidia were mounted in methylene blue and observed through a Leica microscope (40GrX) at 400×. The length and the width of 30 conidia and the diameter of 30 pycnidia were determined per each isolate (Benfradj et al. 2024).

To confirm the characterization, the morphologically identified species were used for the sequence based identification. Genomic DNA was isolated utilizing the Plant Genomic DNA Mini Kit (Viogene), following the instructions provided by the manufacturer. Two loci were amplified through polymerase chain reaction (PCR) and subsequently sequenced, specifically the internal transcribed spacer (ITS) using primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5' TCC TCCGCTTATTGATATGC), as well as the elongation factor TEF1 with primers EF1 (ATGGGTAAGGARGAC AAGAC) and EF2 (5' GGARGTACCAGTSATCATG TT) (Benfradj et al. 2024). Amplification reactions were done in a total reaction volume of 50 µL, consisting of 5 µL PCR buffer (10×), 2 mM MgCl₂, 40 µM dNTPs, 0.08 µM of each forward and reverse primer, 0.5 U of Taq DNA polymerase, and 1–10 ng of DNA. The resulting sequences were deposited in GenBank (Benfradj et al. 2024).

Table 1 Characteristics of *Phyllosticta citricarpa* isolates collected from Citrus lemon orchards in 2021, used in this investigation

Isolates	Organs	Symptoms	Origins	GenBank Accession Number
CBS1	Mature fruit	Hard spot	Bouargoub	OM679448
CBS2	Mature fruit	Hard spot	Bouargoub	OM679449
CBS3	Leaves	Circular spots on both sides	Bouargoub	OM679450
CBS4	Mature fruit	False melanose	Menzel Bouzalfa	OM679451
CBS5			Argentina	

Isolation and characterization of antagonistic fungi

The used antagonistic fungi were isolated from asymptomatic *C. limon* fruits and leaves from different Tunisian orchards (Table 2). These fungal species were identified after a 7-day incubation period on PDA medium at a temperature of 28 °C, following the morphological criteria outlined by Siddiquee (2017) and Shah et al. (2019). Then, genomic DNA was extracted from each isolate using the Plant Genomic DNA Mini Kit (Viogene), adhering to the manufacturer's instructions. The ITS region was amplified using universal primers ITS1 and ITS4, as described by Ristaino et al. (1998). The polymerase chain reaction (PCR) was conducted in a total volume of 50 µl, which included 2 µl of DNA (50 ng/µl), 0.5 µl of Taq polymerase (5 U/µl), 3 µl of MgCl₂ (1.25 mM), 5 µl of PCR buffer (10×), 5 µl of dNTP (1.25 mM), 5 µl of each of 5 µM forward and reverse primers and 24.5 µl of sterile distilled water. The resulting PCR products were purified and subsequently sequenced, with the sequences being submitted to GenBank.

Effect of *Trichoderma* species on *P. citricarpa* mycelial growth

To study the in vitro effect, agar plugs (6 mm in diameter) of each *P. citricarpa* isolate culture were positioned in the opposite side to the *Trichoderma* isolate agar disks. The control plates were only with pathogen culture plug without antagonistic culture (Mannai and Boughalleb-M'hamdi 2023). Since the slowness development of *P. citricarpa* and the fast growth of the *Trichoderma* species, these antagonists were cultivated on PDA three days after *P. citricarpa* isolates. Each treatment was subjected to three replicates. The plates were maintained at a temperature of 25 °C for a duration of seven days, and the experiment was repeated twice. The radius of the pathogen colony was recorded. The inhibition percentage of *P. citricarpa* mycelial growth was determined using the following formula:

$$\% \text{ inhibition} = (1 - P_T/P_C) \times 100$$

where P_T represents the mean radius of colonies observed in the presence of *Trichoderma*, while P_C denotes the mean radius of the control colonies.

Effect of *Trichoderma* species on CBS disease severity on Citrus lemon fruits

Trichoderma species which revealed to be effective in vitro were selected to assess their in vivo antifungal potential. An artificial inoculation of Citrus lemon variety Eureka healthy fruits, close of maturity, was carried out aseptically. Each fruit was surface-disinfected with 70% ethanol and then pricked superficially with sterile toothpicks at three equidistant points, to facilitate the pathogen penetration. Subsequently, pellets (6 mm in diameter) of the cultures of each *P. citricarpa* isolate, incubated on PDA medium for 30 days at a temperature of 25 °C, were placed on the pricked area and covered with filter paper.

Four agar disks from seven-day-old of each *Trichoderma* species culture were incubated, for 6 days at 120 rpm stirring, in an Erlenmeyer with 100 ml of Potato Dextrose Broth (PDB). The suspensions were adjusted to 10⁶ spores/ml prior to use. Fifteen days post-inoculation, these fruits were treated by adding 40 µl of each antagonist suspension to each inoculated point. For each treatment, three fruits were used. Thus, nine replicates were employed per each individual treatment. A positive control was inoculated and treated only with distilled sterile water. Finally, these fruits were incubated for 37 days at 25 °C, maintaining a photoperiod of 12 h of light, followed by 12 h of darkness. The effectiveness of the antagonists was determined by measuring the diameter of the necrotic zone developed around the inoculated point, and then, a re-isolation was carried out in order to verify the presence of *P. citricarpa*, thus confirming the Koch postulates.

Statistical analysis

A completely randomized factorial design was followed in the current study. Data analysis was performed using SPSS, version 23, and mean separations were executed

Table 2 Characteristics of antagonists used to control *Phyllosticta citricarpa* associated with citrus black spot in Tunisian orchards

Antagonists	Codes	Sample site	Organs	Host species	Years	GenBank Accession Number
<i>Trichoderma atroviride</i>	A1	Bouargoub	Fruits	<i>C. lemon</i>	2021	OP962468
<i>Trichoderma kunmingense</i>	A2	Bouargoub	Fruits	<i>C. lemon</i>	2021	OP962469
<i>Trichoderma atroviride</i>	A3	Bnikhaled	Fruits	<i>C. lemon</i>	2021	OP962470
<i>Trichoderma asperellum</i>	A4	Bouargoub	Fruits	<i>C. lemon</i>	2021	

through the Student–Newman–Keuls test at $P \leq 0.01$ for the in vitro test and $P \leq 0.05$ for the in vivo test.

Results

Isolation and characterization of CBS isolates

The morphological and molecular identification confirmed that the pathogen isolates used in this investigation are *Phyllosticta citricarpa* species. The characteristics of these isolates are described in Table 1.

Isolation and characterization of antagonistic fungi

The characterization of antagonistic fungi showed the presence of three species of *Trichoderma*. The characteristics of antagonists used in this study to control *Phyllosticta citricarpa* associated with citrus black spot in Tunisian orchards are mentioned in Table 2.

In vitro impact of antagonistic fungi on the growth of *Phyllosticta citricarpa*

Statistical analyses, according to the direct contact confrontation method, indicated a highly significant relationship between the two factors (*Trichoderma* and pathogen isolates) ($P \leq 0.01$). Indeed, the antagonists reacted differently toward each isolate after 7 days incubation period at 25 °C, as illustrated in (Figs. 1, 2, 3, 4, 5 and 6). *Trichoderma* species reduced the *P. citricarpa* mycelial growth around 27–55%, and *Trichoderma* such as *T. atroviride* (confronted with CBS1), *T.*

asperellum (confronted with CBS4) and *T. kunmingense* (confronted with CBS4) demonstrated a reduction in pathogen development by 51 and 55%, respectively, when compared to the control group. After 6 days of incubation, the *Trichoderma* isolates fully covered the surface of the culture in the Petri dishes (Figs. 1, 2, 3, 4, 5 and 6).

Trichoderma species effect on the disease severity on *Citrus lemon* fruits

Variance analysis of the citrus black spot disease severity index recorded 37 days after inoculation of lemon fruits variety Eureka showed a significant effect of each factor and a significant interaction between the two tested factors (isolates and antagonists) ($P \leq 0.05$).

The present results demonstrated that the antagonists, namely *T. atroviride*, *T. kunmingense*, and *T. aspergillum*, were effective in diminishing the progression of lesions induced by the four isolates with values oscillating between 28.28 and 72.83% in the case of the pathogen isolate CBS3 treated with *T. kunmingense* and *T. atroviride* (A1), respectively (Figs. 7 and 8).

For the isolate CBS1, the application of *T. atroviride* (A1) recorded the highest percentage of lesion reduction (62.28%). The other antagonists showed an activity of 47.74% for *T. kunmingense* and 43% for the two isolates *T. atroviride* (A3) and *T. aspergillum* (A4). However, *T. aspergillum* decreased the progression of lesions

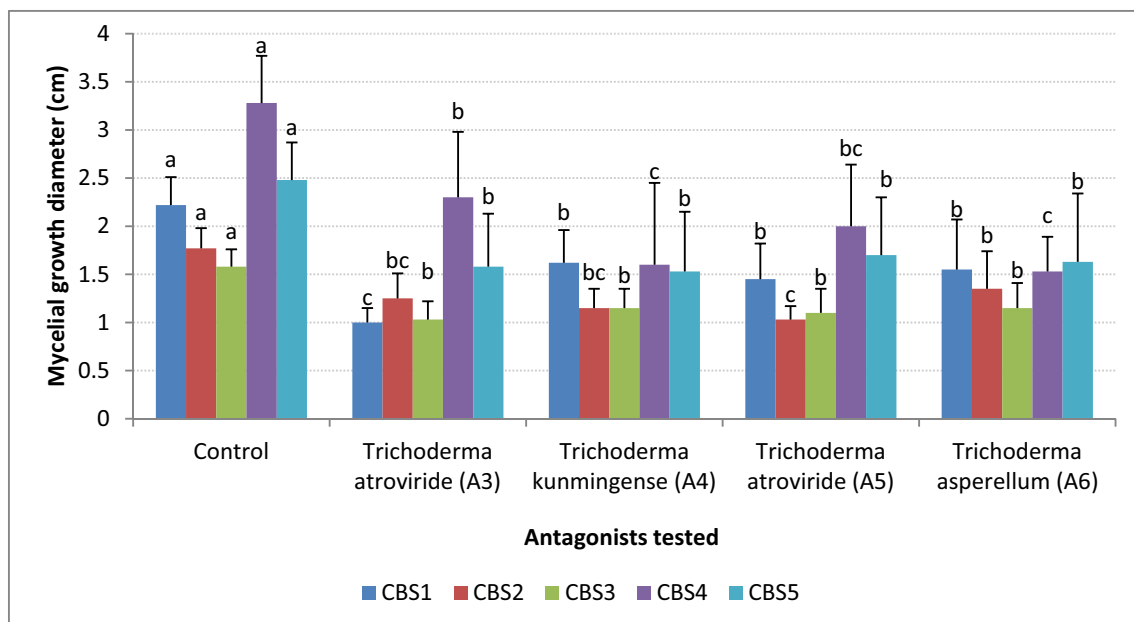


Fig. 1 Inhibition percentage of *Phyllosticta citricarpa* mycelial growth by antagonists tested (%) recorded after seven days of *P. citricarpa* incubation at 25 °C. For each isolate of *P. citricarpa*, bars that share the same letter do not exhibit significant differences as determined by the SNK test ($P \leq 0.05$). CBS1, CBS2, CBS3 and CBS5: isolates of *P. citricarpa* used

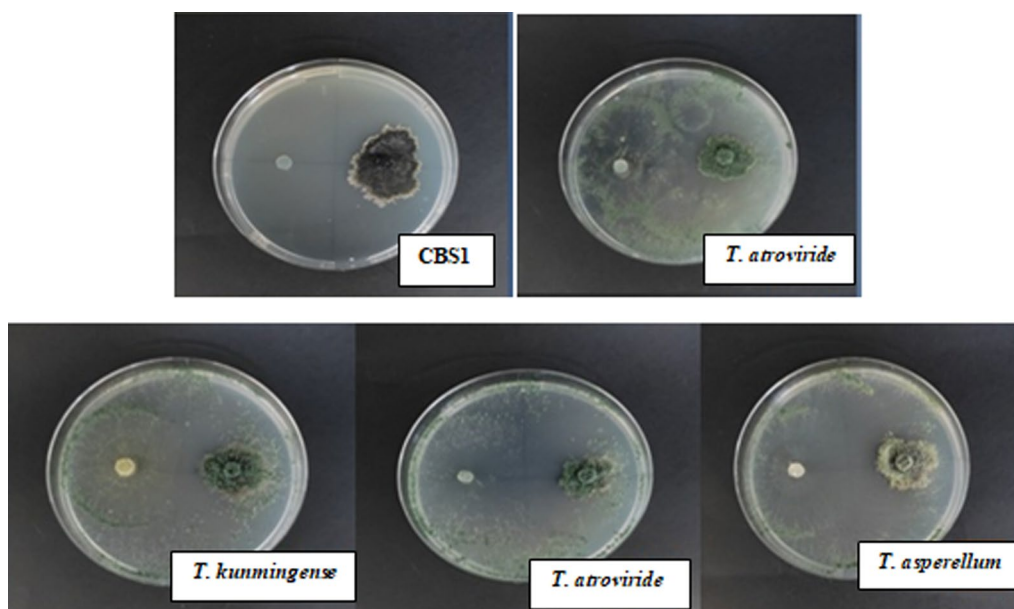


Fig. 2 Comparisons between colonies of *Phyllosticta citricarpa* isolate 1 control (T) and the pathogen confronted with *Trichoderma atroviride* (A1 and A2), *T. kunmingense* (A3) and *T. asperellum* (A4) following a period of incubation lasting 7 days at a temperature of 25 °C

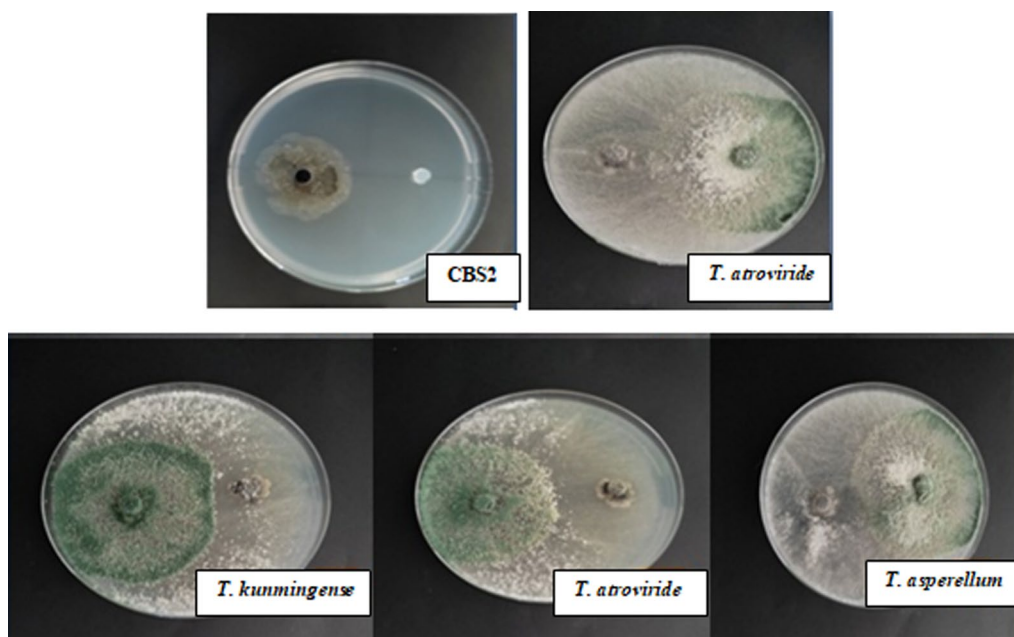


Fig. 3 Comparisons between colonies of *Phyllosticta citricarpa* isolate 2 control (T) and the pathogen confronted with *Trichoderma atroviride* (A1 and A2), *T. kunmingense* (A3) and *T. asperellum* (A4) following a period of incubation lasting 7 days at a temperature of 25 °C

caused by the isolate CBS2 with value of 64.81. For the CBS5 isolate, *T. atroviride* (A3) recorded a significant percentage reduction (67.80%). According to these results, the isolates *T. atroviride* (A1 and A3) and *T. asperellum* (A4) were more effective than *T. kunmingense* against *P. citricarpa* (Figs. 7 and 8).

Discussion

The use of the microbial antagonists is quite gaining popularity. Microbial bioagents can help to management postharvest diseases in two ways. Microorganisms that are already on the produce or ones that are added later can help protect it against

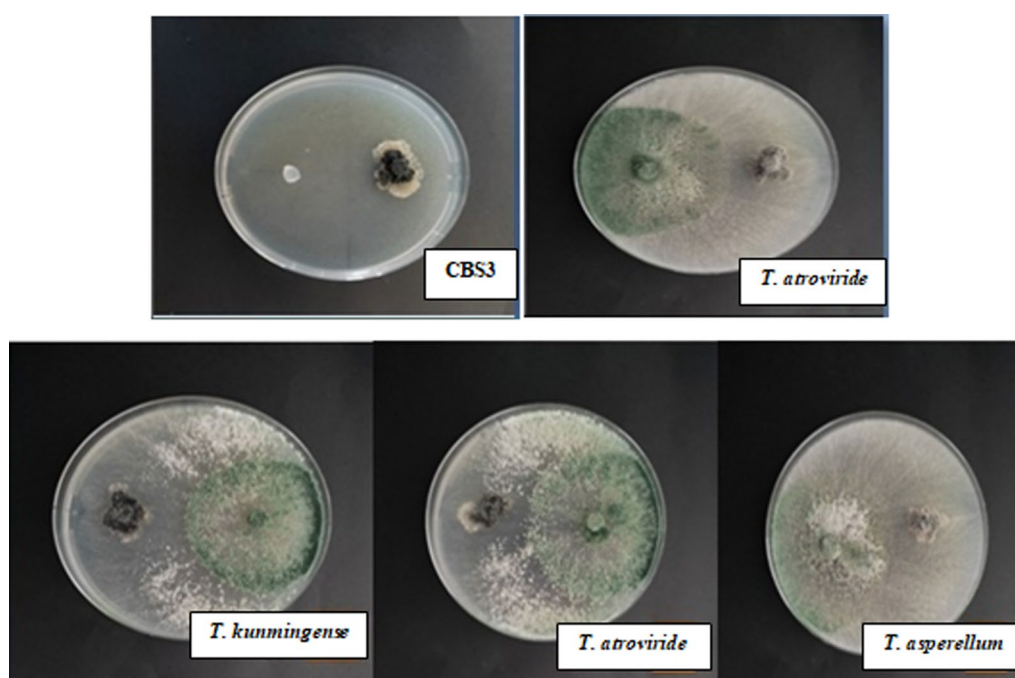


Fig. 4 Comparisons between colonies of *Phyllosticta citricarpa* isolate 3 control (T) and the pathogen confronted with *Trichoderma atroviride* (A1 and A2), *T. kunmingense* (A3) and *T. asperellum* (A4) following a period of incubation lasting 7 days at a temperature of 25 °C

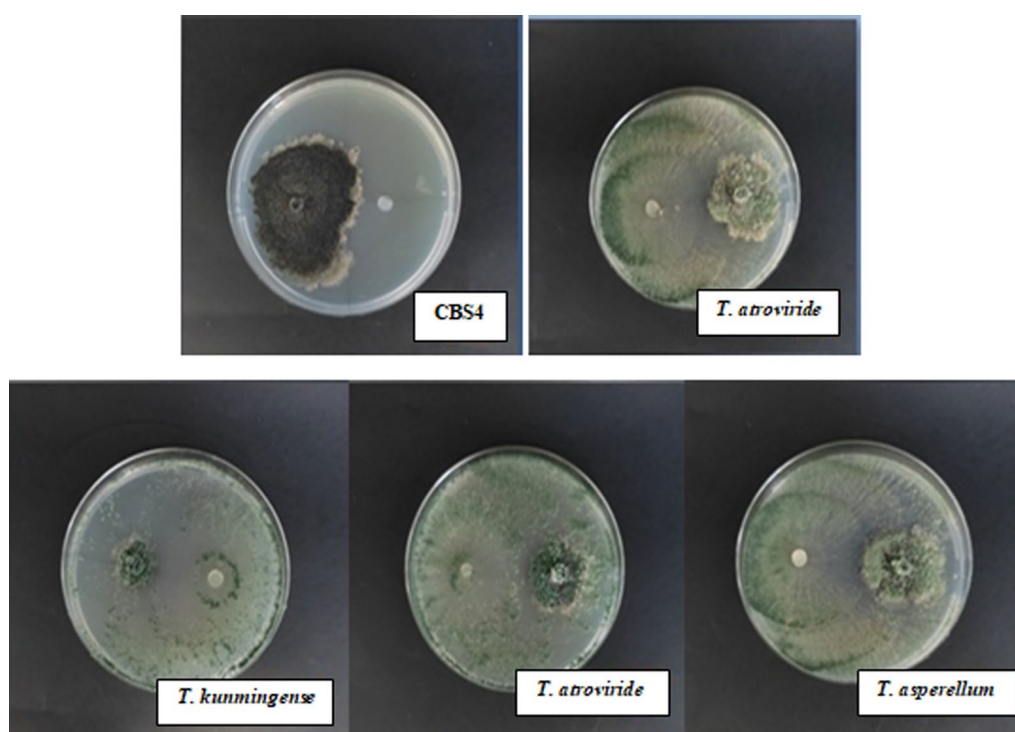


Fig. 5 Comparisons between colonies of *Phyllosticta citricarpa* isolate 4 control (T) and the pathogen confronted with *Trichoderma atroviride* (A1 and A2), *T. kunmingense* (A3) and *T. asperellum* (A4) following a period of incubation lasting 7 days at a temperature of 25 °C

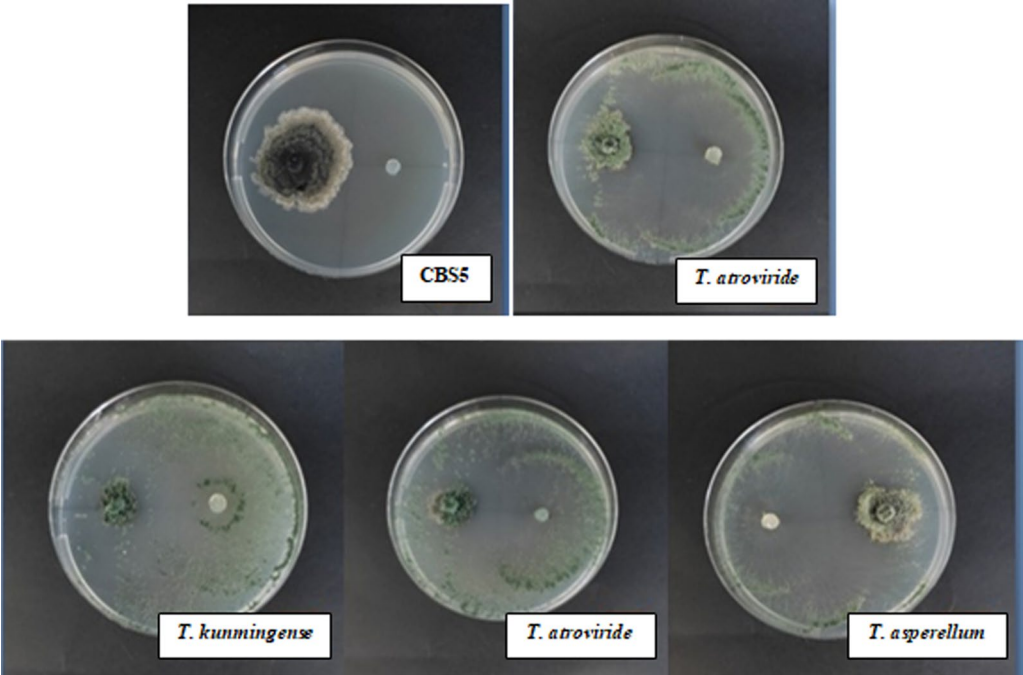


Fig. 6 Comparisons between colonies of *Phyllosticta citricarpa* isolate 5 control (T) and the pathogen confronted with *Trichoderma atroviride* (A1 and A2), *T. kunmingense* (A3) and *T. asperellum* (A4) following a period of incubation lasting 7 days at a temperature of 25 °C

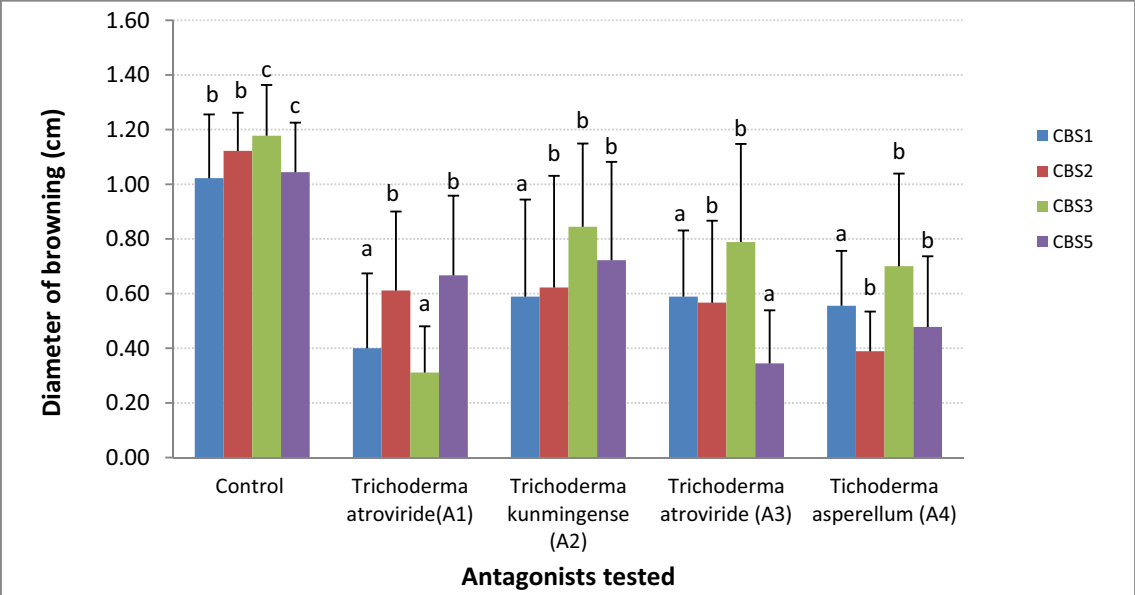


Fig. 7 Effect of *Trichoderma* species on the diameter of browning caused by *Phyllosticta citricarpa* in vivo on “Eureka” lemon fruits (cm) recorded 37 days after inoculation and incubation at 25 °C. For each isolate of *P. citricarpa*, bars that share the same letter do not exhibit significant differences as determined by the SNK test ($P \leq 0.05$). CBS1, CBS2, CBS3 and CBS5: isolates of *P. citricarpa* used

postharvest pathogens (Sharma et al. 2009). Among the most studied antagonistic microorganisms, species of *Trichoderma*, *Chaetomium*, *Aspergillus* genera and *Bacillus subtilis* (Mukherjee et al. 2019), *Trichoderma* species are effective agents used widely against many diseases caused by phytopathogenic fungi (Mukherjee et al. 2019). Some species of *Trichoderma* parasitize plant pathogens like *Botrytis cinerea*, *Rhizoctonia*



Fig. 8 Effect of *Trichoderma* isolates on browning caused by *Phyllosticta citricarpa* on "Eureka" lemon fruits recorded 37 days after inoculation and incubation at 25 °C. CBS1, CBS2, CBS3 and CBS5: isolates of *P. citricarpa* used; C: control; A3: *Trichoderma atroviride*; A4: *T. kunmingense*; A5: *T. atroviride*; A6: *T. asperellum*

solani, *Fusarium* spp., *Macrophomina phaseolina*, *Monosporascus cannonballus*, *Pythium ultimum* and *Phytophthora* spp. and promote plant growth (Mannai and Boughalleb-M'Hamdi 2023). Those characteristics increased the interest on these fungi. *Trichoderma harzianum* is a powerful species employed to manage different pathogens like *A. alternata*, *F. oxysporum*, *Cochliobolus heterostrophus*, *R. solani* and *S. rolfii*, which are responsible for agricultural losses (Rao et al. 2015). Due to worries about food safety and the environmental impact, more people are using biological control agents instead of chemicals to protect plants (Martinez-Medina et al. 2014).

This work is included to the assessment of many management approaches against citrus black spot disease in Tunisia. The control approach presented in the present study was the search of the biological agents including different *Trichoderma* species. Our focus was to determine the inhibitory potency of some antagonists in vitro and in vivo.

Trichoderma asperellum, *T. kunmingense* and *T. atroviride* showed in vitro and in vivo efficacies to control citrus black spot. Previous studies showed that *Trichoderma* spp. were very effective in vitro against *P. citricarpa* (Shirley and Mapotso 2017). In fact, *T. harzianum* reduced the mycelial growth of *P. citricarpa* in vitro, by 85% compared to *Bacillus subtilis* (4%) (Shirley and Mapotso 2017). Additionally, Kupper et al. (2011) demonstrated that *Trichoderma* spp. inhibited *P. citricarpa* development by 50 and 57%. In the present study, *P. citricarpa* isolates were transferred three days before antagonists since their fast growing potential. These antagonists covered completely the culture surface four days after their incubation. However, Kupper et al. (2011) showed that *Trichoderma* spp. isolates, completely covered the *P. citricarpa* culture surface after 6 days of incubation, when paired with it in Petri dishes. In addition, the same isolates paired with *Colletotrichum acutatum* covered the Petri dishes faster (in 2 days) (Moretto et al. 2001). Thus, the change in *Trichoderma*

spp. growth could be the result of the production of *P. citricarpa* metabolites that stopped or modified the antagonist development. However, *T. harzianum* was evaluated to control citrus black spot in organic systems in Brazil and it was not effective (Bernardo and Bettiol 2010). The fact that *T. harzianum* was not efficient in controlling the citrus black spot disease indicates that the fungus, originating in the soil, could not adapt in the environment of the aerial part of the plants or that the isolate evaluated is not efficient for the path system tested (Bernardo and Bettiol 2010). Recently, Lima et al. (2017) showed that the proteins secreted by *T. harzianum*, like L-amino acid oxidase, glycoside hydrolases, serine protease and Epl 1 protein, are related to mycoparasitism and the plant defense response increase and biocontrol against *G. citricarpa*.

Trichoderma kunmingense, a new soil-inhabiting *Trichoderma* species identified by Qiao et al. (2018), morphologically similar to *T. asperellum* with some differences revealed to be effective against *P. citricarpa* and confirmed that this genus is one of the most effective biological control agents.

The application of single *Trichoderma* species to control CBS has beneficial effect in reducing the disease severity on fruits. It is important to test the combination of two or multiple *Trichoderma* isolates or species in the future. Several recent works showed that the combined use of more than one species of *Trichoderma* can enhance plant development and offer protection against phytopathogenic microorganisms. This effect was achieved through the activation of dormant gene clusters and the production of secondary metabolites. Additionally, these species can increase the levels of amino acids and γ -aminobutyric acid, contributing to further enhancements in plant growth (Kredics et al. 2024).

Conclusions

The present results showed the efficacy of *Trichoderma atroviride*, *T. kunmingense* and *T. asperellum* to reduce *P. citricarpa* mycelial growth in vitro. The in vivo test confirmed their potentials to decrease the development of the lesions caused by *P. citricarpa* on Citrus lemon variety Eureka and showed that *T. atroviride* and *T. asperellum* are more efficient than *T. kunmingense*. Besides, the efficacy varies depending on antagonist species and pathogen isolates. Thus, the used *Trichoderma* species exhibited a promising potential to be used to control CBS.

Abbreviations

CBS	Citrus black spot
<i>C. limon</i>	<i>Citrus limon</i>
<i>P. citricarpa</i>	<i>Phyllosticta citricarpa</i>

<i>T. atroviride</i>	<i>Trichoderma atroviride</i>
<i>T. kunmingense</i>	<i>Trichoderma kunmingense</i>
<i>T. asperellum</i>	<i>Trichoderma asperellum</i>

Acknowledgements

This work was supported by L21AGR05, University of Sousse, Tunisia.

Author contributions

SM, NBM and IBS designed the research, SM, AF and IBS conducted surveys, sampling and analyses. SM wrote and NBM revised the manuscript.

Funding

This work was supported by L21AGR05, University of Sousse, Tunisia.

Availability of data and materials

All data are available in the manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 16 September 2024 Accepted: 13 November 2024

Published online: 11 April 2025

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