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In vitro and in planta potential effect of some indigenous antagonists against *Fusarium* and pythiaceous species associated with peach seedlings decline

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Abstract

Background: The effect of *Aspergillus* spp. and *Trichoderma harzianum* isolates was evaluated against the growth of *Fusarium oxysporum*, *F. solani*, *Pythium ultimum* and *Phytophthora citrophthora* affecting peach seedlings.

Results: The in vitro results revealed the ability of these antagonistic in reducing the radial growth of these pathogens. The most important mycelial growth reduction was of 85.82%, recorded for *F. oxysporum* in confrontation with A5 of *Aspergillus candidus*. *Aspergillus flavus* A4 and *A. niger* A10 were the most effective against *F. solani* with an inhibition percent more than 60%. For *P. citrophthora*, *A. flavus* A4, *A. candidus* A5, *A. terreus* A9 and *A. niger* A10 inhibited the mycelia growth by more than 60%. *Aspergillus nidulans* A1 was the most effective against *Pythium ultimum* (72.07%). *Trichoderma harzianum* isolates T9 and T10, are the most effective with a high inhibition percent of mycelial growth. The inhibition induced after 4 days of incubation, against *F. oxysporum*, *F. solani*, *P. citrophthora* and *Pythium ultimum* by these 2 antagonists exceeded 70, 60, 70 and 80%, respectively. The in planta test showed the efficacy of antagonists tested solo against some pathogens. In fact, *Bacillus subtilis* improved the health status by 62.55% compared to the control inoculated with *P. ultimum*. *Trichoderma harzianum* T9 significantly reduced the root rot index by 87.5% than the control inoculated with *F. solani*. In the same sense, *B. subtilis* significantly reduced this parameter by 62.55 and 88.89% than the control inoculated with *P. ultimum* and *P. citrophthora*, respectively. Furthermore, *B. subtilis* (B) and *Aspergillus niger* A10 improved plants height than the control inoculated with *Pythium ultimum* by 31.52 and 40.49%, respectively. However, the combinations of antagonists (T9 + T10; A5 + A10 and B + T10) did not improve their efficacy.

Conclusions: The isolates *T. harzianum* (T9 and T10), *A. candidus* A5 and *A. niger* A10 were the most effective in vitro against *Fusarium*, *Pythium* and *Phytophthora* species associated with peach seedling decline. The in vivo assay showed the effectiveness of *B. subtilis* against *P. ultimum* and *P. citrophthora* and the potential effect of *T. harzianum* T9 against *F. solani*. Their combinations revealed to be ineffective.

Keywords: *Aspergillus* spp., *Bacillus subtilis*, Biocontrol, Peach seedlings decline, *Trichoderma harzianum*

Background

Peach decline is one of the most destructive soil-borne diseases causing a dramatic reduction in plant growth, roots browning and stunting of tree shoots. Some pathogens agents could affect the tree development by damaging the trees roots and stunting shoots (Mannai

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et al. 2018a). Complexes of fungi and oomycetes were reported to be the main causal agents of fruit trees decline in orchards and also in nurseries (Mannai et al. 2018a). This complex includes *Fusarium* spp., *Cylindrocarpon* spp., *Pythium* spp., *Phytophthora* spp. and *Rhizoctonia solani* (Bent et al. 2009). In Tunisia, previous studies showed that *Phytophthora cactorum* and many *Pythium* species such as *P. indigoferae*, *P. irregulare*, *P. rostratifingens*, *P. sterilum*, *P. undulatum* were associated with apple trees decline in orchards and showed their virulence (Souli et al. 2014). Prior investigations have demonstrated the association of *Fusarium*, *Pythium* and *Phytophthora* species to apple and peach decline in Tunisian seedling nurseries (Mannai 2019).

Chemical products are the most used tools for this disease management. Some fungicides such as fosetyl-Al and metalaxyl showed excellent systemic activity against several diseases caused by *Phytophthora* and *Pythium* (Mazzola et al. 2002). In Tunisia, the fosetyl-Al, hymexazol, carbendazim and chinisol were tested in vitro and in vivo and revealed to be effective against *Fusarium oxysporum* and *F. solani* associated with the peach seedling decline in nurseries (Mannai et al. 2018b).

However, economic and environmental pressures to reduce the reliance on chemicals have led to a renewed interest in using biological agents such as bacteria and antagonistic fungi. Among biocontrol fungi agents, *Trichoderma*, *Gliocladium* and *Aspergillus* species have been proved to be active to control pathogens in vitro and in vivo (Boughalleb-M'Hamdi et al. 2018). *Trichoderma* species are the most widely used antagonists for controlling plant diseases (Yassin et al. 2021). Several bacteria such as *Bacillus*, *Pseudomonas*, *Burkholderia* and *Streptomyces* species have been used to manage soil-borne pathogens of many vegetable crops (Mannai et al. 2020). These species are capable of producing some bioactive secondary metabolites (Khan et al. 2020). Several previous studies showed that these fungal and bacterial antagonists' species efficiently controlled *Fusarium*, *Pythium* and *Phytophthora* species pathogens associated with many crops (Mannai et al. 2020). A combinatory approach has also the potential to resolve problems that occur with individual biocontrol agents. Several previous scientists have tested the combination of different biocontrol strains (Thilagavathi et al. 2007).

The objectives of this study were to: (1) evaluate the in vitro antifungal potential of 10 *Aspergillus* spp. and 10 *Trichoderma harzianum* isolates against the mycelial growth of *F. oxysporum*, *F. solani*, *Pythium ultimum* and *Phytophthora citrophthora* isolates associated with peach seedlings decline and (2) study the ability of these antagonists with *Bacillus subtilis* to suppress and/or reduce

Table 1 Pathogens isolates from peach tested in this study

Species	GenBank accession number
<i>Fusarium oxysporum</i>	MF993097
<i>F. solani</i>	MF993094
<i>Pythium ultimum</i>	MF993110
<i>Phytophthora citrophthora</i>	Nd
Nd not determined	

Table 2 Antagonists used to control growth of *Fusarium* and *Pythiaceus* species associated with peach decline seedlings in the Tunisian nurseries

Host species	Antagonists isolated	Code	Sample site
Peach	<i>Trichoderma harzianum</i>	T1, T2, T5, T6	Ben Arous
		T4	Monastir
		T9, T10	Kasserine
	<i>Aspergillus flavus</i>	A4	Kairouan
		A6	Monastir
		B	Sousse
Apple	<i>T. harzianum</i>	T3, T7, T8	Ben Arous
		A1	Zaghouan
	<i>A. pseudoligans</i>	A2, A3	Zaghouan
		A5	Kairouan
	<i>A. terreus</i>	A7, A8, A9	Kairouan
		A10	Zaghouan

the disease severity and enhance the growth of infected peach plants.

Methods

Pathogens isolates

The used pathogens for in vitro and in planta assays were isolates of *Fusarium* and *Pythiaceae* species obtained from peach tree nurseries in Tunisia. A representative isolate of each species was used (Table 1).

Antagonist's collection

The used antagonistic fungi and the bacterium strain were isolated from the roots and the rhizosphere of apple and peach seedlings from various Tunisian nurseries. The characteristics of all isolates are presented in Table 2.

Evaluation of the effect of the antagonists on mycelial growth of the pathogens associated with peach seedlings decline

Dual culture plate assays were performed in 90 mm Petri dishes containing Potato Dextrose Agar (PDA; Laboratories CONDA; Spain) to study the ability of fungal antagonists to inhibit pathogens mycelial growth. Agar plugs (6

mm in diameter) cut from cultures of each pathogen were placed in the opposite side to those of tested antagonists. Control plates were cultured with pathogen plugs only. Three replicates were considered for each treatment. The incubation was performed at 25 °C for 4 days for *T. harzianum* isolates and 6 days for *Aspergillus* spp. isolates. The experiment was repeated twice. The percentage inhibition of mycelial growth was calculated according to the following formula:

$$\% \text{ inhibition} = (1 - T/C) * 100$$

where *T* is the average colony radius in the presence of the antagonist fungus, and *C*: average radius of control colonies.

Study of the effect of antagonists on the severity of the peach seedlings decline

The method of Ruano-Rosa and Lopez-Herrera (2009), with minor modifications, was adopted to evaluate the in vivo effect of bioagents on the severity of the peach seedlings decline. For this trial, 2 isolates of *Trichoderma* spp. (T9 and T10), 2 isolates of *Aspergillus* spp. (A5 and A10) and *B. subtilis* strain were used. The inoculum for each oomycete isolate was prepared by growing the pathogen in 500-ml flasks containing 200 g of sand, 20 g of oat and 30 mL of distilled water autoclaved at 120 °C for 20 min on 2 consecutive days. This mixture was inoculated with 10 agar plugs of each isolate per flask under aseptic conditions. To prepare the *Fusarium* inoculum, bottles containing 200 g of sterile wheat seeds were inoculated with 10 mycelial disks (6 mm diam.) of each *Fusarium* spp. isolate grown on PDA medium for 2 weeks. Wheat seeds and sand-oat were inoculated with disks of PDA medium served as controls. The flasks were mixed and incubated for one week for oomycetes and 2 weeks for *Fusarium* spp. at 25 °C and shaken every 2 days to ensure thorough colonization. Sand-oat and wheat seeds inoculums were added to a potting mix (peat and sand in 2:1 v/v) at a rate of 1% (v/v) which was then placed in 10 cm diameter plastic pots. The uninoculated control was an uninfected potting mix.

To prepare the inoculum of each antagonist treatment, a few agar disks from 7-day-old antagonist cultures grown on PDA medium were incubated, for one week, in an Erlenmeyer flask containing 150 ml of Potato Dextrose Broth (PDB; Laboratories CONDA; Spain) medium, with stirring (120 rpm). The suspensions were adjusted to 10⁶ spores/ml. *Bacillus subtilis* cell suspensions used were prepared by scraping bacterial colonies, previously grown in nutrient agar (NA) medium for 48 h, in sterile distilled water (SDW) and adjusted to 10⁶ cells/ml. The antagonist treatment was done at the following dates: 1 and 30 days from the start of the experiment (50 ml/

plant). The antagonistic isolates were applied alone and in combinations. The combinations used were: T9 + T10, A5 + A10 and B + T10.

The experiment was conducted according to a completely randomized design, with 3 replicates per elementary treatment. The plants were harvested after 3 months. Four parameters were noted at harvest. These parameters were: the sanitary state index, the seedlings' height, the root weight and the root browning index. The sanitary state index rated onto 0–5 scale (0 = healthy seedlings: 1 = moderate discoloration of plant leaves (≤25%); 2 = moderate discoloration and falling leaves (≤50%); 3 = moderate discoloration of plant collar, stem and leaves (≤75%); 4 = extensive discoloration of plant collar and stem with falling leaves (>); and 5 = dead plant) (Santini et al. 2006). The root browning index was rated according to a 0–5 scale (0 = no obvious symptoms; 1 = moderate discoloration of root tissue; 2 = moderate discoloration of tissue with some lesion; 3 = extensive discoloration of tissue; 4 = extensive discoloration of tissue with girdling lesions; and 5 = dead plant) (Tewoldemedhin et al. 2011).

The pathogen re-isolations were made from roots of inoculated seedlings using PDA (Potato Dextrose Agar) and PARP medium (pimaricin + ampicillin + rifampicin + pentachloronitrobenzene [PCNB]) to confirm Koch's postulates (Jeffers and Martin 1986).

Statistical analysis

Data were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences software (SPSS), version 20.0. The in vitro and in planta tests were analyzed according to a completely randomized factorial model with 2 factors (antagonists and pathogens tested). For all tests, means were compared using Student–Newman–Keuls (SNK) test ($P \leq 0.05$).

Results

Effect of antagonists on mycelial growth of the studied pathogens

In vitro antifungal activities of *Aspergillus* species

The mean radius of pathogens colonies, formed after 6 days of incubation at 25 °C, is dependent on antagonistic treatments tested and pathogen isolates, and a highly significant interaction was observed between both fixed factors at $P \leq 0.001$. In fact, all *Aspergillus* species were able to reduce the peach decline pathogens' radial growth compared to their relative untreated controls (Table 3). The most important mycelial growth reduction, compared to the untreated control, was 85.82%, recorded in the case of *F. oxysporum* treated with *A. candidus* (A5). The isolates A4 (*A. flavus*) and A10 (*A. niger*) were the most effective against *F. solani*, with an inhibition

Table 3 Inhibition percentage of peach seedling pathogens colony growth, noted after 6 days of dual culture with *Aspergillus* spp. isolates

	<i>Fusarium solani</i>	<i>Fusarium oxysporum</i>	<i>Phytophthora citrophthora</i>	<i>Pythium ultimum</i>	P value***
A1	25.23 ± 6.80b*A**	54.48 ± 1.29aB	26.83 ± 2.44aA	20.72 ± 1.56aA	≤ 0.001
A2	51.35 ± 10.81dA	65.67 ± 5.63bA	56.10 ± 13.58cdA	47.75 ± 1.56bcA	0.171
A3	39.64 ± 1.56cA	66.42 ± 2.24bC	43.09 ± 3.73bA	48.65 ± 2.70bcB	≤ 0.001
A4	64.86 ± 2.70eA	63.43 ± 3.42bA	65.85 ± 4.88dA	47.75 ± 4.13bcB	≤ 0.001
A5	51.95 ± 4.13aA	85.82 ± 1.29cC	63.41 ± 0.00 dB	48.05 ± 12.19bcA	≤ 0.001
A6	39.64 ± 4.13cAB	50.75 ± 2.24aC	46.34 ± 4.88bcBC	32.43 ± 5.41abA	0.004
A7	40.54 ± 2.70cA	64.18 ± 6.72bB	58.54 ± 4.88 dB	38.74 ± 1.56bcA	≤ 0.001
A8	37.84 ± 2.70cA	70.90 ± 2.24bB	40.65 ± 3.73bA	45.95 ± 16.22bcA	0.005
A9	36.04 ± 4.13cA	55.22 ± 2.24aB	60.16 ± 3.73 dB	53.15 ± 4.13cB	≤ 0.001
A10	70.27 ± 0.00eA	72.39 ± 3.42bA	69.92 ± 6.14dA	72.07 ± 4.13dA	0.838
P value	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	

*Means ± standard error in the column for each parameter followed by the same lowercase letter were not significantly different according to SNK test at $P \leq 0.01$

**Means ± standard error in a row followed by the same capital letter were not significantly different according to SNK test at $P \leq 0.05$

***Probabilities associated with individual F tests

percentage greater than 60%. For *Ph. citrophthora*, the isolates A4 (*A. flavus*), A5 (*A. candidus*), A9 (*A. terreus*) and A10 (*A. niger*) inhibited the mycelia growth by more than 60%. The antagonist *Aspergillus nidulans* (A1) was the most effective against *P. ultimum* (72.07%) (Table 3; Fig. 1).

In vitro antifungal activity of *Trichoderma harzianum* isolates

The *in vitro* evaluation of the efficiency of different *T. harzianum* isolates on pathogens hyphal growth showed a highly significant interaction between both fixed factors (antagonists*pathogens) at $P \leq 0.001$ (Table 4). The isolates T9 and T10 of *T. harzianum*, originating from the Kasserine region, exhibited the highest inhibition percent of mycelial growth for different pathogens. The inhibition rates induced after 4 days of incubation against *F. oxysporum*, *F. solani*, *P. citrophthora* and *Pythium ultimum* by these 2 antagonists were 70, 60, 70 and 80%, respectively (Table 4; Fig. 2). The antagonists A5, A10, T9 and T10 were chosen for the *in vivo* assays because of their effectiveness against the different tested pathogens.

Effect of antagonists on the severity of peach seedlings decline

The analysis of the relative variance of the health status of the inoculated vegetative part of the plants showed a highly significant difference ($P \leq 0.001$) between the different treatments and a significant difference ($P \leq 0.05$) between pathogens. *Bacillus subtilis* (B) improved the health status by 62.55% than the control inoculated with *P. ultimum*. Other treatments did not significantly

improve this parameter for all the studied pathogens (Table 5; Fig. 3).

Analysis of the variance relative to the severity of root rot, noted 90 days after planting, showed the existence of a significant interaction ($P \leq 0.05$) of the 2 fixed factors (pathogens x antagonists) and a highly significant difference ($P \leq 0.001$) between the effect of the tested antagonists. The *T. harzianum* isolate (T9) and *B. subtilis* (B) were the most effective. *T. harzianum* T9 significantly reduced the root rot index by 87.5% than the control inoculated with *F. solani*. For *B. subtilis*, this parameter was significantly decreased by 62.55 and 88.89% compared to the control inoculated with *P. ultimum* and *Ph. Citrophthora*, respectively (Table 5; Fig. 4). However, no effect was noted on the root rot index when used the other treatments (*T. harzianum* isolate T10, *A. candidus* A5 and *A. niger* A10, A5 + A10, T9 + T10 and B + T10) (Table 5).

Statistical analysis of the relative variance of plant height showed a significant interaction between fixed factors (pathogens*antagonists) and a highly significant difference ($P \leq 0.001$) between different antagonists. The treatments *A. niger* (A10) and *B. subtilis* (B) improved the plant height compared to the control inoculated with *P. ultimum* by 40.49 and 31.52%, respectively (Table 5; Fig. 3). However, all treatments did not improve this parameter for peach seedlings inoculated by *F. oxysporum*, *F. solani* or *Ph. citrophthora*. The seedlings heights were statistically comparable to that of inoculated control (Table 5).

The analysis of the variance of root weight data revealed a highly significant difference ($P \leq 0.001$) according to different treatments and a significant difference between

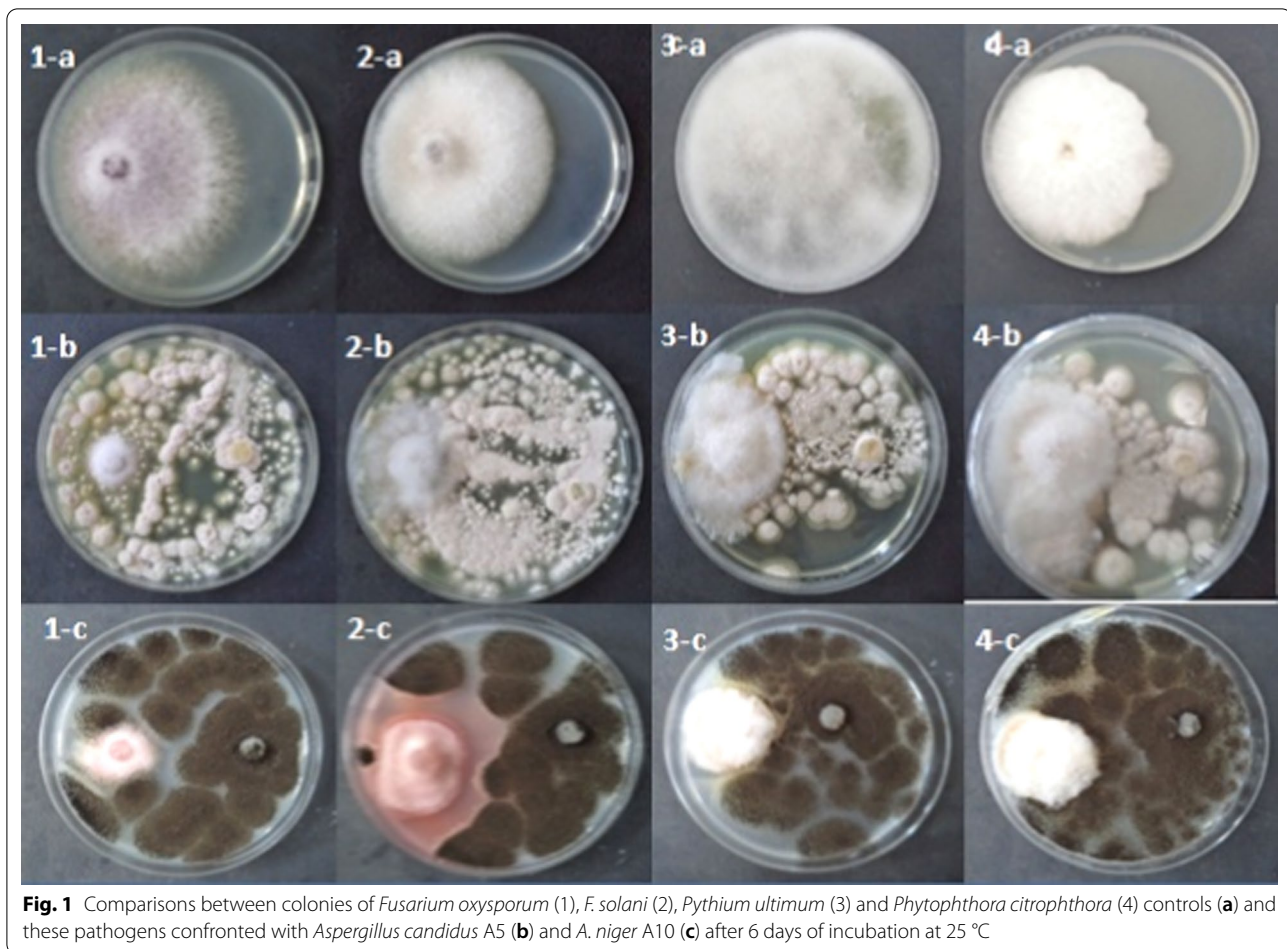


Table 4 Inhibition percentage of peach seedling pathogens' colony growth, noted after 4 days of dual culture with *Trichoderma harzianum* isolates

	<i>Fusarium oxysporum</i>	<i>Fusarium solani</i>	<i>Pythium ultimum</i>	<i>Phytophthora citrophthora</i>	P value***
T1	50.87 ± 4.19ab*A**	58.06 ± 2.79cA	42.11 ± 1.75aA	67.48 ± 2.01bA	0.378
T2	65.39 ± 3.15abcA	50.00 ± 2.79abcB	47.95 ± 4.05aB	64.00 ± 2.01bA	≤ 0.001
T3	70.86 ± 3.15bcA	53.23 ± 7.39abcB	67.25 ± 6.16bA	64.00 ± 2.01bA	0.015
T4	70.86 ± 8.35bcA	56.45 ± 12.80bcA	74.85 ± 12.45bcA	65.16 ± 6.97bA	0.234
T5	56.28 ± 4.46abAB	51.61 ± 9.68abcB	75.44 ± 1.75bcA	60.51 ± 4.02bAB	0.053
T6	54.46 ± 3.75abA	59.68 ± 2.79cA	73.68 ± 5.26bcA	67.48 ± 2.01bA	0.059
T7	50.82 ± 5.46abA	35.48 ± 7.39aB	45.03 ± 4.42aAB	46.57 ± 2.01aAB	0.036
T8	43.53 ± 8.35aA	37.10 ± 8.38abA	39.77 ± 2.68aA	45.41 ± 5.32aA	0.460
T9	70.86 ± 3.15bcB	61.29 ± 8.38cB	86.55 ± 1.01cA	69.80 ± 2.01bB	≤ 0.001
T10	81.79 ± 3.15cA	77.42 ± 10.07dA	80.70 ± 6.33bcA	70.96 ± 5.32bA	0.268
P value	0.044	≤ 0.001	≤ 0.001	≤ 0.001	

*Means ± standard error in the column followed by the same lowercase letter are not significantly different according to SNK test at $P \leq 0.01$

**Means ± standard error in a row followed by the same capital letter are not significantly different according to SNK test at $P \leq 0.05$

***Probabilities associated with individual *F* tests

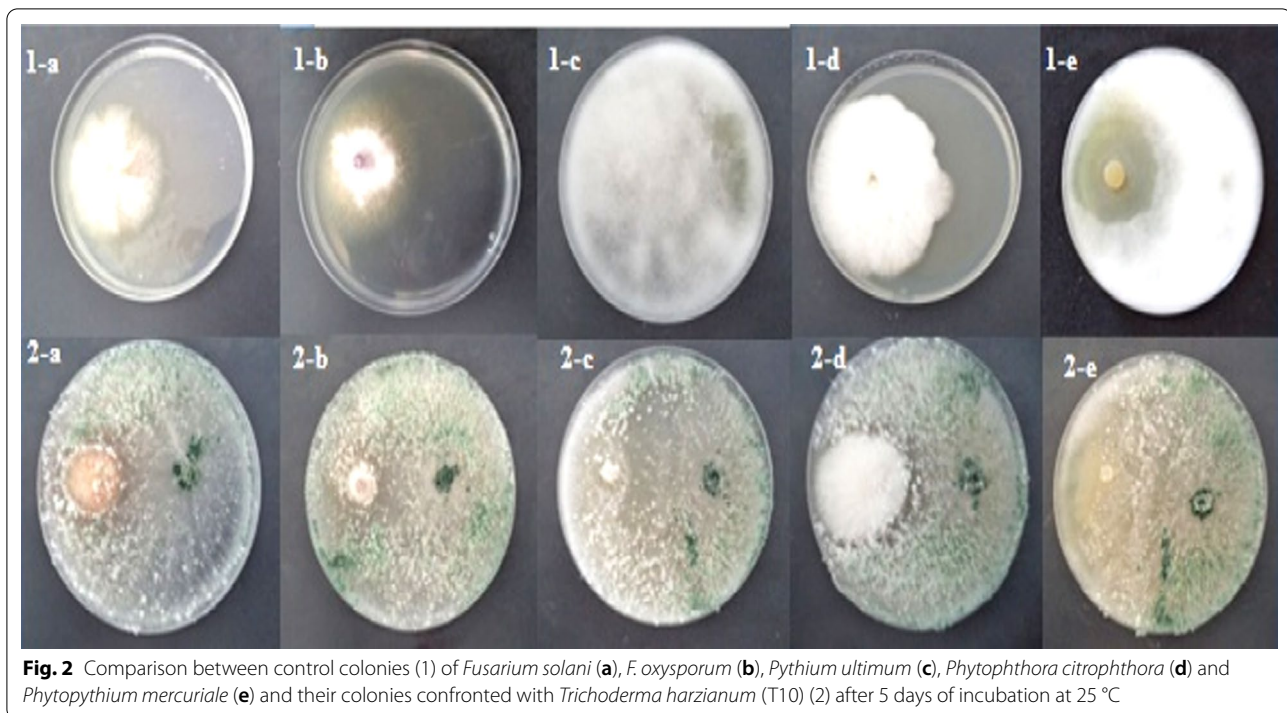


Fig. 2 Comparison between control colonies (1) of *Fusarium solani* (a), *F. oxysporum* (b), *Pythium ultimum* (c), *Phytophthora citrophthora* (d) and *Phytophthium mercuriale* (e) and their colonies confronted with *Trichoderma harzianum* (T10) (2) after 5 days of incubation at 25 °C

pathogens. However, all treatments did not significantly improve this parameter. They were statistically comparable to the inoculated control (Table 5; Fig. 4).

Discussion

Trichoderma harzianum isolates T9 and T10, native to the Kasserine region, were the most effective on the different pathogens in vitro. The in planta assays demonstrated that T9 significantly reduced the root rot index of peach plants inoculated with *F. solani*. Several previous studies have shown that the genus *Trichoderma* is among the most studied biological fungal agents marketed as biopesticides (Jamil et al. 2021). Use of *Trichoderma* spp. in agriculture could offer many benefits such as the colonization of the rhizosphere allowing rapid establishment in stable microbial communities of the rhizosphere, control of pathogens using various mechanisms, improving plant health and stimulating growth root (Harman et al. 2004). However, Kandula et al. (2010) studied the effect of *Trichoderma* spp. bio-inoculants on specific apple replant disease (SARD) symptoms in apple rootstocks in New Zealand and proved that the plants grown in the SARD soils treated with the *Trichoderma* sp. pellet formulations exhibited a significant improvement in growth, with no reduction in root disease symptoms. Similar responses were noted when dead (autoclaved) *Trichoderma* sp. pellet formulations or unformulated pellets were applied. So, Kandula et al. (2010) proved that no disease reduction or growth improvement was observed

in the plants grown in soil treated with a *Trichoderma* sp. spore powder.

A previous study assessed the potential of four *Trichoderma asperellum* strains in vitro and in vivo to control *Pythium myriotylum*, the causal agent of cocoyam root rot disease in Cameroon, showed their efficacy, although differences were found among the strains. In in vivo trials, pretreatment of cocoyam plants with 2 *T. asperellum* strains reduced *P. myriotylum* infection by 50% (Mbarga et al. 2012). Moreover, a recent study on the efficacy of *T. harzianum* and *T. viride* against *Pythium* damping-off of pepper showed that these antagonists inhibited *P. ultimum* radial growth by 18.54, 17.52 %, respectively, in dual culture assay and reduced the pre- and post-emergence pepper damping-off infections (Mannai et al. 2020). Besides, a study established in Saudi Arabia to evaluate the antagonistic efficacy of 2 species of *Trichoderma* against the most common causative agents of stalk rot disease of maize showed that *Trichoderma viride* was effective against *F. proliferatum* and *F. verticillioides* with mycelial inhibition rates of 80.17 and 70.46% and *T. harzianum* exhibited rates of 68.38 and 60.64%, respectively (Yassin et al. 2021).

Aspergillus candidus and *A. niger* were among the most effective isolates to reduce pathogens mycelia growth. In planta, *A. niger* stimulated the growth of peach plants inoculated with *P. ultimum*. These results are in agreement with previous studies reporting that several *Aspergillus* species are capable of producing a number

Table 5 Effect of two *Trichoderma* and *Aspergillus* species isolates and *Bacillus subtilis* on the severity of decline disease and seedlings growth three months after the inoculation on peach seedlings 'Garnem'

	<i>Fusarium oxysporum</i>	<i>Fusarium solani</i>	<i>Phytophthora citrophthora</i>	<i>Pythium ultimum</i>	P value***
<i>Sanitary state</i>					
A5	2.67 ± 0.58a*A*	3.67 ± 0.58abA	3.33 ± 0.58cA	3.33 ± 0.58aA	≥ 0.05
A10	2.00 ± 1.00aA	3.33 ± 1.53abA	3.67 ± 0.58cA	2.67 ± 0.58abA	≥ 0.05
T9	1.33 ± 0.58aA	1.67 ± 0.58aA	2.00 ± 0.00abA	2.33 ± 0.58abA	≥ 0.05
T10	3.67 ± 0.58aA	4.00 ± 0.00bA	3.33 ± 0.58cA	3.33 ± 0.58aA	≥ 0.05
B	1.67 ± 1.15aA	2.00 ± 0.00abA	1.33 ± 0.58aA	1.00 ± 0.00cA	≥ 0.05
T9+T10	3.33 ± 1.53aA	3.33 ± 0.58abA	3.67 ± 0.58cA	3.00 ± 0.00abA	≥ 0.05
A5 + A10	2.67 ± 0.58aAB	3.67 ± 0.58abB	3.00 ± 0.00bcAB	2.33 ± 0.58abA	0.055
B+T10	1.33 ± 0.58a	2.33 ± 0.58ab	2.33 ± 0.58abc	2.67 ± 0.58ab	≥ 0.05
NIC	1.67 ± 0.58a	1.67 ± 0.58a	1.67 ± 0.58a	1.67 ± 0.58ab	nd
IC	1.67 ± 0.58a	2.00 ± 1.00ab	2.33 ± 0.58abc	2.67 ± 0.58ab	nd
P value	≥ 0.05	≤ 0.05	≤ 0.001	≤ 0.001	
<i>Root browning</i>					
A5	2.33 ± 0.58aA	2.33 ± 0.58bA	2.33 ± 0.58bA	2.33 ± 0.58abA	≥ 0.05
A10	2.67 ± 0.58aA	2.33 ± 0.58bA	2.67 ± 0.58bA	2.33 ± 0.58abA	≥ 0.05
T9	1.67 ± 0.58aB	0.33 ± 0.58aA	2.00 ± 0.00bB	1.67 ± 0.58abB	0.015
T10	2.33 ± 0.58aA	2.33 ± 0.58bA	1.33 ± 0.58abA	2.67 ± 0.58aA	≥ 0.05
B	1.33 ± 0.58aA	1.33 ± 0.58abA	0.33 ± 0.58aA	1.00 ± 0.00bA	≥ 0.05
T9+T10	2.33 ± 0.58aA	1.00 ± 1.00abA	2.33 ± 0.58bA	2.67 ± 0.58aA	≥ 0.05
A5 + A10	1.67 ± 0.58aA	1.33 ± 0.58abA	2.67 ± 0.58bA	1.67 ± 0.58abA	≥ 0.05
B+T10	1.67 ± 0.58aA	2.33 ± 0.58bA	1.67 ± 0.58abA	2.00 ± 0.00abA	≥ 0.05
NIC	1.33 ± 0.58a	1.33 ± 0.58ab	1.33 ± 0.58ab	1.33 ± 0.58ab	nd
IC	3.00 ± 1.00a	2.67 ± 0.58b	3.00 ± 1.00b	2.67 ± 0.58a	nd
P value	≥ 0.05	≤ 0.05	≤ 0.001	0.005	
<i>Height (cm)</i>					
A5	54.67 ± 5.97aA	57.50 ± 6.38aA	59.00 ± 0.50ab A	60.67 ± 6.25abA	≥ 0.05
A10	59.83 ± 8.35aA	64.67 ± 4.37aA	53.17 ± 10.20aA	87.67 ± 10.50cA	≥ 0.05
T9	73.67 ± 7.87aA	61.83 ± 8.74aA	73.83 ± 4.25bA	64.83 ± 1.26abA	≥ 0.05
T10	47.90 ± 9.84aA	53.00 ± 7.00aA	73.67 ± 4.75bB	59.00 ± 9.00abA	0.016
B	71.33 ± 5.74aAB	65.00 ± 9.00aAB	56.33 ± 3.75abA	82.07 ± 7.40bcB	0.06
T9+T10	52.83 ± 4.83aA	62.67 ± 5.35aA	55.17 ± 3.06abA	57.50 ± 6.50abA	≥ 0.05
A5 + A10	71.67 ± 6.48aA	56.50 ± 2.00aA	62.50 ± 10.00abA	70.57 ± 13.10abcA	≥ 0.05
B+T10	68.00 ± 4.84aA	65.67 ± 9.29aA	68.00 ± 3.50abA	60.67 ± 13.50abA	≥ 0.05
NIC	64.07 ± 0.40a	62.40 ± 2.26a	62.40 ± 2.26ab	62.40 ± 2.26ab	nd
IC	63.17 ± 1.15a	55.00 ± 10.11a	54.17 ± 14.63ab	46.00 ± 16.39a	nd
P value	≥ 0.05	≥ 0.05	≤ 0.05	0.002	
<i>Root weight (g)</i>					
A5	2.68 ± 0.55aA	2.64 ± 1.38aA	3.83 ± 0.53aA	2.85 ± 0.03bA	≥ 0.05
A10	4.47 ± 2.98aA	3.02 ± 1.21aA	3.07 ± 0.51aA	6.96 ± 1.81abA	≥ 0.05
T9	5.42 ± 0.84aA	6.49 ± 1.84bA	4.70 ± 0.97aA	5.12 ± 0.17abA	≥ 0.05
T10	2.93 ± 1.63aA	1.98 ± 0.49aA	3.58 ± 1.18aA	3.11 ± 0.99bA	≥ 0.05
B	3.73 ± 1.30aA	4.30 ± 0.91abA	4.97 ± 0.39aAB	7.56 ± 2.23aB	0.040
T9+T10	3.12 ± 1.41aA	3.82 ± 0.62abA	2.74 ± 1.00aA	4.24 ± 0.90abA	≥ 0.05
A5 + A10	4.71 ± 0.93aA	3.69 ± 0.68abA	4.55 ± 1.54aA	5.22 ± 1.29abA	≥ 0.05
B+T10	3.82 ± 0.60aA	4.47 ± 1.38abA	3.37 ± 0.31aA	3.56 ± 1.19abA	≥ 0.05
NIC	4.79 ± 0.37a	4.79 ± 0.37ab	4.79 ± 0.37a	4.79 ± 0.37ab	nd
IC	3.63 ± 0.78a	4.50 ± 0.55ab	2.84 ± 1.04a	3.73 ± 3.00ab	nd
P value	≥ 0.05	≤ 0.05	≥ 0.05	0.012	

Table 5 (continued)

*Means \pm standard error in the column for each parameter (root browning index, sanitary state index, height and root weight) followed by the same lowercase letter were not significantly different according to SNK test at $P \leq 0.05$

**Means \pm standard error in a row followed by the same capital letter were not significantly different according to SNK test at $P \leq 0.05$

***Probabilities associated with individual F tests; Nd: not determined; NIC: uninoculated control; IC: inoculated control; A5: *Aspergillus candidus*; A10: *A. niger*; T9, T10: *Trichoderma harzianum* isolates; B: *B. subtilis*

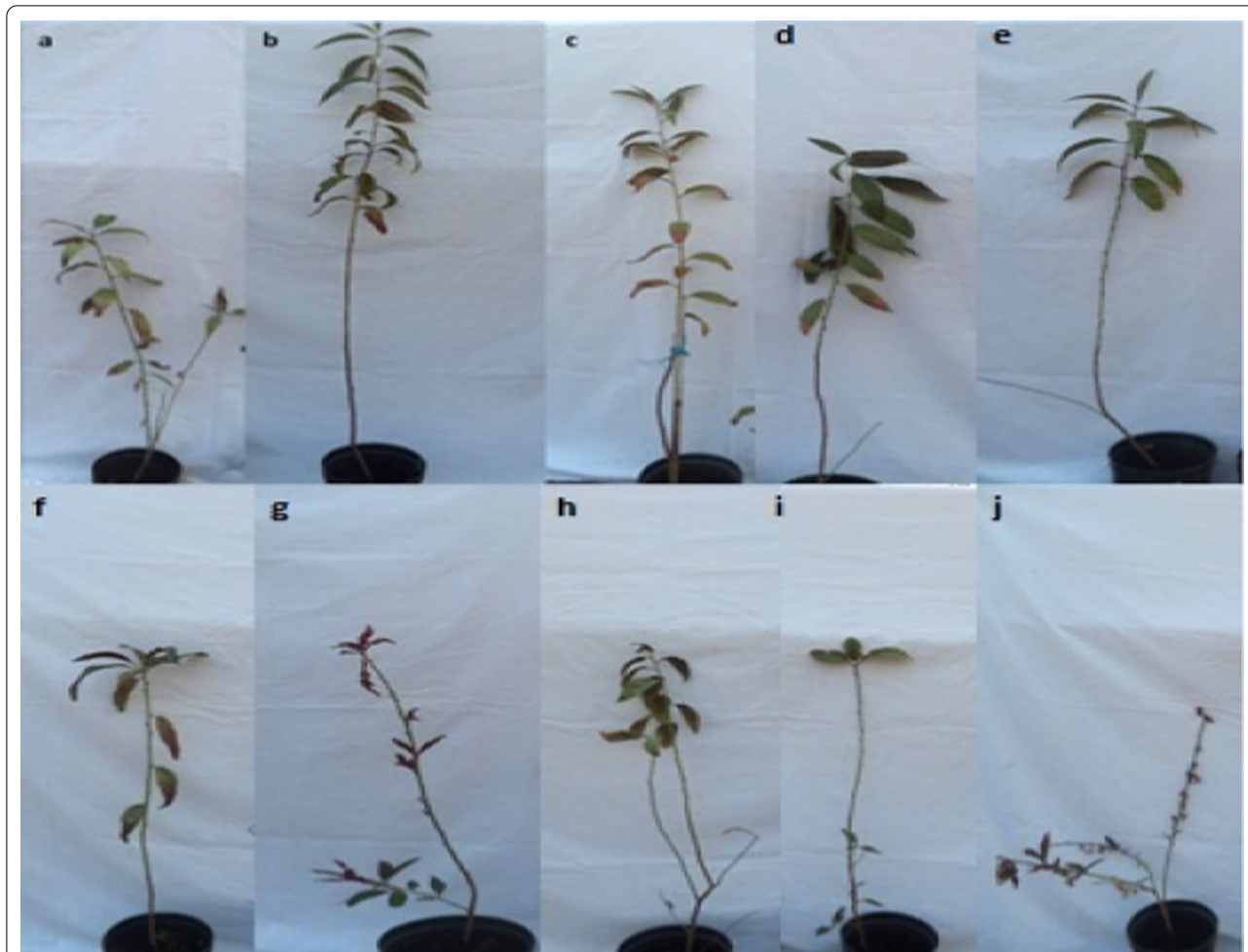


Fig. 3 Peach plants recorded three months after inoculation with *Pythium ultimum* and their treatment: inoculated control (a), *Aspergillus niger* A10 (b), *Bacillus subtilis* (B) (c), *Trichoderma harzianum* T9 (d), uninoculated control (e), *A. candidus* A5 + *A. niger* A10 (f), B + *T. harzianum* T10 (g), *T. harzianum* T9 + *T. harzianum* T10 (h), *T. harzianum* T10 (i), *A. candidus* A5 (j)

of bioactive secondary metabolites (Ngo et al. 2021). In addition to their antagonistic capacity, several members of this genus have demonstrated the ability to confer plant resistance to diseases and other known benefits to contribute to general soil suppression (Urja and Meenu 2010).

A previous study proved the efficacy of *Aspergillus flavus*, *A. niger*, *A. terreus* and *A. fumigatus* to reduce the mycelial growth of *F. oxysporum* and *F. solani* in vitro and decrease the disease severity and improve the growth of melon and watermelon seedlings inoculated

by these pathogens (Boughalleb-M'Hamdi et al. 2018). In the same sense, Dwivedi (2013) confirmed that 4 *Aspergillus* species (*A. flavus*, *A. niger*, *A. sulphureus* and *A. luchuensis*) had a fungi toxicity against *F. solani*. Zhao et al. (2018) proved that a strain of *A. tubingensis* significantly protected tomato against gray mold, obviously enhanced plant length, dry mass and fresh mass of tomato plants and successfully colonized in the rhizosphere.

The isolate of *B. subtilis* tested in vivo was also very effective against some pathogens. In fact, this antagonist



Fig. 4 Peach plants roots three months after inoculation with *Pythium ultimum* and their treatment: uninoculated control (a), inoculated control (b), *Aspergillus candidus* A5 + *A. niger* A10 (c), *Trichoderma harzianum* T10 (d), *A. candidus* A5 (e), *A. niger* A10 (f), *Bacillus subtilis* (B) (g), *T. harzianum* T9 (h), *T. harzianum* T10 + B (i) and *T. harzianum* T9 + *T. harzianum* T10 (j)

reduced the index of root rot and the severity of the sanitary status and increased the height of the peach plants inoculated with *P. ultimum*. It also reduced the root rot index of peach plants inoculated with *Ph. citrophthora*. Previous studies have found that the genus *Bacillus* is an important microbial antagonist of pathogens (Devi et al. 2022). It improves plant growth and reduces fungal pathogens in apple orchards infested with dieback disease (replantation) (Van Schoor and Bezuidenhout 2014). Efficacy of *B. subtilis* capacity produces a number of bioactive secondary metabolites. The *Bacillus* species are the most frequent producers of bioactive microbial metabolites (Saxena et al. 2020). The present results were also in concordance with previous studies showing that the use of *B. subtilis*, as a soil treatment, might be used to control *Pythium* and *Phytophthora* root rot on several crops (Mannai et al. 2020). In addition, *B. subtilis* cell suspensions decreased the severity of pepper root rot caused by *Phytophthora* by 55% (Sid Ahmed et al. 2003). In addition, the application of the *B. subtilis* TM3 formulation was able to suppress *F. verticillioides* infection in corn plants in Indonesia (Suriani et al. 2021).

By contrast, the combinations between antagonists neither reduced the disease severity nor improved the growth of seedlings. Each strain used individually was more efficient than its combination with another antagonist strain. This may be due to an incompatible reaction among strains (Thilagavathi et al. 2007).

Thus, as an eco-friendly alternative to chemical fungicide, the management of the peach seedling decline through biocontrol agents is possible and the results are

promising as some of the tested biocontrol agents were effective in reducing disease severity and in improving seedling growth.

Furthermore, in nurseries, seedlings can be attacked by more than one soil-borne pathogen and the disease severity could be due to fungal complexes (Moein et al. 2019). For this reason, it will be important to test these antagonists in nurseries without combinations.

Conclusions

In vitro test of *T. harzianum* and *Aspergillus* species proved that the isolates *T. harzianum* isolates T9 and T10 and *A. candidus* isolate A5 and *A. niger* isolate A10 were the most effective against *Fusarium*, *Pythium* and *Phytophthora* species associated with peach seedling decline. The test in planta showed the efficacy of *B. subtilis* against *P. ultimum* and *P. citrophthora*. It reduced the disease severity indexes and improved plants growth. Furthermore, *T. harzianum* isolate (T9) significantly reduced the root rot index induced by *F. solani*. *Aspergillus niger* improved the height of peach seedlings inoculated by *P. ultimum*. However, their combination uses were ineffective.

Abbreviations

A. candidus: *Aspergillus candidus*; *A. niger*: *Aspergillus niger*; ANOVA: Analysis of variance; *B. subtilis*: *Bacillus subtilis*; *F. solani*: *Fusarium solani*; *P. ultimum*: *Pythium ultimum*; *Ph. Citrophthora*: *Phytophthora citrophthora*; *T. harzianum*: *Trichoderma harzianum*; SPSS: Statistical Package for the Social Sciences software; SNK: Student–Newman–Keuls.

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Author contributions

NBM and SM designed the research and conducted surveys, sampling and analyses. SM wrote and NBM revised the manuscript. All authors read and approved the final manuscript.

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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.

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