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Research Article

Efficiency of secondary metabolites produced by *Trichoderma* spp. in the biological control of *Fusarium* wilt in chickpea

Dahou Moutassem[1, 2*], Lakhdar Belabid[1] and Yuva Bellik[2]

1. Faculty of Nature and Life Sciences, Mustapha Stamboli Mascara University (29000), Algeria.
2. Laboratory of Characterization and Valorization of Natural Resources (L. C. V. R) SNV-STU Faculty-Bordj Bou Arreridj University (34000)-Algeria.

Abstract: The present study was aimed to investigate the effects of secondary metabolites produced by five *Trichoderma* spp. on the control of *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (FOC) in chickpea. *In vitro* biocontrol potentialities of *Trichoderma* spp. against FOC was tested. *Trichoderma* secondary metabolites were extracted by solvent extraction methods and evaluated against FOC. *In vitro* tests showed very good inhibitory effects by all *Trichoderma* spp. against FOC along with an inhibitory rate up to 73.8% and 27.8%, for direct and indirect contacts, respectively. Additionally, *Trichoderma* spp. caused a significant decrease in *Fusarium* wilt disease severity, in particular, *T. polysporum* showing 64.2% of disease severity reduction. The tested secondary metabolites were also effective against FOC with a significant decrease of mycelial growth from 6% to 76.9%. Similarly, *in vivo* tests revealed that secondary metabolites were very active in reducing disease severity. It was found that *T. polysporum* was the most active with 56.9% of disease severity reduction. Chickpea resistance is mostly attributed to polyphenolic compounds. The studied *Trichoderma* spp. and their secondary metabolites could be used as potential and promising antifungal agents in preventing the occurrence of *Fusarium* wilt in chickpea.

Keywords: Biological control, *Trichoderma*, *Fusarium oxysporum*, secondary metabolites, Chickpea

Introduction

Chickpea, *Cicer arietinum* L., is one of the most important food legumes worldwide (Sharma *et al*., 1994). The chickpea cultivation around the world is economically important because of its high nutritional value richness in essential proteins (Al-Snafi, 2016), therapeutic effect (Jukanti *et al*., 2012) and agricultural interest in biological nitrogen fixation (Gaur and Sen, 1979). Unfortunately, this crop faces several biotic constraints, especially pathogenic fungi, affecting its yield and quality. Vascular wilt caused by *Fusarium oxysporum* f. sp *ciceris* (FOC) has been reported as the most destructive disease of chickpea cultivation in the world. The annual yield losses, caused by the disease were estimated at 10 to 15% (Jiménez-Díaz *et al*., 2015). However, this pathogen can completely destroy the crop under the favorable conditions for disease development (Trapero-Casas and Jim´enez-D´ıaz, 1985).

In general, the disease can be reduced by means of chemical, biological or physical disease control methods (Jiménez-Díaz *et al*., 2015). However, the intensive pesticide

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* **Corresponding author**, e-mail: moutassemdahou@gmail.com
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applications in the conventional production, mainly with fungicides can initiate many emerging problems, including environmental pollution, development of fungicide-resistant pathogens in addition to inducing variety of human and animal health problems (You *et al*., 2016). Recently, a great research interest has been paid to the alternative management measures, particularly the fungal biological control agents (BCAs) for the protection of worldwide crops. So far, the antagonistic fungi are mainly known by their potential ability to decrease inoculum density of pathogenic fungi (Dennis and Webster, 1971a), and among which fungi of the genus *Trichoderma*, as biocontrol agents (BCAs), are successfully used as biopesticides worldwide (Vinale *et al*., 2008). Furthermore, the tested *Trichoderma* formulations on biological management have proved satisfactory for control of vascular disease agents, eg, *Fusarium oxysporum* (Vinale *et al*., 2009). However, these types of formulations have certain disadvantages, such as limited shelf life, high dose efficacy, and present low stability in an adverse environment (Keswani *et al*., 2014). Since the secondary metabolites are a promising solution for these problems, the conventional formulations of bio-pesticides can be either improved or replaced, and thus the next-generation of secondary metabolites based formulations may be developed for management of phytopathogens (Keswani *et al*., 2014). In addition, many species of *Trichoderma* are commonly able to produce secondary metabolites with a good antibiotic activity (Vinale *et al*., 2008, 2009, 2012). The present work, therefore, aimed to provide further understanding on the biological control agents belonging to the genus of *Trichoderma* and their secondary metabolites, targeting the FOC. This biological control model would be based on analyses of these microorganisms, using i), *in vitro* and *in vivo* studies of the antagonistic effect of some *Trichoderma* spp. against FOC, ii), the extraction of secondary metabolites and *in vitro* as well as *in vivo* determinations of their antifungal activity against FOC.

Materials and Methods

Fungal strains

The biocontrol agents *Trichoderma harzianum, T. viride, T. polysporum, T. virens,* and *T. atroviride* were isolated from soil rhizosphere of chickpea. *Trichoderma* spp. were identified based on visual macroscopic and microscopic observations according to Gams and Bissett, (1998). Chickpea pathogen (*Fusarium oxysporum* f. sp. *ciceris*) (FOC) was obtained from the infected plant parts of chickpea collected in the region of Mascara, North-Western Algeria.

In vitro effect of *Trichoderma* spp. on FOC

Dual and opposite culture test

The dual culture test was performed in Petri dishes (9 mm in diameter) containing PDA medium as described by Dennis and Webster (1971b). Mycelial plugs (5 mm in diameter) of *Trichoderma* spp. and FOC taken from advancing edge of 7-days PDA culture were placed at equal distance from the periphery (2 cm). Inoculated plates were incubated at 25 ± 3 °C. Control without *Trichoderma* was used. The radial growth inhibition of FOC by *Trichoderma* spp. were determined and compared with the control. Four replications were achieved for each species.

Microscopic examination of the hyphal interactions in dual culture plates was performed. The method was done by taking small portion from the contact hyphal region between *Trichoderma* spp. and FOC on a glass slide and mounted in methylene blue (El-Debaiky, 2017). The contact regions between the antagonistic *Trichoderma* spp. and FOC were investigated using an optical microscope.

In another experiment, the five *Trichoderma* spp. were evaluated for their volatile inhibitory effect according to the techniques described by Dennis and Webster (1971a). In brief, 5mm discs of *Trichoderma* spp. from 7-days old cultures were transferred to the center of PDA Petri dishes, the top of each Petri dish was replaced with bottom of a PDA plate inoculated centrally with FOC, and then sealed with Parafilm for incubation at 25 ± 3 °C. The same setup without *Trichoderma* spp. was used as

control. Four replications were maintained for each species. The diameter of pathogen colony was measured 4, 6 and 8 days after incubation and the inhibition of mycelial growth were determined.

Extraction and evaluation of secondary metabolites against FOC

Isolation of secondary metabolites produced by *Trichoderma* spp. were carried out as previously described (Vinale *et al*., 2008). Briefly, two 7 mm diameter plugs of each *Trichoderma* strain were obtained from actively growing margins of PDA cultures and inoculated to 2 L conical flasks containing 250 mL of sterile potato dextrose broth (PDB). Then the stationary cultures were incubated for 31 days at 25 ± 3 °C, followed by vacuum filtration onto filter paper. The filtered culture broth (2 L) of *Trichoderma* spp. was after wards extracted exhaustively with ethyl acetate. The antifungal activity of the *Trichoderma* secondary metabolites was tested against FOC following the direct solubilization in PDA medium (Dubey *et al*., 2011). Secondary metabolites derived from five species of *Trichoderma* were tested at various concentrations (50, 100, 250, 500 and 1000ppm) prepared by DMSO (3%).The medium was poured into Petri dishes after flask shaking, and then pathogen plugs were centrally inoculated by making 5mm discs taken from 7-days old culture on the PDA plates. Control received the same quantity of DMSO (3%) mixed with PDA. Also, four replicate plates were tested. The pathogen growth was determined daily by measuring the colony diameter.

The percentage of mycelial growth inhibition in all above experiments was calculated by the formula:, $MGI(\%) = \frac{(C-T)}{C} \times 100$, where MGI: percent growth inhibition, C = growth in control and T = growth in treatment.

***In vivo* effect of *Trichoderma* spp. on *Fusarium* wilt severity**

The preparation of FOC inoculum was performed according to the technique of Nene and Haware (1980). A mixture containing sieved sand (90 g) and corn flour (10 g) moistened with distilled water (20 ml), according to the proportion of 9/1/2 (w/w/v), respectively, was prepared in plastic bags. The mixture was sterilized three times at 121 °C for 30 min and then inoculated with 25discs of 5 mm diameter taken from an 8-days FOC culture. The incubation was carried out for 21 days at 25 ± 3 °C. Agitation of the bags every day was carried out in order to allow a homogeneous colonization of the medium by FOC. The obtained inoculum was then incorporated into pots containing the sterilized culture substrate at the rate of 100 g of inoculum per 1 kg of substrate, which is composed of a mixture of sand, soil, and organic matter in the proportions of 1/1/1 (v/v/v) sterilized at 121 °C for 24 h.

Chickpea line (Guab 5) seeds were surface disinfected using sodium hypochlorite (1%) for 3 min and rinsed in sterile water. Coating of chickpea seeds with the five species of *Trichoderma* was carried out according to the method of Mohammad *et al*. (2011). The chickpea seeds were immersed in 10 ml of the *Trichoderma* spore suspension at the concentration of 5×10^8 spore/ml for 30 min and placed (10 seed/ dishes) in sterile Petri plates containing two sterile filter papers. Then Petri dishes containing seeds were incubated at 25 ± 3 °C, and the 8-day old plants coated with spore suspension of *Trichoderma* were carefully transferred into the soil inoculated with FOC.

Severity of *Fusarium* wilt disease was assessed at 2 day intervals, on a scale ranging from 0 to 4 according to the percentage of foliage with yellowing or necrosis, where 0 = 0%, 1 = 1 to 33%, 2 = 34 to 66%, 3 = 67 to 100%, and 4 = dead plant). Also, the evaluation was carried out according to the method of Traperos Cases and Jim´enez-D´ıaz (1985), since disease incidence (DI) was assessed by counting the number of plants showing symptoms. Hence, incidence symptoms (0 or 1) and severity data (ranged from 0 to 4) were used to calculate disease index intensity (*Dis*) using the equation $Dis = (I \times S)/4$. In addition, the AUDPC value was mainly used to estimate

the area under disease progress curve of each treatment:

$$AUDPC = \sum_{t+1}^{n} [(x_i + x_{i+1})/2](t_{i+1} - t_i)$$

In vivo effect of secondary metabolites produced by _Trichoderma_ spp.

To test the *in vivo* effects of *Trichoderma* extracts, 1000 ppm of secondary metabolites was prepared in 1 L of sterile distilled water. Chickpea line (Guab 5) seeds were surface disinfected for 3 min using sodium hypochlorite (1%), rinsed in sterile water for 3 min and then germinated (10 seed/ disc) for 8 days in Petri plates containing 2 sterilized filter paper. Petri plates containing seeds were incubated under laboratory conditions (25 ± 3 °C). Germinated seed were transferred to peat substrate, sterilised at 121 °C/ 30 min. Two-week-old roots of chickpea plants grown on peat were immersed in each extract to ensure better application and carefully transferred into plastic pots of 20 cm diameter and 30 cm height) filled with the substrate composed of the mixture soil-sand-compost (1v: 1v: 1v), sterilized in autoclave for 40 min at 120 °C and then sprayed again by the different *Trichoderma* extracts. Five seedlings were planted per pot. Two control treatments were used: (*i*) negative control pots uninoculated with FOC and treated with sterile distilled water; (*ii*) positive control pots inoculated with FOC and treated with aqueous DMSO (3%).

Determination of total polyphenol and flavonoid contents

Total polyphenol content was evaluated according to the Folin–Ciocalteu procedure as described by Ardestani and Yazdanparast (2007). Briefly, a host plant extract was prepared by adding10 ml of 80% methanol to 250 mg of the dried-milled chickpea under a slow shaking. The obtained solution was filtered and 0.5 ml of the methanolic extract was mixed with 2.5 ml of Folin-Ciocalteu's reagent (1:10 diluted with distilled water) and 2 mL of 7.5% Na_2CO_3 solution in a test tube under shaking. Thereafter, the mixture was incubated at 30 °C in a hot water bath for 90 min, and the absorbance of the mixture was measured at 765 nm using a spectrophotometer. Total polyphenol content was given as milligram gallic acid equivalents/g dried extract, using the blank sample composed of water and reagents. All the measurements were replicated four times.

Total flavonoid content was evaluated according to the aluminum chloride colorimetric method (Chua *et al*., 2011). A volume of 1 ml of the methanolic extract was added to 2 ml of the methanolic solution containing 2% $AlCl_3$. The absorption of pink color of mixture was measured after 15 min at 430 nm. Total flavonoid content was given in milligram of quercetin equivalents (QE) per gram of extract.

Statistical Analysis

In the present study each experiment was performed four times and displayed as mean ± SE. The statistical analysis was performed by one and two-way analysis of variance (ANOVA). Comparisons between results of each treatment were compared with Tukey post hoc test ($p < 0.05$). Statistical tests were performed using software package STATISTICA 8.

Results

***In vitro* antagonistic activity of *Trichoderma* spp. against FOC**

Results of *in vitro* tests showed that *Trichoderma* spp. exhibited strong inhibitory effects on mycelial growth of FOC (Fig. 1). We noticed significant differences in terms of inhibitory activity among the tested plates (Fig. 2). After eight days, the highest inhibitory effect was obtained with *T. harzianum* and *T. polysporum* (83.47% and 83.05%, respectively).Whereas, *T. viride*, *T. atroviride* and *T. virens* showed an inhibition activity which ranged from 73.81% to 77.68%.

Microscopic observations of the contact zone between the two protagonists showed profound changes in the mycelium of FOC manifested by massive winding of *Trichoderma* mycelium over that of FOC, transformation in cords of the mycelial filaments, lysis of pathogen mycelia,

vacuolation and early aging by wall thickening and chlamydospore formation (Fig. 3).

The obtained results revealed an inhibitory effect of volatile substances on the mycelial growth of FOC as compared to control (Fig. 2). Here, FOC was found to be more sensitive to the volatile substances of *T. harzianum*, at 8 days of incubation, leading subsequently to maximum mycelial growth inhibition of FOC (54.79%). However, the lowest rate of mycelial growth inhibition was observed with *T. atroviride* (27.86%).

In vitro effects of *Trichoderma* spp. secondary metabolites

The *in vitro* antifungal activity of *Trichoderma* spp. secondary metabolite was investigated against FOC (Fig. 4). We noticed significant differences in antifungal activity for the tested concentrations of secondary metabolites ($p \leq 0.05$), in addition to the different degrees of antifungal activity of each species (Table 1). In this study, the incorporation method showed a significant reduction of mycelial growth of FOC and the rate of reduction was gradually increased by increasing the concentration of extract. *T. polysporum* and *T. harzianum* were the most effective in inhibiting FOC mycelial growth for all tested concentrations. The level of mycelial growth inhibition by *T. polysporum* ranged from 45.8% to 76.94%. *Trichoderma virens* showed the lowest antifungal activity with percentages ranging from 6.08% to 33.94%.

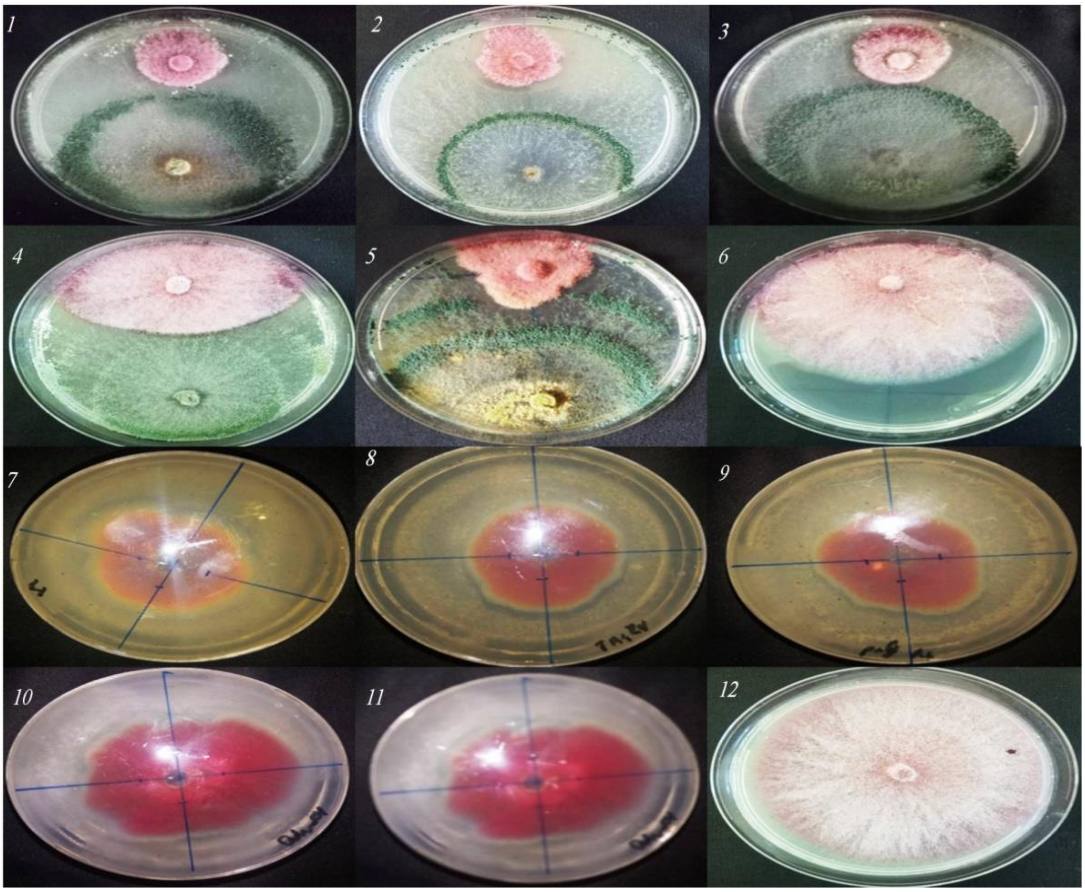

Figure 1 *In vitro* antagonistic effect of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *ciceris* in both dual culture (1 to 6) (1- *Trichoderma viride*, 2-*T. harzianum*, 3- *T. polysporum*, 4- *T. virens*, 5- *T. atroviride*, 6- Control) and opposite cultures (7 to 12) (7- *Tricoderma viride*, 8-*T. harzianum*, 9- *T. polysporum*, 10- *T. virens*, 11- *T. atroviride*, 12- Control).

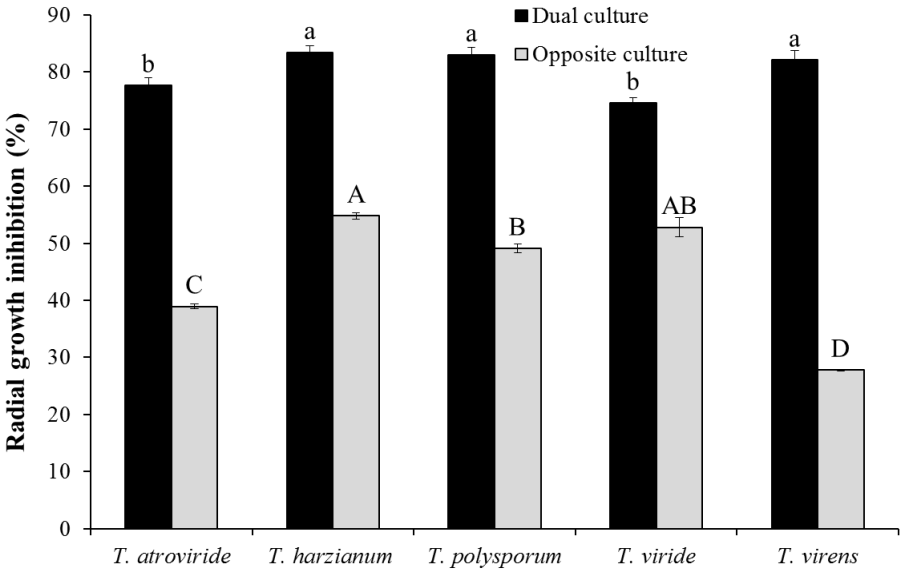

Figure 2 Inhibition of *Fusarium oxysporum* f. sp. *ciceris* mycelial growth in dual and opposite culture. Values represent the mean of four replicates ± SE. Data marked by different letters in a bar indicate significant difference (Tukey's test, $P < 0.05$).

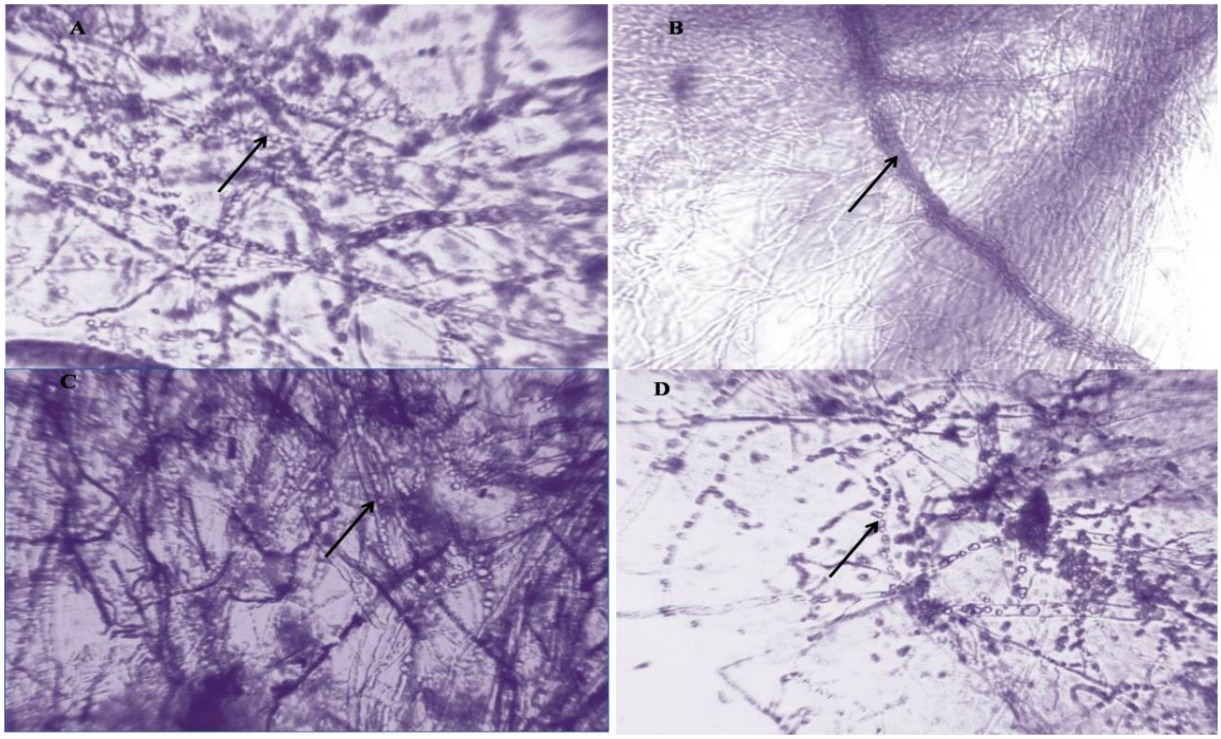

Figure 3 Morphological abnormalities caused by *Trichoderma* spp. on mycelia of *Fusarium oxysporum* f. sp. *ciceris* (8 days treatments). A) Massive winding of *Trichoderma* mycelium over that of *Fusarium oxysporum* f. sp. *ciceris*, B) transformation in cords of the mycelial filaments, C) vacuolation, D) lysis of pathogen mycelia.

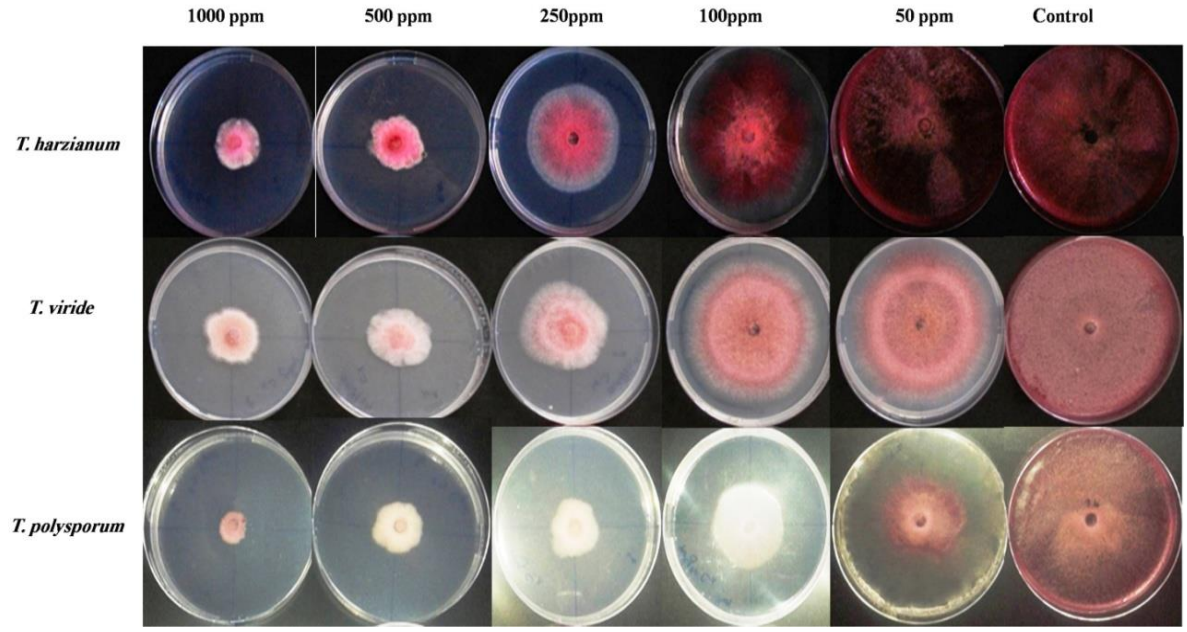

Figure 4 Effects of different concentrations of *Trichoderma* spp. secondary metabolites on mycelial growth of *Fusarium oxysporum* f. sp. *ciceris* after 8 days of incubation at 25 ± 3 °C. Control was treated with DMSO (3%).

Table 1 Effect of secondary metabolites isolated from culture filtrates of various species of *Trichoderma* on mycelial growth of *Fusarium oxysporum* f. sp. *ciceris*.

Species	Mycelial growth inhibition (Mean ± SE) (%)				
	1000 ppm	500 ppm	250 ppm	100 ppm	50 ppm
T. harzianum	71.69 ± 0.35^{ab}	60.17 ± 2.07^{bcde}	52.99 ± 1.06^{def}	17.94 ± 2.79^{klmn}	8.98 ± 1.72^{mno}
T. polysporum	76.94 ± 1.09^{a}	68.54 ± 2.08^{abc}	61.33 ± 4.46^{bcd}	53.29 ± 1.79^{def}	45.8 ± 2.47^{efgh}
T. viride	53.88 ± 0.92^{cdef}	48.20 ± 1.22^{defg}	40.75 ± 6.16^{fghi}	21.53 ± 2.90^{jklm}	8.38 ± 2.95^{mno}
T. atroviride	52.83 ± 3.21^{def}	32.29 ± 1.87^{hijk}	$13.39 \pm 2.13l^{mno}$	4.70 ± 0.34^{no}	00 ± 00^{o}
T. virens	33.94 ± 7.59^{ghij}	26.79 ± 2.53^{ijkl}	$14.75 \pm 1.6l^{mno}$	6.08 ± 0.61^{no}	00 ± 00^{o}

Values represent the mean of four replicates ± SE (standard errors). Data marked by different letters in a column indicate significant difference (Tukey's test, $P < 0.05$).

In vivo antagonistic activity of *Trichoderma* spp. and their secondary metabolites on *Fusarium* wilt severity

The effect of the different *Trichoderma* spp. on severity of *Fusarium* wilt, achieved after 40 days of treatment, are displayed in Figures 5-6. Chickpea plants treated with different species of *Trichoderma* exhibited AUDPC values ranging from 730.63 to 1468.88, whereas the untreated control showed an AUDPC value of 2045, which is significantly higher than all treated plants. Importantly, the *in vivo* study showed that *T. polysporum*, *T. harzianum* and *T. viride* were the most effective against wilt disease in which the AUDPC values were significantly reduced ($P \leq 0.05$). The mean values of the AUDPC were 730.63, 835.25 and 960 representing 64.27%, 59.15% and 53.05% of disease severity reduction, respectively. Treatment of chickpea plants with *T. virens* and *T. atroviride* showed the lowest effect against *Fusarium* wilt severity.

Plants treated with secondary metabolites of different *Trichoderma* spp. showed AUDPC values ranging from 880.5 to 1329, which related

to 56.94% and 35.01% of reduction in disease severity, respectively, compared to the AUDPC of positive control (2045) (Fig. 6). *Trichoderma polysporum* and *T. viride* secondary metabolites showed AUDPC values of 880.5 and 942.75 which correspond to 35.01% and 47.33% of disease reduction, respectively.

Determination of total polyphenolic and flavonoid content in plants treated with *Trichoderma* spp.

Plants treated with all *Trichoderma* spp. induced production of polyphenol compounds in chickpea plants with rate ranging from 25.57 to 34.77 mg/g, while positive and negative controls exhibited average polyphenol levels of 9.25 and 23.29 mg/g, respectively. The highest level of polyphenols was recorded in the plants treated with *T. harzianum (*34.77 mg/g) (Fig. 7A). In terms of flavonoids, plants treated with various *Trichoderma* spp. also showed high values of flavonoid contents ranging from 7.93 to 13.22 mg/g, which are significantly higher than those of the positive and negative controls (1.57 and 5.45 mg/g, respectively). Here too, the highest levels of flavonoids were recorded in plants treated with *T. harzianum* (13.22 mg/g) (Fig. 7B).

To explore the potential effect of secondary metabolites of *Trichoderma* spp. in inducing plant defense system, polyphenolic and flavonoid contents were measured in chickpea seedling treated with different secondary metabolites of *Trichoderma* spp. (Fig. 7). Marked decreases in polyphenol and flavonoid contents of seedling treated with secondary metabolites were noticed. As shown in figure 7A, plants treated with secondary metabolites of *T. polysporum* and *T. harzianum* showed high polyphenol levels (36.2 and 27.8 mg/g, respectively). Lower values were recorded for *T. atroviride* 18.7 mg/g. Flavonoid contents were found to vary within plants treated with secondary metabolites of *Trichoderma* spp. (Fig. 7B). The high values were recorded in plants treated with secondary metabolites of *T. viride* (9.09 mg/g), whereas, the low values were found in plants treated with *T. harzianum* (7.48 mg/g).

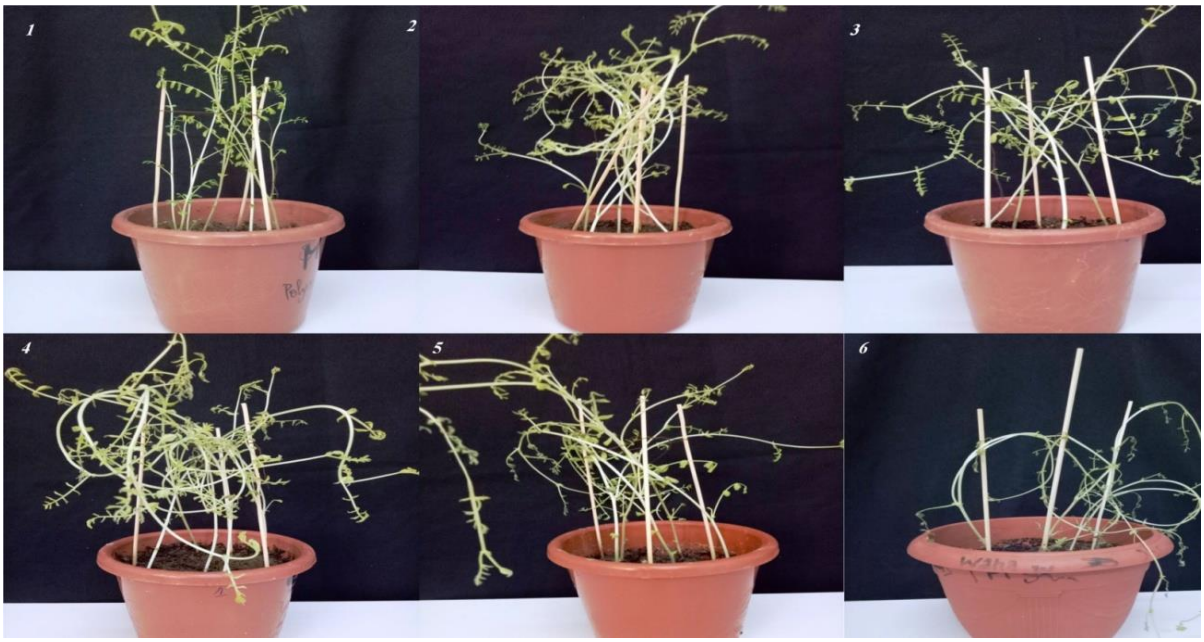

Figure 5 Effects of *Trichoderma* spp. (1- *Trichoderma harzianum*, 2- *T. polysporum*, 3- *T. atroviride*, 4- *T. viride*, 5- *T. virens*, 6- Control) on the reduction of disease severity caused by FOC.

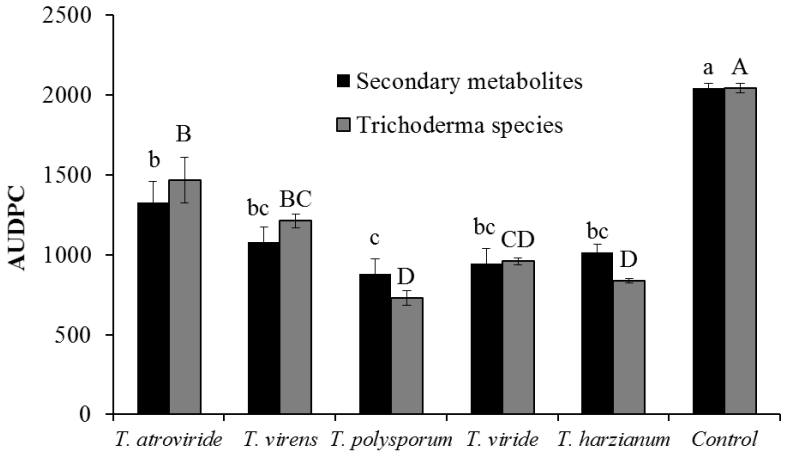

Figure 6 Effects of *Trichoderma* spp. and their secondary metabolites on the reduction of *Fusarium* wilt disease severity. Values represent the mean of four replicates ± SE. Data marked by different letters in a bar indicate significant difference (Tukey's test, $P < 0.05$).

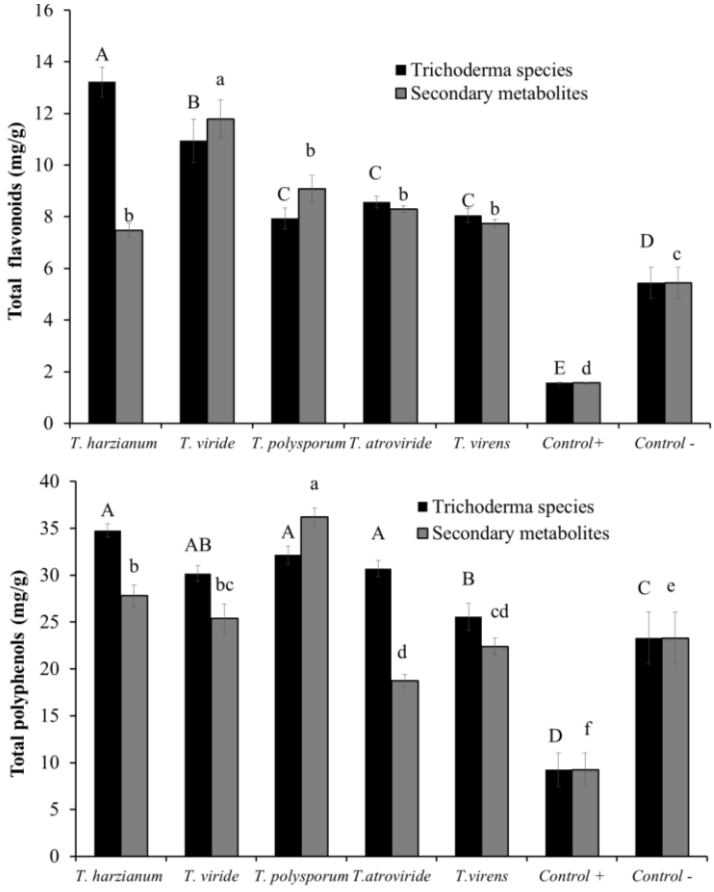

Figure 7 Total polyphenol and flavonoid contents in chickpea seedlings treated with different species of *Trichoderma* spp. and their secondary metabolites. Values represent the mean of four replicates ± SE. Data marked by different letters in a bar indicate significant difference (Tukey's test, $P < 0.05$).

Discussion

The main objective of this study was to investigate biological control of chickpea against FOC through application of different species of *Trichoderma* spp. Our results demonstrated the potential effect of *Trichoderma* spp. and their secondary metabolites in decreasing the severity of wilt disease of chickpea. Several studies have demonstrated the successful use of *Trichoderma* spp. as biological control agents against a wide range of economically important soil borne phytopathogens, particularly *Sclerotinia sclerotiorum* (Zhang *et al*., 2016), *Verticillium dahliae*, *Penicillium verrucosum*, *Aspergillus carbonarius* (Kumar *et al*., 2014) and *Fusarium oxysporum* f. sp *ciceris* (Dubey *et al*., 2007).

In this work, five species of *Trichoderma* showed an important inhibitory zone of mycelial growth of FOC at about 73.81% to 83.47% in dual culture experiments. Microscopic observations of the contact zone between *Trichoderma* spp. and FOC showed a marked alteration in the mycelium of FOC, evidenced by an important lysis, transformation in cords of the mycelial filaments and a coil of the *Trichoderma* mycelium on FOC. These results are in agreement with previous studies (Saravanakumar *et al*., 2016; Toghueo *et al*., 2016; El-Debaiky, 2017). Our results corroborate those of Dubey *et al*. (2007) who reported that *T. viride*, *T. harzianum* and *T. virens* inhibited FOC mycelial growth from 50 to 60% in dual culture. Moreover, Zhang *et al*. (2016) reported that *T. harzianum* inhibited mycelial growth of *S. sclerotiorum* with an effectiveness of 56.3% in dual culture tests. Nonetheless, *T. atroviride* and *T. harzianum* caused over 28.8% inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *Phaseoli* in dual culture (Otadoh *et al*., 2011).

The result of the present study revealed that five *Trichoderma* spp. could significantly inhibit mycelial growth of FOC in opposite culture. Thus, we could also anticipate that the antagonistic *Trichoderma* inhibited the mycelial growth of FOC through production of nonvolatile substances. These are in agreement with those obtained by Dubey *et al*. (2007), who reported that *T. viride*, *T. harzianum* and *T. virens* showing a highest ability to inhibit mycelial growth of FOC from 50 to 60% in indirect tests. The volatile metabolites from various *Trichoderma* spp. were effective in inhibiting *S. sclerotiorum*, *F. solani* and *R. solani* growth with inhibitory zone ranging from 33 to 71% (Qualhato *et al*., 2013).

The antagonistic activity of *Trichoderma* as biocontrol agent against various plant diseases can be achieved by a number of bioactive compounds including secondary metabolites (Zeilinger *et al*., 2016; Li *et al*., 2018). In the present study, *Trichoderma* secondary metabolites showed a significant reduction of mycelial growth of FOC. It was found that the rate of reduction gradually increased by increasing the concentration of secondary metabolites. The observed variation in fungicidal activity among the five *Trichoderma* spp. could be attributed to the different types of chemical compounds produced in the species. As reported, *Trichoderma* spp. secreted various volatile and non-volatile compounds like, alkylpyrones, peptaibols, polyketides, sesquiterpenes, siderophores and terpenoids which are crucial factors for mycoparasitism and antibiosis by *Trichoderma* spp. (Zeilinger *et al*., 2016). These substances involve a very large variety of action mainly referred to a function of their chemical composition. In this regard, Vinale *et al*. (2013) reported that Harzianic acid is a *T. harzianum* secondary metabolite having antifungal activities. Also, *T. polysporum* produces common families of secondary metabolites, such as trichopolyns, alamethicins, hypelcins, trichosporins, trichocellius, trichokindins and aibellin, possessing variety of biological activities derived from their membrane modifying properties, such as the formation of voltage-gated ion channels, hemolysis, uncoupling of oxidative phosphorilation (New *et al*., 1996; Iida *et al*., 1999). Obtained results corroborate those of Dubey *et al*. (2011) who demonstrated that *T. viride*, *T. virens* and *T.*

harzianum secondary metabolites exhibited pronounced antifungal activity against FOC ranging from 47.8% to 78% at 75 ppm. Similar effect was recorded by Toghueo *et al*. (2016) when investigating the antagonistic effects of secondary metabolites secreted by *T. atroviridae* at different concentrations showing a high mycelial growth inhibition of *F. solani*. Moreover, Bae *et al*. (2016) reported significant inhibitory effects of the metabolites of *T. atroviride* and *T. virens* against *Phytophthora* isolates. It is worth noting that in this study, only the concentrations of 3, 50 and 100 µl/ml of bioactive extracts of *Trichoderma* significantly increased fungicidal effect against FOC.

The results from this study demonstrated that *Trichoderma* spp. exhibited antifungal activity *in vivo* and reduced severity of *Fusarium* wilt of chickpea. The efficacy of *Trichoderma* spp in biocontrol of *Fusarium* wilt could be attributed to the reduction of soil fungal populations and induction of host resistance, thereby confirming the antifungal activity of *Trichoderma* spp. *in vivo*. Indeed, the *Trichoderma* biocontrol agents present numerous advantages to their linked host plants which are useful in combating biotic stresses. A wide range of biologically active metabolites are deployed by *Trichoderma* spp. for pathogen exclusion such as antibiosis (Sharma *et al*., 2017). It is assumed that *Trichoderma* spp. induced the production of secondary metabolites causing direct antimicrobial effect, or indirect defense by stimulating host plant defense (Vinale *et al*., 2012). Therefore, the results of the experiments indicated *Trichoderma* secondary metabolites are directly implicated in the establishment of plant defense genes, as previously reported for peptaibols (Viterbo *et al*., 2007). In particular, peptaibols induced an over-expression of defense related genes implicated in the systemic responses of plant (Vinale *et al*., 2008).

One of the main goals of this work was to examine the potential involvement of secondary metabolites in the induction of systemic resistance through *Trichoderma*-plant interaction. Biochemical analysis revealed a strong accumulation of polyphenols and flavonoids in the plant treated with various *Trichoderma* spp. and their secondary metabolites. This is likely due to the positive effect of *Trichoderma* spp. in reducing the severity of chickpea wilt disease. Interestingly, polyphenol compounds play a major role owed to their accumulation, basically known as important compounds in resistant plants and may be involved in the crosslinking, suberification and lignifications aiming in particular to limit the action of compressive forces and that of parasite hydrolases (Clérivet *et al*., 1996). As previously reported, phenolic and flavonoid compounds were identified to be involved in cell wall lignifications and in the decrease of *Fusarium* wilt severity by limiting pathogen penetration (Jin *et al*., 2012). Mona *et al*. (2017) also reported that *T. harzianum* increased the rate of phenol and flavonoid contents in tomato seedling. A very recent study showed accumulation of 25 abiotic and biotic stress-responsive metabolites including flavonol and flavonoid compounds in seedling of onion treated with *T. harzianum* (Abdelrahman *et al*., 2018). Additionally, El-Sharkawy *et al*. (2018) reported that application of *Trichoderma* spp. significantly reduced disease severity by inducing peroxidase and polyphenol oxidase enzymes, and increasing the total phenol content in host plant. The use of *T. polysporum* exhibited the highest effectiveness in biocontrol of melon wilt by about 44.85% (Gava and Pinto, 2016). Sunpapao *et al*. (2018) studied three species of *Trichoderma* and recorded more than 66.21% inhibition in disease severity in leaf spot caused by *Curvularia oryzae* in oil palm seedlings. In terms of disease resistance induction, Pascale *et al*. (2017) investigated the effect of two *Trichoderma* strains and their secondary metabolites against powdery mildew caused by *Uncinula necator;* they found that *T. harzianum* and *T. atroviride* increased polyphenols rate in grapes.

Overall, this study provided solid evidence that *Trichoderma* spp. are the most potential biocontrol agents against FOC. The use of some

Trichoderma secondary metabolites reduced significantly disease severity and enhanced systemic resistance. This could have a significant beneficial impact on the management of diseases in crop plants.

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References

Abdelrahman, M., Abdel-Motaal, F., El-Sayed, M., Jogaiah, S., Shigyo, M., Ito, S. and Tran, L. S. P. 2016. Dissection of *Trichoderma longibrachiatum* -induced defense in onion (*Allium cepa* L.) against *Fusarium oxysporum* f. sp. *cepa* by target metabolite profiling. Plant Science, 246: 128-138.

Al-Snafi A. E., 2016. The medical Importance of *Cicer arietinum* - A review. IOSR Journal of Pharmacy, 6 (3): 29-40.

Ardestani, A. and Yazdanparast, R. 2007. Antioxidant and free radical scavenging potential of *Achillea santolina* extracts. Food Chemistry, 104 (1): 21-29.

Bae, S. J., Mohanta, T. K., Chung, J. Y., Ryu, M., Park, G., Shim, S. and Bae, H. 2016. *Trichoderma* metabolites as biological control agents against *Phytophthora* pathogens. Biological Control, 92: 128-138.

Chua, L. S., Latiff, N. A., Lee, S. Y., Lee, C. T., Sarmidi, M. R. and Aziz, R. A. 2011. Flavonoids and phenolic acids from *Labisia pumila* (Kacip Fatimah). Food Chemistry, 127 (3): 1186-1192.

Clérivet, A., Alami, I., Breton, F., Garcia, D. and Sanier, C. 1996. Les composés phénoliques et la résistance des plantes aux agents pathogènes. Acta Botanica Gallica, 143 (6): 531-538.

Dennis, L. and Webster. J. 1971. Antagonisme properties of species-groups of *Trichoderma*. III. Hyphal interaction. Transactions of the British Mycological Society, 57: 363-369.

Dennis, L. and Webster. J. 1971. Antagonistic properties of species-groups of *Trichoderma* I. production of non-volatile antibiotics. Transactions of the British Mycological Society, 57 (I): 25-39.

Dubey, S. C., Suresh, M., and Singh, B. 2007. Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. Biological Control, 40 (1): 118-127.

Dubey, S. C., Tripathi, A., Dureja, P. and Grover, A. 2011. Characterization of secondary metabolites and enzymes produced by *Trichoderma* species and their efficacy against plant pathogenic fungi. Indian Journal of Agricultural Sciences, 81 (5): 455-61.

El-Debaiky, S. A. 2017. Antagonistic studies and hyphal interactions of the new antagonist Aspergillus piperis against some phytopathogenic fungi in vitro in comparison with *Trichoderma harzianum*. Microbial Pathogenesis, 113: 135-143.

El-Sharkawy, H. H. A., Rashad, Y. M. and Ibrahim, S. A. 2018. Biocontrol of stem rust disease of wheat using arbuscular mycorrhizal fungi and *Trichoderma* spp. Physiological and Molecular Plant Pathology, 103: 84-91.

Gaur, Y. D. and Sen, A. N. 1979. Cross inoculation group specificity in *Cicer*-Rhizobium symbiosis. New Phytologist, 83 (3): 745-754.

Gava, C. A. T. and Pinto, J. M. 2016. Biocontrol of melon wilt caused by *Fusarium oxysporum* Schlect f. sp. *melonis* using seed treatment with *Trichoderma* spp. and liquid compost. Biological Control, 97: 13-20.

Iida, A., Mihara, T., Fujita, T. and Takaishi, Y. 1999. Peptidic immunosuppressants from the fungus *Trichoderma polysporum*. Bioorganic and Medicinal Chemistry Letters, 9 (24): 3393-3396.

Jiménez-Díaz, R. M., Castillo, P., Jiménez-Gasco, M. del M., Landa, B. B. and Navas-Cortés, J. A. 2015. *Fusarium* wilt of chickpeas: Biology, ecology and management. Crop Protection, 73: 16-27.

Jin, P., Wang, S. Y., Gao, H., Chen, H., Zheng, Y. and Wang, C. Y. 2012. Effect of cultural system and essential oil treatment on antioxidant capacity in raspberries. Food Chemistry, 132 (1): 399-405.

Jukanti, A. K., Gaur, P. M., Gowda, C. L. L. and Chibbar, R. N. 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. British Journal of Nutrition, 108 (1): 11-26.

Keswani, C., Mishra, S., Sarma, B. K., Singh, S. P. and Singh, H. B. 2013. Unraveling the efficient applications of secondary metabolites of various *Trichoderma* spp. Applied Microbiology and Biotechnology, 98 (2): 533-544.

Kumar, S., Thakur, M. and Rani, A. 2014. *Trichoderma*: Mass production, formulation, quality control, delivery and its scope in commercialization in India for the management of plant diseases. African Journal of Agricultural Research, 9 (53): 3838-3852.

Li, Y. T., Hwang, S. G., Huang, Y. M. and Huang, C. H. 2018. Effects of *Trichoderma asperellum* on nutrient uptake and *Fusarium* wilt of tomato. Crop Protection, 110: 275-282.

Mohammad, A., Hadi, G. and Masoud, A. 2011. Evaluation of different combinations of *Trichoderma* species for controlling *Fusarium* rot of lentil. African Journal of Biotechnology, 10 (14): 2653-2658.

Mona, S. A., Hashem, A., Abd_Allah, E. F., Alqarawi, A. A., Soliman, D. W. K., Wirth, S. and Egamberdieva, D. 2017. Increased resistance of drought by *Trichodermaharzianum* fungal treatment correlates with increased secondary metabolites and proline content. Journal of Integrative Agriculture, 16 (8): 1751-1757.

Nene, Y. L. and Haware, M. P. 1980. Screening chickpea for resistance to wilt. Plant Disease, 64 (4): 379.

New, A. P., Eckers, C., Haskins, N. J., Neville, W. A., Elson, S., Hueso-Rodrfguez, J. A. and Rivera-Sagredo, A. 1996. Analytical Structures of Polysporins A-D, Four New Peptaibols Isolated from *Trichoderma polysporum*. Tetrahedron Letters, 37 (17): 3039-3042.

Otadoh, J. A., Okoth S. A., Ochanda, J. and Kahindi, J. P. 2011. Assessment of *Trichoderma* isolates for virulence efficacy on *Fusarium oxysporum* f. sp. *phaseoli*. Tropical and Subtropical Agroecosystems, 13: 99-107.

Pascale, A., Vinale, F., Manganiello, G., Nigro, M., Lanzuise, S., Ruocco, M. and Lorito, M. 2017. *Trichoderma* and its secondary metabolites improve yield and quality of grapes. Crop Protection, 92: 176-181.

Qualhato, T. F., Lopes, F. A. C., Steindorff, A. S., Brandão, R. S., Jesuino, R. S. A. and Ulhoa, C. J. 2013. Mycoparasitism studies of *Trichoderma* species against three phytopathogenic fungi: evaluation of antagonism and hydrolytic enzyme production. Biotechnology Letters, 35 (9): 1461-1468.

Saravanakumar, K., Yu, C., Dou, K., Wang, M., Li, Y. and Chen, J. 2016. Synergistic effect of *Trichoderma*-derived antifungal metabolites and cell wall degrading enzymes on enhanced biocontrol of *Fusarium oxysporum* f. sp. *cucumerinum*. Biological Control, 94: 37-46.

Sharma, S. B., Sikora, R. A., Greco, N., Di Vito, M. and Caubel, G. 1994. Screening techniques and sources of resistance to nematodes in cool season food legumes. Euphytica, 73: 59-66.

Sharma, V., Salwan, R. and Sharma, P. N. 2017. The comparative mechanistic aspects of *Trichoderma* and Probiotics: Scope for future research. Physiological and Molecular Plant Pathology, 100: 84-96.

Sunpapao, A., Chairin, T. and Ito, S. 2018. The biocontrol by *Streptomyces* and *Trichoderma* of leaf spot disease caused by *Curvularia oryzae* in oil palm seedlings. Biological Control, 123: 36-42.

Toghueo, R. M. K., Eke, P., Zabalgogeazcoa, Í., de Aldana, B. R. V., Nana, L. W. and Boyom, F. F. 2016. Biocontrol and growth enhancement potential of two endophytic *Trichoderma* spp. from *Terminalia catappa* against the causative agent of Common Bean Root Rot (*Fusarium solani*). Biological Control, 96: 8-20.

Trapero-Casas, A. and Jiminez-Diaz, R. M. 1985. Fungal wilt and root rot diseases of chickpea in Southern Spain. Phytopathology, 57: 1146-1151.

Vinale, F., Ghisalberti, E. L., Sivasithamparam, K., Marra, R., Ritieni, A., Ferracane, R. and Lorito, M. 2009. Factors affecting the production of *Trichoderma harzianum* secondary metabolites during the interaction with different plant pathogens.Letters in Applied Microbiology. doi:10.1111/j.1472-765x.2009.02599.x.

Vinale, F., Nigro, M., Sivasithamparam, K., Flematti, G., Ghisalberti, E. L., Ruocco, M. and Lorito, M. 2013. Harzianic acid: a novel siderophore from *Trichoderma harzianum*. FEMS Microbiology Letters, n/a–n/a. doi:10.1111/1574-6968.12231.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Barbetti, M. J., Li, H., Woo S. L. and Lorito, M. 2008. A novel role for *Trichoderma* secondary metabolites in the interactions with plants. Physiological and Molecular Plant Pathology, 72 (1-3): 80-86.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Ruocco, M, Woo, S. and Lorito, M., 2012. *Trichoderma* Secondary Metabolites that Affect Plant Metabolism. Natural Product Communications, 7 (11): 2012.

Viterbo, A., Wiest, A., Brotman, Y., Chet, I. and Kenerley, C. 2007. The 18mer peptaibols from *Trichoderma virens* elicit plant defence responses. Mol Plant Pathol, 8: 737-46.

You, J., Zhang, J., Wu, M., Yang, L., Chen, W. and Li, G. 2016. Multiple criteria-based screening of *Trichoderma* isolates for biological control of *Botrytis cinerea* on tomato. Biological Control, 101: 31-38.

Zeilinger, S., Gruber, S., Bansal, R. and Mukherjee, P. K. 2016. Secondary metabolism in *Trichoderma*-Chemistry meets genomics. Fungal Biology Reviews, 30 (2): 74-90.

Zhang, F., Ge, H., Zhang, F., Guo, N., Wang, Y., Chen, L., Ji, X. and Li, C. 2016. Biocontrol potential of *Trichoderma harzianum* isolates T-aloe against *Sclerotinia sclerotiorum* in soybean. Plant Physiology and Biochemistry, 100: 64-74.

کارایی متابولیت‌های ثانویه تولید شده توسط *Trichoderma* spp. در کنترل بیولوژیکی پژمردگی فوزاریومی نخود

داهو موتسم [۱،۲*]، لخدر بلابید[۱] و یووا بلیک[۲]

۱- دانشکده علوم طبیعی و زیستی، دانشگاه مصطفی استامبولی ماسکارا (۲۹۰۰۰)، الجزایر.
۲- آزمایشگاه تعیین مشخصات و اعتبارسنجی منابع طبیعی (ال. سی. وی. آر) دانشکده اس ان وی- اس تی یو-دانشگاه بورج بو اریج (۳۴۰۰۰)-الجزایر.
پست الکترونیکی نویسنده مسئول مکاتبه: moutassemdahou@gmail.com
دریافت: ۱۸ شهریور ۱۳۹۷؛ پذیرش: ۱۶ آذر ۱۳۹۸

چکیده: تحقیق حاضر به‌منظور ارزیابی اثر متابولیت‌های ثانویه تولید شده توسط پنج گونه *Trichoderma* spp. بر کنترل پژمردگی فوزاریومی ناشی از *Fusarium oxysporum* f. sp. *ciceris* (*FOC*) در نخود انجام شد. توانایی بیوکنترلی گونه‌های *Trichoderma* spp. علیه *FOC* در شرایط درون شیشه‌ای آزمایش شد. متابولیت‌های ثانویه *تریکودرما* با روش استخراج به‌کمک حلال استخراج شد و علیه *FOC* مورد ارزیابی قرار گرفت. آزمایش‌های درون شیشه‌ای اثرات بازدارندگی خیلی خوبی را در تمام گونه‌های *Trichoderma* spp. علیه *FOC* نشان داد به‌طوری‌که نرخ بازدارندگی در تماس مستقیم و غیرمستقیم به‌ترتیب تا ۷۳/۸ و ۲۷/۸ درصد بود. به‌علاوه، گونه‌های *Trichoderma* spp. موجب کاهش قابل‌توجهی در شدت بیماری پژمردگی فوزاریومی شدند. به‌طور ویژه گونه *T. polysporum* به‌میزان ۶۴/۲ درصد شدت بیماری را کاهش داد. هم‌چنین متابولیت‌های ثانویه مورد آزمایش موجب کاهش قابل توجه رشد میسلیومی *FOC* از شش تا ۷۶/۹ درصد شدند. به‌همین ترتیب آزمایش‌های گلخانه‌ای نشان داد که متابولیت‌های ثانویه در کاهش شدت بیماری بسیار فعال بودند. گونه *T. polysporum* بیش‌ترین فعالیت را در کاهش شدت بیماری با ۵۶/۹ درصد کاهش نشان داد. مقاومت نخود غالباً به ترکیبات پلی‌فنولی نسبت داده می‌شود. گونه‌های *Trichoderma* spp. مطالعه شده و متابولیت‌های ثانویه آنها می‌توانند به‌عنوان عوامل ضدقارچی بالقوه و امیدبخش در جلوگیری از وقوع پژمردگی فوزاریومی نخود باشند.

واژگان کلیدی: کنترل بیولوژیکی، تریکودرما، *Fusarium oxysporum*، متابولیت‌های ثانویه، نخود