Biocontrol of *Verticillium dahliae* by native *Trichoderma* strains isolated from Algeria

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Abstract

Trichoderma species are known as excellent biocontrol agents against soil-borne pathogens that cause considerable crop losses. Among them, *Verticillium dahliae* Kleb is the causative agent of Verticillium wilt. In this study, the ability of *Trichoderma* strains to antagonize *V. dahliae* was evaluated. *In vitro* dual culture tests were conducted in a completely randomized design using 53 *Trichoderma* isolates, collected from different locations in Algeria. Among these, only 15 of significantly inhibited the mycelial growth of 2 isolates of *V. dahliae*. Antagonist isolates of *Trichoderma* were characterized through phenotypic and molecular means. The isolates were divided into three groups: *T. harzianum*, *T. longibrachiatum* and *T. artoviride*. The results revealed that *T. artoviride* (T2) exhibited the highest antagonistic ability against *V. dahliae* (V1 and V2) with inhibition percentage of 74.41% and 69.50%, respectively. *T. harzianum* (T4) exhibited the lowest antagonism against V2 (41.07%). *Trichoderma* isolates showed a very high activity in both bioassay distance culture and antibiotic assay. The three *Trichoderma* strains (T2, T7 and T10) successfully controlled Verticillium wilt on tomato with disease reduction of 95.98%, 90.71% and 92.98%, respectively.

Keywords: Algeria, Antagonism, Biological control, *Solanum lycopersicum*, *Trichoderma* strains, Verticillium wilt.

Introduction

Verticillium wilt disease incited by the soil-borne *Vertcillium dahliae* caused several symptoms such as wilting, dieback, defoliation and death of susceptible plants. However, symptoms can differ considerably between hosts and *Verticillium* species (Fradin and Thomma, 2006; Weiland *et al.,* 2018). *V. dahliae* causes severe yield reductions in a variety of important annual crops worldwide (Uppal *et al.,* 2008; Yan *et al.,* 2018). It is a very difficult disease to control and it can survive in the soil for long periods of time in the form of resting structures such as microsclerotia, chlamydospores and dark mycelia (Zhang *et al.,* 2018; Hu *et al.,* 2019). The use of resistant cultivars is one of the most practical and cost-effective strategies for managing plant diseases (Bautista-Jalon *et al.,* 2021). Angelopoulou *et al.* (2014) showed that the control of Verticillium wilt has relied on soil fumigation; however, the use of the main soil fumigant, methyl bromide, has been banned in the European Union since 2010, creating a demand for novel crop protectants.

The use of biocontrol agents is a promising tool and alternative to chemical control (Javaid *et al.,* 2017, 2018). The biocontrol efficiency of soil-borne pathogens, and especially Verticillium species, depends mainly on the antagonist establishment and development in the rhizosphere and on its ability to colonize the subterranean plant tissues for avoiding infection (Jabnoun-Khiareddine *et al.,* 2009). It is estimated that 90% of all antagonistic fungi used in plant protection belong to the genus *Trichoderma* (Benitez *et al.,* 2004). The genus *Trichoderma* belongs to: the phylum Ascomycetes, class Sordariomycetes, order Hypocreales, family Hypocreaceae. The systematic and taxonomy of these fungi have evolved since 1794 when Persoon (1794) introduced the name *Trichoderma* (Błaszczyk *et al.,* 2014). It is one of the most ubiquitous genera worldwide. This genus has an excellent ability to adapt to diverse environments, even under poor nutritional conditions. It is a successful biocontrol agent of some soil-borne pathogens (Khan and Javaid, 2020). The ability of this fungus occupying rhizosphere of olive, tomato, cotton, and eggplant, and decreasing germination of microsclerotia of *V. dahliae* was reported by Uppal *et al.* (2008); Başay *et al.* (2011) and Jiménez-Díaz *et al.* (2012). A well-known biocontrol using antagonistic *Trichoderma* spp. is effective against *V. dahliae* (Mouria *et al.,* 2013; Carrero-Carron *et al.,* 2016). The objectives of this study were to investigate the antagonistic effects of endogenous *Trichoderma* species for the biocontrol of the tomato Verticillium wilt *in vitro* and *in vivo* trials.

Materials and methods

Isolation of *Trichoderma* spp.

The rhizosphere soil samples had been collected from nine locations in Algeria (Table 1). Rhizosphere soil samples of olive trees were collected during the period from 2017 to 2018 from different departments of agricultural fields in North-Western Algeria.

The fungal antagonists were isolated using the soil dilution plate method according to Zachow *et al*. (2009) on two different media: Potato dextrose agar (PDA) and a selective medium TSM for *Trichoderma*. One gram of dried soil samples was suspended in 9 mL sterile distilled water, agitated for 1 min, and allowed to settle for 1 h. The suspension was subsequently diluted to10^{-1} at 10^{-7}. The 0.1 mL soil dilutions of 10^{-3} to 10^{-7} were separately streak-plated on PDA and TSM medium, in triplicate. The plates were then incubated at 25 ºC for 48 h at 72 h. From the countable plates, the representative colonies with different morphological appearances were selected and re-streaked on a new plate containing the same medium, to obtain pure colonies. They were transferred and purified by single spore method on PDA at 25 ºC until fungi colonies become visible.

Morphological characterization of the isolates further established using slide culture. *Trichoderma* isolates were identified to species level based on morphological and microscopical features following the taxonomic key of Samuels *et al*. (2002, 2006, 2010), Watanabe (2005) and Bissett *et al*. (2015). For long-term storage, the *Trichoderma* strains was transferred to 20% glycerol and kept at –20 °C.

Molecular identification of antagonistic *Trichoderma* spp.

For DNA extraction, fungal isolates were cultivated on PDA at 25 ºC for 6 days. The total genomic DNA was extracted from each *Trichoderma* isolate following the protocol of the NucleoSpin® Food commercial kit from Macherey Nagel (Germany). An ITS region of the 5.8S rDNA gene was amplified by PCR with universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3′) and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (White *et al.,* 1990) in a Icycler Bioradtype thermocycler (Thermal Cycler) (Biorad, USA) using the following programme: initial denaturation for 2 min at 95 ºC; followed by 30 cycles of denaturation for 30 s at 95 ºC, primer annealing for 30 s at 55 ºC, and extension for 1min at 72 ºC; and a final extension period of 10 min at 72 ºC.

Each PCR product was electrophoresed on 1.5% agarose gels. Following the manufacturer's instruction, the PCR products were purified using Wizard® SV Gel kit and Promega's PCR Clean-Up System. Following the manufacturer's instruction, the PCR products were purified using ExoSAP-IT (USB, Ohio, and USA). The PCR amplicons corresponding to the ITS region of rDNA were then sequenced by Applied Biosystems Big Dye v3.1 kit. The sequences obtained were analyzed and cleaned using the CHROMAS PRO software then identified by BLAST Program (https://blast.ncbi.nlm.nih.gov /Blast.cgi). The sequences were deposited in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). All isolates were morphologically characterized, and the ITS sequences were used for phylogenetic analysis.

***In vitro* screening of *Trichoderma* isolates by dual culture assay**

All isolates of *Trichoderma* spp. were subjected to dual culture assay described by Dennis and Webster (1971a); Coskuntuna and Ozer (2008); a preliminary assay to screening mycoparasitisms potential through antagonistic activity against *V. dahliae* (V1 and V2). A 5 mm agar disc from fresh PDA cultures of 7-days-old *Trichoderma* and pathogen were taken and co-inoculated at 3 cm from each other on Petri dishes containing PDA. Plates without *Trichoderma* were used as control. All plates were incubated at 25 °C and mycoparasitism was determined by measuring the diameter of phytopathogenic fungi colonies after 6 days post-inoculation. The percentage growth inhibition was calculated in comparison with that of control (Bekkar *et al.,* 2016). Each test was replicated three times. After 6 days of incubation, the interface region was observed under light microscope, noting any morphological changes.

The growth inhibition (GI) of *V. dahliae* strains was measured using the formula as follows: GI = (C-T/C) ×100. Where; GI = percent inhibition; C = radial growth of pathogen (mm) alone (control); T = radial growth of pathogen (mm) in the presence of *Trichoderma* isolates (Royse and Ries, 1978).

***In vitro* screening of *Trichoderma* isolates by distance culture assay**

Trichoderma inhibition through production of the volatile antifungal substances was detected following the sealed plate method used by Dennis and Webster (1971b).

A Petri dish containing PDA with a 6 mm plug of the test fungus, was placed in the center of the plate, inverted and placed over the *Trichoderma* culture.

The two culture plates were placed face-to-face preventing any physical contact and sealed together with double layer of paraffin film to prevent the loss of volatiles produced and then they were incubated at 25±2 °C in dark for 10 days. As positive control PDA plate without *Trichoderma* culture was covered and sealed with PDA plate inoculated with *V. dahlia* (V1/V2). Each test was replicated three times.

The percentage inhibition was calculated according to the formula mentioned previously.

Antagonistic effects of non-volatile extracts of *Trichoderma*

For determine the effect of diffusible antifungal metabolites produced by *Trichoderma* spp. against *V. dahliae*, three agar discs (5 mm) obtained from 6-days-old of *Tichoderma* cultures were transferred into 250 mL Erlenmeyer vials containing 100 mL Potato dextrose broth (PDB) and incubated at 25 °C in a shaker (180 rpm) by alternating light and dark (12h/12h) for 15 days. Cultures were filtered twice, first by a sterilized Whatman No.1 filter paper to remove the mycelial mass and then were filtered through a 0.22 µm Millipore membrane. Cultures filtrates were incorporated at 50% (v/v) in the PDA medium, and then were poured into Petri dishes, in order to input a disc (5 mm) of *V. dahlia* in the center of each Petri dish then incubated at 25 °C in darkness. For the control, the culture filtrates were replaced by sterile distilled water. The percentage of inhibition was evaluated 10 days after incubation (Zhang *et al*., 2016; Idan *et al*., 2017).

Effect of *Trichoderma* isolates on tomato Verticillium wilt in pots

The efficiency of *Trichoderma* spp. against *V. dahliae in vivo* was evaluated on wilt severity and plant growth in comparison to the untreated controls.

Fungal pathogen and spore preparation

The two pathogenic fungal of *V. dahliae* (strain V1 MN325001 and V2) (Unpublished data), originated from Mostaganem and Mascara (Algeria), respectively were used in the present study. *V. dahliae* was maintained on potato dextrose agar (PDA) medium at 25±2 °C in darkness and there after stored at 4 °C.

The *V. dahliae* spore suspension was prepared by incubating *V. dahliae* in potato dextrose agar (PDA) at 25±2 °C for 10 days. The spore suspensions were prepared by removing the spores from the sporulating edges of the culture with a sterile rod and adding 5 mL of sterile distilled water for better spore separation. These suspensions were sieved through two paper filters, subsequently, were determined to 3×10^7 spores mL^{-1} using a Malassez cell (Zaim *et al.,* 2018; Carrero-Carron *et al.,* 2016).

After pathogenicity test; observing visual symptoms of Verticillium wilt and death of young seedling, V2 was selected as the most virulent isolate, and was used as a target pathogen in this experiment.

***Trichoderma* inoculum preparation**

Trichoderma spp. was grown in on PDA plates at 25 °C till the colonies became green. Then, conidial concentration was adjusted to10^7 conidia mL^{-1} with sterile distilled water (Carrero-Carron *et al.,* 2016).

Pot experiment

A pot experiment was conducted in 2018 and 2019 to evaluate the performance of *Trichoderma* as a bio-control agent against Verticillium wilt. Four treatments and three replicates were done. We sowed each of three susceptible tomato cultivar seeds in a 7 cm diameter sterilized surface (3% sodium hypochlorite) plastic pot, filled 2/3 full with a sterilized soil mixture and peat (V/V). Sterilization was done at 120 °C for 1 h three times in three days.

A *Verticillium* susceptible tomato cultivar Saint Pierre was used in this experiment. Tomato seeds were surface sterilized with 1% sodium hypochlorite for 3 min and rinsed three times with sterile distilled water, dried, and then germinated (Wang and Zhuang, 2019). After 2 weeks of culture, the experiment was replicated four times. Tomato seedling was inoculated at the 1st true leaf stage. Preparation 1included the non-inoculated control, plant roots were immersed in sterile distilled water without fungal inoculum (without *Trichoderma* and the pathogen). In preparation 2, plant roots were immersed only with conidia of *V. dahlia*V2 (with pathogen and no *Trichoderma*). Preparation 3consisted of plants pre-inoculated with *Trichoderma* (10^7 mL^{-1} conidial suspension) were immersed in *V. dahliae* V2 conidial suspension (with *Trichoderma* and pathogen). Preparation 4 containing separately immersed plants with effective *Trichoderma* ssp. but without pathogen conidia (*Trichoderma* + no pathogen). Pots were kept under observation of symptoms for 40 days, and compared after transplanting with the control pots.

Disease assessment

In order to evaluate the antagonistic activity of *Trichoderma* strains, *in vivo* trials were carried out against *V. dahliae*, on tomato plants. The three *Trichoderma* strains (T2, T7, and T10) selected for their different antagonistic behaviors *in vitro* were tested *in vivo*.

Five weeks after inoculation of the plants, percentage of disease incidence (DI) was scored according to the following formula:

Disease incidence (%) = (Number of infected plants⁄Total number of inoculated plants) × 100

Disease severity was assessed through the index of leaf damage. The symptoms were evaluated on a score ranging of 0 to 5 according to Mouria *et al.* (2014) as follows: **0** = leaves with healthy appearance; **1** = wilting or chlorosis of the cotyledons; **2** = loss of the cotyledons; **3** = wilting or chlorosis of the true leaves; **4** = necrosis of the true leaves; **5** = loss of the true leaves. An average of 13 plants was calculated for each treatment.

Disease reduction (DR) percentage per treatment was calculated using the following formula: DR (%) = [1 – (DT/DC)] × 100, where DT: disease incidence percentages in treatment; DC: disease incidence in control.

***Verticillium dahliae* re-isolation**

At the end of the experiment, pathogenicity test was confirmed by isolating the *V. dahliae* from stems in affected plants and controls. Samples were washed in running tap water, the bark was removed and tissue surface was disinfected in 1% sodium hypochlorite for 1 min. Stems segments were transferred on PDA plates and incubated in the dark at 25 °C for a week.

Statistical analysis

The statistical analyses were performed using STATISTICA (version 10.0). Significant treatment results were determined by F values ($P \leq 0.05$). The treatment mean was separated by Tukey's HSD test.

Results and Discussion

Morphological and molecular identification of *Trichoderma* isolates

A total of 53 *Trichoderma* isolates were obtained, by dilution isolation technique, from rhizosphere samples of olive trees which were collected from nine locations in North-Western Algeria. Colony morphology and morphological features of the isolates typically resembled of those of genus *Trichoderma*.

Fifteen *Trichoderma* isolates, with antagonist activity more than 40% against *V. dahliae*, were selected to morphological observations and characterization by ITS sequences analyses (Table 1).

Combining the results of morphological characters and molecular identification, the 15 selected isolates were belonged to the genus *Trichoderma* with three different species: *T. harzianum, T. atroviride* and *T. longibrachiatum* (Table 1). *T. harzianum* was the most dominant specie isolated which accounted for 73.33% of all the isolates. The remaining *Trichoderma* species obtained in this study were less represented (13.33% for each species). *Trichoderma* are free living fungi that are highly interactive in root, soil and foliar environment can parasitize other fungi (Harman *et al.,* 2004). *T. harzianum* is known for its dominance in the majority of soil ecosystems in the world (Druzhinina *et al.,* 2010). This abundance was also been reported in various studies (Kumar *et al.,* 2012; Bader *et al.,* 2019; Feitosa *et al.,* 2019). Morphological taxonomy of *Trichoderma* is primarily based on macro- and micro-morphological characteristics, which includes colony color, shape, appearance on specific culture media, and spore-forming structures (Samuels *et al.,* 2002).

Based on morphological characteristics on PDA, the eleven isolates corresponded to *T. harzianum* were T3, T4, T7, T8, T9, T11, T12, T13, T14 T15 and T16 (Chaverri *et al*., 2015). The isolates T9 and T17 were also morphologically distinguishable and showed similarity with *T. longibrachiatum* (Bissett, 1991). The others, T2 and T10 were grouped as the isolates presumed to be *T. artoviride* (Rifai, 1969).

The three different species of *Trichoderma* showed different cultural characteristics on PDA (Fig. 1). *T. harzianum* formed 1–2 concentric rings with green conidial production. Colonies in culture ranged in color from yellowish green to pale green with colorless reverse side of plates. The conidia production was denser in center then towards the margins. Hypha is septate, hyaline. Conidia are globose to sub-globose, smooth and green. Conidiophores are irregular branching. The phialides are thick or swollen, relatively short (Fig. 2A). This species formed abundant chlamydospores producing both terminally and intercalary.

T. artoviride colony showed dark green sporulation, colony reverse was amber or uncolored. *T. artoviride* formed white mycelia with on conidial formation. Conidiophores are jagged and branches are not compact (Fig. 2B). Terminally and intercalary chlamydospore were frequent producing.

T. longibrachiatum produced yellowish green pigmentation in the bottom of the culture dish. The phialids which were mostly solitary, cylindrical and conical at the neck. The conidia are smooth and ellipsoid (Fig. 2C). Chlamydospores are common, terminal appearing oval to globose or intercalated and cylindrical to swollen. The same observations and characteristics of the species *T. harzianum* (sect. *Pachybasium*), *T. longibrachiatum* (sect. *Longibrachiatum*) and *T. atroviride* (sect. *Trichoderma*) were recorded by Bisset (1991), Games and Bisset (1998) and Samuels *et al.* (2002, 2004). For molecular identification and based on the phylogenetic analyses of internal transcribed spacer (ITS) sequences, all the fifteen *Trichoderma* isolates are closely related to three different species: *T. harzianum*, *T. longibrachiatum* and *T. atroviride* (Fig. 3).

The results of molecular identification hence proved identical to those of the morphological identification. According to the BLAST results and compared with the sequences of the GenBank database from NCBI, all sequenced isolates were at least 99% similar to the corresponding GenBank sequences. The determined ITS sequences of these *Trichoderma* isolates were deposited in NCBI GenBank.

The identification of *Trichoderma* spp. is difficult due to the similarity of morphological characteristics (Samuels *et al.,* 2010). The molecular method allowed a more reliable and precise identification based on sequence analysis of several genes (Błaszczyk *et al.,* 2011; Kubicek *et al.,* 2011; Dou *et al.,* 2019). The internal transcribed spacer (ITS) regions of rDNA are the most widely used DNA regions in fungi (Maurya *et al.,* 2019; Zhou *et al.,* 2019).

In this study ITS regions of rDNA were used

with success because the 5.8S rRNA gene is highly conserved at the genus level and capable of differentiating from closely related fungal species (Feitosa *et al.,* 2019; Maurya *et al.,* 2019; Zhou *et al.,* 2019).

In vitro* screening of *Trichoderma* isolates for antagonism against *V. dahliae

All tested *Trichoderma* strains reduced V1 and V2 development where inhibition percentage varied from 54.09 to 74.41% and from 41.07 to 69.50%, respectively (Table 2).

The highest inhibitory effect was noted by *T. atroviride* (T2) with a 74.41% rate. On the other hand, the lowest inhibitory effect towards V1 was noted by *T. longibrachiatum* (T17) with a 54.09% rate. The lowest inhibitory effect towards V2 was by *T. harzianum* (T4) with a 41.07% rate. The control plates without *Trichoderma* were completely covered by pathogen mycelia showing no fungus growth inhibition.

Similar results were obtained by Fotoohiyan *et al.* (2017). Their results indicated that all 20 isolates of *T. harzianum* were capable of inhibiting the mycelial growth of *V. dahliae* of pistachio significantly.

After 6 days of incubation, the colonies of *V. dahliae* were fully invaded by the majority of *Trichoderma* sp. with intensive sporulation; some of them formed several aggregates of spores' adherent to the colonies of the host. This means that *Trichoderma* used its ability for faster growth as an essential antagonistic mechanism as a way of competing for space and nutrients with pathogenic fungi. Carrero-Carrón *et al.* (2016) reported that all *T. asperellum* strains overgrew on *V. dahlia* isolates, and extracellular compounds from strains Bt3 and T25 showed higher antagonistic activities.

T. atroviride (T2) overgrew on *V. dahliae* and turned dark green due to sporulation below it but mycelium of the pathogen was clearly visible and showed a gradual loss of the typical dark color and mass form of microsclerotia which became completely disintegrated compared to untreated control.

Microscopic observations showed clear modification in hyphae morphology showing parasitic behavior against *V. dahliae*. Malformations, vacuolations and swellings occurred in hyphae treated with *Trichodema* spp. However, the mycelia from the control were intact, smooth with no swellings or vacuolation. Occasionally *T. harzianum* hyphae formed a hook or bunch like structures around the hyphae of the pathogen from where penetration took place. We also noticed that *T. atroviride* (T2) coiled around the pathogen and formed appressorium-like structures suggesting mechanical activity before penetration in the host hyphae where eventually disintegrated and lysis. The hyphae of *T. harzianum* (T3, T7, T8 and T12) attached to the mycelium of *V. dahliae* by hooks or haustorium. Coiling on *V. dahliae* hyphae without penetrating cells has been observed in *T. harzianum* (T4, T9, T11 and T14). Cheng *et al.* (2012) demonstrated that a *T. harzianum* ETS 323 effectively inhibited *Botrytis cinerea* hyphal growth, caused cytosolic vacuolization in the hyphae, and led to hyphallysis. Direct antifungal activity may be based on the production of siderophores, lytic enzymes and antibiotics (Howell 2003; Mahdizadehnaraghi *et al.,* 2015).

In vitro* effects of volatile metabolite of *Trichoderma* isolates on *V. dahliae

In vitro studies on the inhibitory mechanisms showed that *Trichoderma* cultures apparently produced volatile substances in the growth medium that suppressed the pathogen growth. Volatile metabolites were produced by isolates *T. atroviride* (T2), *T. atroviride* (T10) and *T. harzianum* (T7) where V1 and V2 at a level of 69.33, 66.66 and 64%, respectively (Table 2). Other isolates (T3, T9, T11, T13 and T17) moderately inhibited its growth from 46.38 to 57.33%. The lowest volatile metabolite activity was observed by T14 and T8.They inhibited the target pathogen V1 from less than 45 and 49.33%, respectively. In addition, T14 and T8 isolates inhibited V2 from 37.50% and 41.77%, respectively. The antagonistic potential was also noted to vary through volatile metabolites, and direct parasitism on the pathogen among different isolates of an antagonist *Trichoderma* isolate.

The identified species in our study, *T. harzianum, T. longibrachiatum* and *T. atroviride* are recognized for biological control, and this confirms their antagonism through competition, enzyme production, antibiosis and induced resistance (Verma *et al.,* 2007; Hermosa *et al.,* 2012).

Antagonistic effects of non-volatile extracts antibiosis proprieties

The Results showed that all *Trichoderma* culture filtrates inhibit significantly ($P \leq 0.005$) mycelial growth of both strains of *V. dahliae* with different inhibition rates. Maximum inhibitory effect of V1 and V2 was mediated by isolates T2, T10 followed by T7 with 60.92%, 58.76% and 48.92%; and 49.30%, 48.11% and 46.67%, respectively. On the other hand, minimum inhibitory effect was caused by *T. harzianum* T14 and T15 with 17.53%.The other isolates showed an average inhibition rate between 20.13% and 43.32% (Table 2).

The antifungal activity was supported by the excretion of glucanase and siderophores; Berg and Ballin (1994) observed the ultrastructural changes of *V. dahliae* during the antagonism *in vitro* and found an abnomal branching, an abnormal growth of the hyphae with swelling and degradation of mycelia. Several *Trichoderma* species can produce the

extracellular of antibiotics (Rajendiran *et al.,* 2010). In the same context, Nakkeeran *et al.* (2002) reported that *Trichoderma* species could secrete some metabolites, such as trichodermin, trichovirdin, dermadin and heptalic acid.

Three isolates T2, T7 and T10 highly efficient towards two isolates of *V. dahliae*, were finally selected based on the performance of *in vitro* isolates selected for their efficiency on individual pathogens. These selected efficient strains were used for further studies.

Effect of *Trichoderma* spp. on tomato Verticillium wilt

After 5 weeks, all the tomato plants, inoculated with *V. dahliae* showed typical Verticillium wilt symptoms. However, uninoculated and untreated plants were symptomless.

Significant differences in wilting symptoms and vascular discoloration were observed between plants inoculated with *V. dahliae* and those non-inoculated. Severe Verticillium symptoms with chlorosis of the leaves, necrosis, wilt, and defoliation were noted in plants inoculated with *V. dahlia* (V2). However, 5 weeks after inoculation, there was 97.3% more disease on wilted plants showing reduced growth and vigour. This disease corresponds to 4.66% of severity.

In contrast, all plants inoculated with V2 and treated with *T. atroviride* (T2 and T10) and *T. harzianum* T7 showed significantly less number of yellowish leaves and low disease severity to 1.11, 1.26 and 1.34, respectively. On the other hand, all *Trichoderma* tested significantly ($P \leq 0.05$) reduced the percentage of wilted plants (Fig. 4). Ali *et al.* (2020) reported significant decrease in collar rot of chickpea by application of *T. harzianum.* Hermosa *et al.* (2013) reported that *Trichoderma* spp. can reduce the severity of plant diseases by inhibiting plant pathogens through their highly potent antagonistic and mycoparasitic activity. For example, Sunpapao *et al.* (2018) showed that the endophytic *Trichoderma* V76-12 can inhibit mycelial growth *in vitro*, and reduce *Curvularia oryzae* symptoms *in vivo* and in natural fields.

In addition to their good activity against fungal phytopathogen, *Trichoderma* can also promote plant growth. *T. atroviride* promoted greater height of *Capsicum annuum* seedlings (Herrera-Parra *et al.,* 2017). *T. atroviride* could also regulate root architecture and promote plant growth (Garnica-Vergara *et al.,* 2016). Another study showed exposure to either *T. virens* or *T. atroviride* increased the biomass production of wild-type Arabidopsis seedlings and stimulated lateral root development (Contreras-Cornejo *et al.,* 2009).

Conclusion

The possibility of replacing chemical control of tomato Verticillium wilt with a biological alternative remains an exciting and challenging objective. Therefore it is necessary to combine the basic research involving *in vitro* and in pot experimentation on antifungal mechanisms and their genetic origin with tests under field conditions and the ecological consequences.

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Table 1: Sampling locations and source of potentially antagonistic *Trichoderma* isolates.

No.	Isolates	Identity	Accession number	Department	Location	Year collected
1	T2	*T. atroviride*	MN319566.1	Mascara	Sig	2017
2	T3	*T. harzianum*	MT929295.1	Relizane	Bellassel	2017
3	T4	*T. harzianum*	MT940803.1	Bejaia	Bejaia	2017
4	T7	*T. harzianum*	MN319584.1	AinTemouchent	Terga	2017
5	T8	*T. harzianum*	MT940827.1	AinTemouchent	Terga	2017
6	T9	*T. harzianum*	MT940828.1	SidiBel Abbes	Sidikhaled	2018
7	T10	*T. atroviride*	MN320383.1	Mostaganem	HassiMameche	2018
8	T11	*T. harzianum*	MT940829.1	Mascara	Mohamadia	2018
9	T12	*T. harzianum*	MT940830.1	SidiBel Abbes	Sfisef	2018
10	T13	*T. harzianum*	MT940831.1	Oran	Tafraoui	2018
11	T14	*T. harzianum*	MT940832.1	Oran	Tafraoui	2018
12	T15	*T. harzianum*	MT940851.1	Tlemcen	AinFezza	2018
13	T16	*T. harzianum*	MT940854.1	Mostaganem	Mazagran	2018
14	T17	*T. longibrachiatum*	MT940855.1	Saida	Saida	2018
15	T19	*T. longibrachiatum*	MT940857.1	Tlemcen	Maghnia	2018

Table 2: Mycelial inhibition of *V. dahliae* in dual culture, volatile and non-volatile metabolites.

Isolates	Mycelial inhibition of *V. dahliae*(%)					
	Dual culture		Volatile		Nonvolatile	
	V. dahliae (V1)	*V. dahliae* (V2)	*V. dahliae* (V1)	*V. dahliae* (V2)	*V. dahliae* (V1)	*V. dahliae* (V2)
T. atroviride T2	74.41 ± 2.33 a	69.50 ± 1.70a	69.33 ± 2.08a	57.23 ± 2.05a	60.92 ± 5.24 a	49.30 ± 1.80 a
T. harzianum T3	70.03 ± 0.58 ad	57.04 ± 3.16 b	57.33 ± 8.02 bc	48.02 ± 3.98abc	30.46 ± 11.28 bde	20.13 ± 4.14 b
T. harzianum T4	56.90 ± 1.54 c	60,98 ±2,04 bc	56.33 ± 3.78 bc	46.38 ± 15.29 abc	28.92 ± 4.61 bd	37.35 ± 2.71 c
T. harzianum T7	72.05 ± 5.91 ae	67.54 ± 4.28 ac	64.00 ± 4.00 ab	55.59 ± 3.55 ab	48,92 ± 8.06cf	46.67 ± 6.42 a
T. harzianum T8	63.97 ± 0.58 df	61.63 ± 5.47 bc	49.33 ± 2,08 cd	41.77 ± 6.90 bc	38.46 ± 4.16be	38.30 ± 2.15 c
T. harzianum T9	72.39 ± 1.16 ae	59.67 ± 3.40 bc	57.00 ± 5,29 bc	49.67 ± 7.45 abc	31.38 ± 1.06bde	30.41 ± 1.24 de
T. atroviride T10	74.07 ± 3.82 a	67.21 ± 1.13 ac	66.66 ± 3.05 a	55.92 ± 0.56 a	58.76 ± 6.98 ac	48.11 ± 1.80 a
T. harzianum T11	65.99 ± 5.83 de	61.31 ± 4.85 bc	56.33 ± 6.65 bc	47.36 ± 5,78 abc	30.15 ± 1.92 bd	27.30 ± 1.49 de
T. harzianum T12	71.38 ± 1.54 ae	63.27 ± 2.27 cd	55.33 ± 4.61 bc	45.39 ± 10.55 abc	24.30 ± 5.53 dg	25.39 ± 4.97 d
T. harzianum T13	69.02 ± 2.10 ad	58.03 ± 1.50 bd	57.00 ± 5.29 bc	46.38 ± 9.48 abc	40,30 ± 7.51 ef	32.08 ± 0.82 e
T. harzianum T14	56.56 ± 2.67 c	60.32 ± 5.76bd	45.00 ± 7.81 d	37.50 ± 18.68c	17.53 ± 5.64 g	27.54 ± 0.71 de
T. harzianum T15	55.21 ± 10.61 c	62.29 ± 1.50 bc	52.33 ± 5,50 cd	43.42 ± 7,13 abc	17.53 ± 2,82 g	31.77 ± 3,97 e
T. harzianum T16	58.58 ± 1.74 cf	59.01 ± 2.04 bd	54.00 ± 1.73 c	44.07 ± 1.50 abc	34.76 ± 4.64bf	37.35 ± 0.82 c
T. longibrachiatum T17	54.09 ± 3.08 c	61.63 ± 3.40bd	54.66 ± 3.78 c	50.32 ± 2.05 abc	29.23 ± 7.95bd	29.69 ±1.24 de
T. longibrachiatum T19	69.02 ± 2.10 ad	60.32 ± 1.50 bd	57.00 ± 9.53 bc	45.72 ± 6.47 abc	38.46 ± 3.84 be	43.32 ± 3.28 a

Different letters within columns indicate significant differences at $P \leq 0.05$, according to Tukey's HSD test.

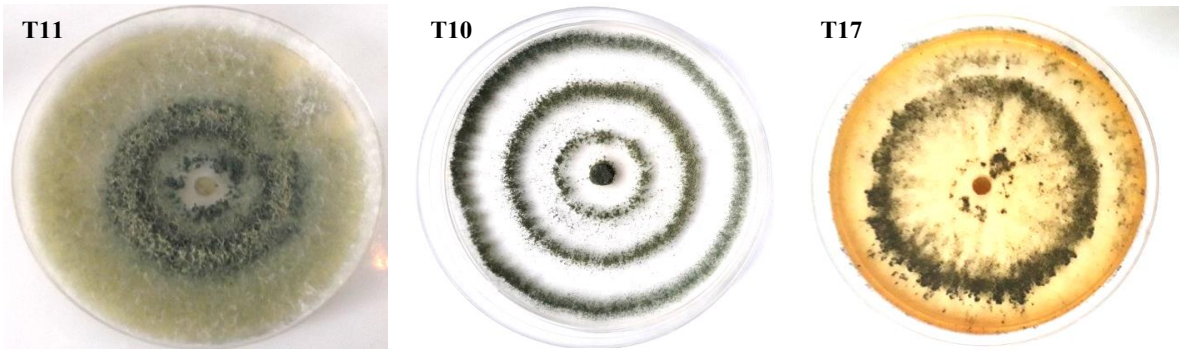

Fig. 1: colony appearance of three different species of *Trichoderma* grown for 6 days at 25 °C on PDA, *T. harzianum* (T11), *T. artoviride* (T10), *T. longibrachiatum* (T17).

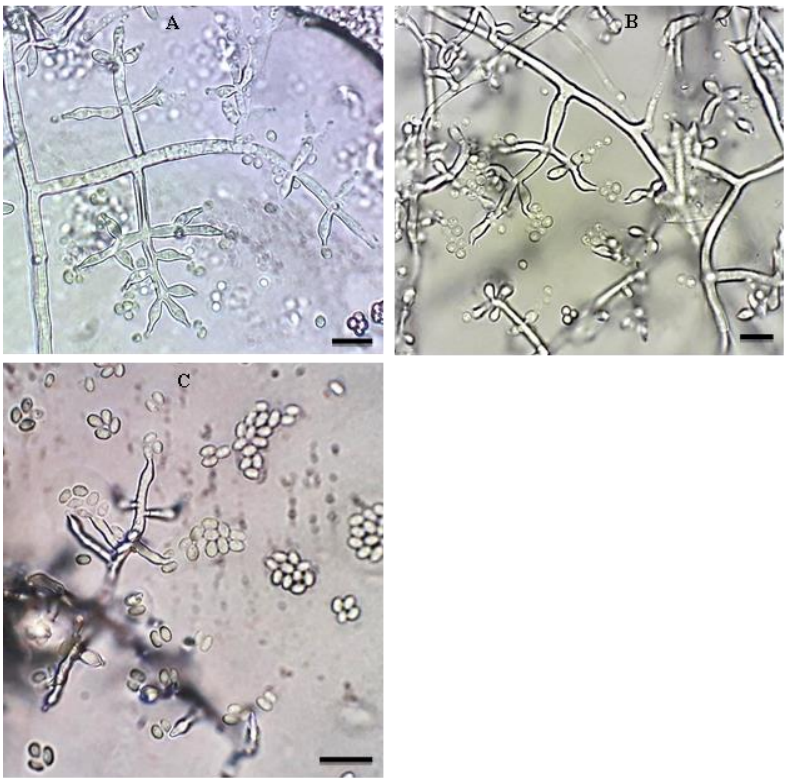

Fig. 2: Microscopic features of *Trichoderma* (scale bar = 10 µm): (A) *T. harzianum* (T11). (B) *T. artoviride* (T10). (C) *T. longibrachiatum* (T17)

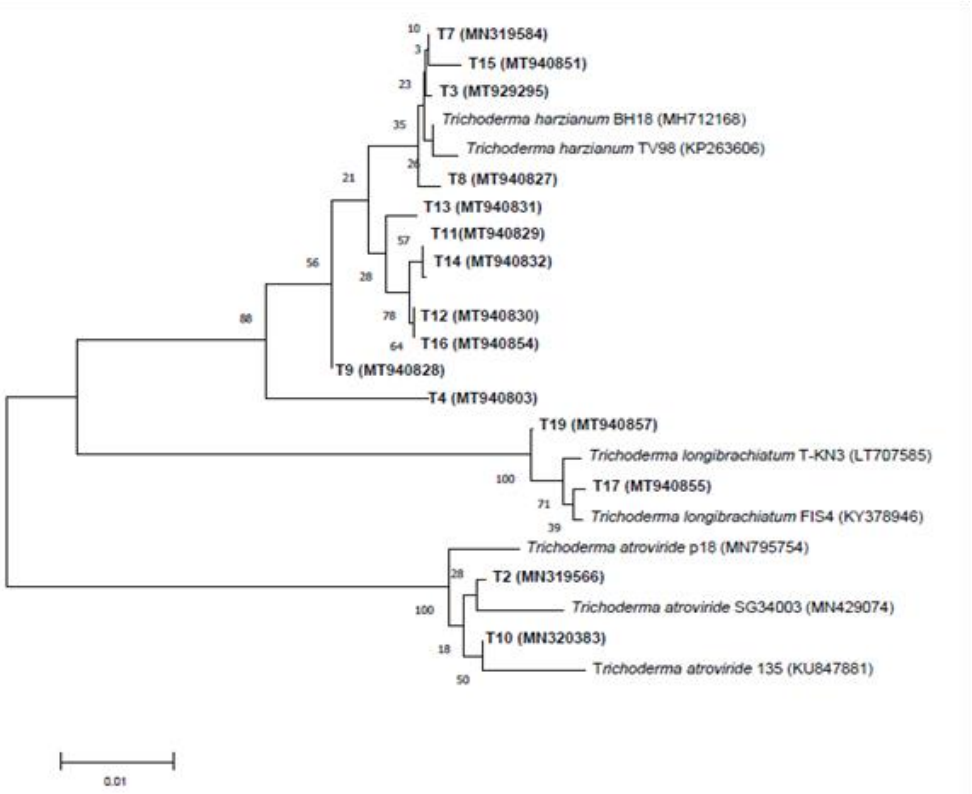

Fig. 3: Phylogenetic tree showing the relative position of *Trichoderma* isolates based on ITS gene sequences by the neighbor-joining method in the MEGA program. Numbers above branches represent bootstrap values with 1,000 replications. The scale bar corresponds to 0.01 units of the number of base substitutions per site.

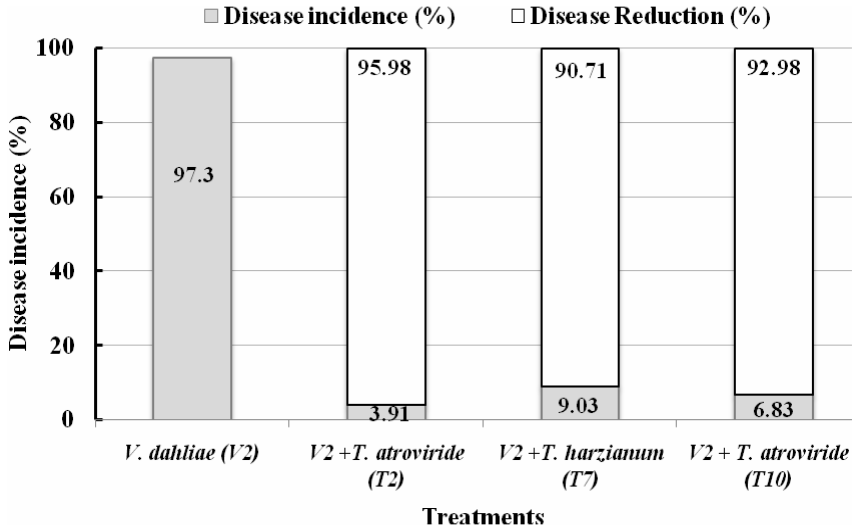

Fig. 4: Incidence and reduction percentages of Verticillium wilt (V2) on tomato plants treated with *T. atroviride* T2 or *T. Harzianum* T7 or *T. atroviride* T10.

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