Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

EFFICACY OF *TRICHODERMA* SPP. AGAINST DEATH, WILT AND DIEBACK OF YOUNG OLIVE TREES IN THE NURSERIES

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A B S T R A C T

Surveys of three nurseries in northwestern Algeria have shown the presence of several cases of withering on olive seedlings.This study aims to confirm the pathogenesis of isolated species mycoflora associated with the young trees in nurseries with the symptom of dieback and wilting. In order to minimize the danger of the use of chemical substances in olive cultivation and establish a successful control strategy for this disease, the biological control potential of *Trihoderma* spp. isolates derived from rhizospheric soils of intact olive trees against *Fusarium oxysporum* (*FO*), *Fusarium solani* (FS) and *Rhizoctonia solani* (RS) under laboratory and greenhouse conditions was estimated. The results indicated that the symptoms observed in nursery were the result of a fungal complex comprising several primary and secondary pathogens contributing to the appearance of various symptoms. The results revealed the presence of dieback ranging from 13 to 26%. As regards the biological control test, all isolates of *Trihoderma* spp. were capable to inhibit pathogens mycelial growth significantly *in vivo*, the maximumwas recorded with the T4 isolate (78.89%) antifungal activity against FO. Results of greenhouse (*in vivo*) tests showed that plants injected only with antagonists or in combination with a fungal pathogen had a lower incidence of wilt disease than plants inoculated with a single pathogen. The decrease in the incidence of wilt disease was 80%, 58% and 50% FO, RS and FS respectively compared to the uninoculated control. Our results also showed that the four mixed *Trichoderma* isolates were able to stimulate plant growth parameters, which mainly resulted in better axial growth and higher biomass.The results obtained under greenhouse, shows the incorporation of *Trichoderma* directly into soil or composts for nursery soils as preventive and curative treatments.

Keywords: Biocontrol, dieback, nurseries, Olivier (*Olea europaea*),*Trichoderma* spp.,wilt.

INTRODUCTION

The olive (*Olea europaea*) is a tree of economic importance in the world. In the Mediterranean basin, the cultivation of this tree occupies approximately 10.5 million hectares. Epidemiological studies carried out in recent years in the main olive groves have shown the presence of several cases of dieback on seedlings of olive trees stemming from herbaceous cuttings in nurseries and in open fields and also on aged olive trees. Olive growers have been concerned about the increased incidence of wilt, dieback and death of young trees, the symptoms of wilting of olive branches and death or dying of trees have been associated with different number of fungi **(**Merzoug *et al.,* 2018**).**The studies of Chliyeh *et al*. (2014) showed that soil fungi such as *Fusarium spp.*and *Rhizoctonia* spp., might be cause wilting and partial or total dieback of the olive tree. In several Mediterranean countries, extensive damage has been reported as a result of attacks by these soil fungi (FAO, 2012). The problem of withering increases every year. Biological control has certain advantages that respond to human questions both on economic issues and on biological and environmental stability. In addition to reducing the risks of pollution, the use of

Submitted: April 04, 2022
Revised: May 17, 2022
Accepted for Publication: June 13, 2022
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antagonistic microorganisms makes it possible to deal with the problems of the appearance of resistant races. Several species of Trichoderma spp. have some antagonistic activity against several pathogens agents and shown some efficacy against several common soil-borne pathogens (Kapoor, 2008). A significant number of *Trichoderma spp*. stimulates plant growth and protects them from fungal and bacterial pathogens through induced systemic resistance, antibiosis, mycoparasitism and enhanced nutrient efficiency (Harman *et al*., 2004; Contreras-Cornejo *et al.,* 2009; Vos *et al*., 2015). More than 60% of registered biopesticides are manufactured by Trichoderma species (Verma *et al.*, 2007; Moya *et al*., 2018).

To conduct this study, three nurseries different in northwestern Algeria were selected for prospecting and sampling from the soil and plants of olive trees affected. The objective of this study was to (i)confirm the pathogenesis of the isolated species of the mycoflora associated with young olive trees in the nursery with the symptom of dieback (ii) perform a biological control test *in vivo* and *in vitro* with *Trichoderma* spp. isolates. (iii) Test the efficacy of *Trichoderma* spp. strains (consortium) against vegetative growth parameters.

MATERIALS AND METHODS

Survey, Isolation, Purification and Culture of fungal colonies: In order to determine, the incidence of olive dieback diseases, three nurseries in northwestern Algeria are selected. In addition, we visited several olive orchards with dieback problems. During these visits, percentages of attack were determined by determining the number of diseased plants relative to the total number of plants. Samples of roots were removed carefully by washing them with running water, then cutting into small fragments (5 to 10 mm). Superficially disinfected by soaking for 5 minutes in sodium hypochlorite diluted to 10%. We cut the rootlets and roots at various levels to young shoots ascending to the vascular system. The dried fragments are placed in Petri dishes on PDA (Potato dextrose agar). The prepared culture were then put in the dark at 24-26°C. Purification of isolates was obtained by single spore technique on PDA. The Isolates of *Trichoderma* spp. Were obtained from the rihzosphere of olive trees situated in different regions of northwestern Algeria between 2018 and 2019. Then develop a selective medium (Davet, 1979) the use of standard identification keys has allowed us to to characterize these isolates morphologically and microscopically (Rifai, 1969; Samuels, 2006; Samuels *et al.,* 2015).

Pathogenicity assay: Pathogenicity trials were conducted with tree fungal species: *Fusarium oxysporum*, *Fusarium solani*, and *Rhizoctonia solani* collected mainly from the surveyed in olive nurseries .The preparation of inoculums was conducted by two techniques, the first technique is used for *Rhizoctonia solani*, which was consisted to grind in a blender the contents of the plate in 100 ml of sterile distilled water. The second technique was adopted for *Fusarium oxysporum* and *Fusarium solani*, by preparing a suspension of the inoculums in a flask hold in 200 ml PDB (potato-dextrose broth) then stored at 25 ° C in a rotative agitator for 3-4 days.After filtration, the concentration is adjusted to10^6 spores / ml. The roots of young plants (6 months old, cv. Sigoise) were cleaned under tap water and immersed in the inoculum suspension for five minutes and then placed in seedling pots(14 cm diameter x 10 cm heighteach pot contains only one plant) included autoclaved soil (peat, soil and sand V/V/V) plus 50 ml of inoculum. Then these plants were posed in glasshouses (25-30 °C, 60-70% RH) and irrigated as required (Sanchez Hernandez *et al.,* 1998). In the case of the fungus *Rhizoctonia solani* the plants were inoculated by transplanting them into pots containing a sterile substrate inoculated with mycelium. Severity of symptoms were noted weekly for each plant (Hibar *et al*., 2017). The experimental protocol was a complete randomized experiment with one variety, four treatments and 3replicates for each treatment. Pathogenicity essay was conducted by performing different combinations of inoculation: - *Fusarium solani* - *Fusarium oxysporum* -*Rhizoctonia solani* and a negative control. The transplants previously infected and showing symptoms of the disease were subject to the re-isolation of fungi. At the end, the morphological and microscopic characters of these fungi were compared to the original isolates used. Disease severity was evaluated two months after inoculation. Using the scale between zero and five(zero – healthy plants, 5 – dead plants) (Huang *et al.*, 2006).

Estimate of the antagonistic effect of *Trihoderma* spp*.*: The dual culture technique: Using the dual culture method, the antagonistic activities of *Trichoderma* spp. isolates tested contrarily FS -FOand RS. At the border of separates Petri plate encompassing PDA culture medium, small piece (5 mm in diameter) of pathogens (FS, FO and RS) have been place, Then

isolates of *Trichoderma* spp. were transferred to the contrarily place of the dish and stored at 25°C for 7 days. Pathogen colony diameters were measured every 24 hours from 3 to 7 days. In each test, 3 treatments and 4 repetitions for each test were performed. The percentage of development interdiction was estimated considering the formulary (Datta *et al.,* 2004**):** I = (C–T)/C × 100, where: C – pathogens development in witness petri dish, T – Pathogens growth on *Trichoderma* plates. Mycoparasitism activities were examined microscopically for any morphological variations in the mycelial development of pathogens caused by *Trihoderma* spp. In the beginning each fungi were develop in the same time on petri dish containing PDA medium.(Morsi *et al.*, 2009; Fotoohiyan *et al.,* 2017). Four local *Trichoderma* isolates (T1, T2, T3 and T4) have been chosen for testing their biocontrol efficacy against the pathogens (FO, FS and RS).

Greenhouse experiment: Antagonistic effect of *Trichoderma harzianum* on pathogenic isolates *F. oxysporum*, *F. solani*, *and R. solani:* *In vitro*, *T. harzianum* (T4) offer the highest inhibition percentage against FO, FS and RS, it was chosen for test hits ability to reduce the incidence of olive seedling dieback diseases.As previously mentioned in pathogenicity tests, inoculum of the isolate of FO, FS and RS was prepared. Inoculum of T4 was cultured in Potato Dextrose Broth and for two weeks at 26 ± 2 °C in the dark and readjusted to 10^6 conidia ml−1. Seedling pots were injected with pathogen and antagonist in 4 tests as follows: 1. Control (pathogen + antagonist); 2. T4only; 3. T4+ FO, FS, and RS separately; 4. Pathogen only. In treatments containing the antagonist, T4 was injected1 hour previously treatment with pathogens (FO, FS, and RS) separately to the plants. These plants were posed in glass houses **(**25-30 °C, 60-80% RH) for 3 months and watered as needed. Four treatments were used in this experiment, each with three replicate. After inoculation, symptoms were noted every 10 days for 3 months. A scale from 0 to 5 was used to estimate the disease severity (Huang *et al*., 2006). The reduction in disease incidence (DS) was evaluated: DS Reduction (%) = (control oliveplants DS - treated oliveplants DS / control oliveplants DS) × 100.

Efficacy of *Trichoderma spp.* strains against vegetative growth parameters: The objective of this experiment was to evaluate the preventive and curative ability of *Trichoderma spp.* strains. This experiment was carried out on olive plants (1-year-old, cv. Sigoise), under greenhouse conditions during 2019.With a suspension 50 ml(5,5 × 105 spore ml−1) of *Trichoderma spp*. mixed (consortium) (T1, T2, T3 and T4), the soil was treated carefully as close as possible to the root system with a sterile syringe and the control olive plants were treated with sterile distilled water taking with same condition as above. The experiment was repeated twice. The plants were regularly irrigated once a week. After three months of experience, the following parameters were evaluated: plant height (cm), stem diameter (cm), number of plant/ leaves (newly formed leaves), fresh weight and dry weight (g./Plant).

STATISTICAL STUDY

All data were analyzed using Statistica v. Software. 5.5 (Statsoft, Ed'99) and Biostat. 2009 when determining the ANOVA, the differences were significant for a $P \leq 0.05$. Newman - Keuls test at $p < 0.05$ were used for multiple comparisons of means.

RESULTS

Disease Survey andPathogenicity assay: Various symptoms were observed such as yellowing, wilting and leaf drop and dieback of twigs in olive tree nurseries and some olive orchards surveyed (Fig.1) with an infection rate 11, 66 to 28, 12%. The isolation results of each part show that the highest frequency of fungal isolates is present in the rhizosphere. After 25 days of inoculation, macroscopic symptoms were observed on olive seedlings of Segoise varieties, by comparing the inoculated and the non-inoculated plants.Isolates tested significantly infected olive trees, causing symptoms of wilt and dieback and differed in their pathogenic capacity. FS, RS and FO were the most severe isolate*.* In the case of FO,the leaves of the base of the plants show in particular a symptom of yellowing and wilting and their windings downwards and take a grey colour. Wilting progresses from the lowest leaves to the top of the plant, a symptom that can be generalized to the whole plant. The roots do not seem to be affected, but a longitudinal section of the stem shows an abnormal orange to red staining that progress in the vascular tissue from the root to the stem. Symptoms began to appear 1 month after inoculation with *Rhizoctonia solani*, the symptoms of the disease consist of partial wilting, browning of leaves that begin to necrotic with their tips and eventually dry out and defoliation followed by dieback of twigs.It is associated with root rot.Olive seedlings inoculated with isolates of *Fusarium solani* showed leaf

curl, browning of the apical zone of the leaves, which ended with dieback and presence of collar necrosis. Leaves dried attach to young branches for a long time. Symptoms appear 40 days after inoculation. Uninoculated controls showed no symptoms. The Koch postulate was verified. The reisolate from the inoculated plants allowed recovering the isolates inoculated at the beginning.

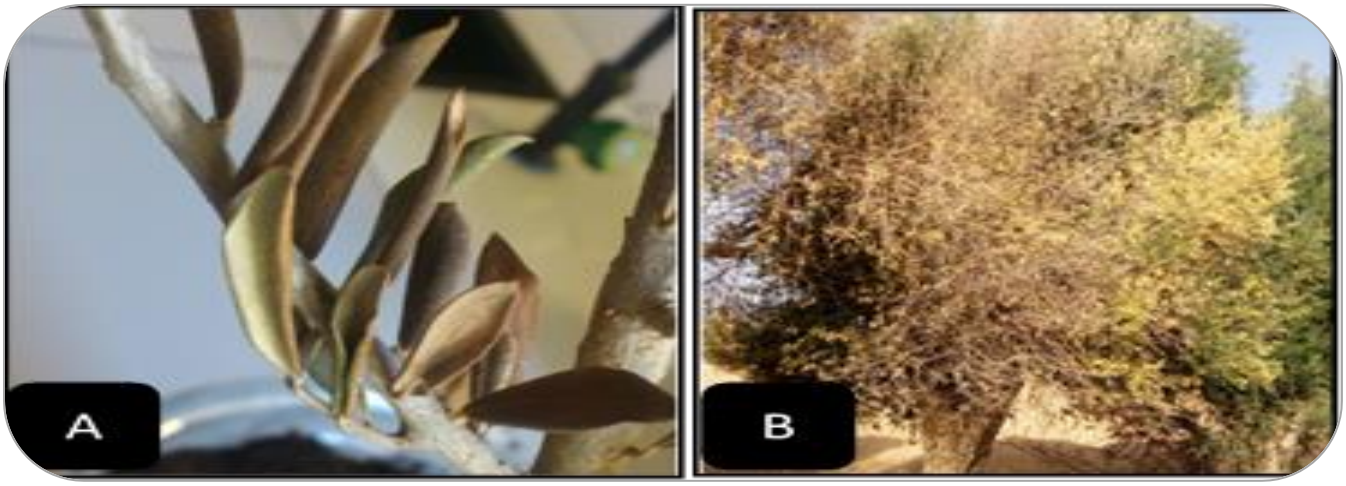

Figure 1. Symptoms of dieback of olive seedlings in nursery A and the field B

Test results of the antagonistic action of *Trihoderma* spp.: The dual culture:

Tests showed that the four isolates T1, T2, T3 and T4 inhibited mycelial growth of FO, FS and RS varying between 52.71% and 78.89% growth inhibition. The "*in vitro*" test results indicate that the four *Trichoderma spp*. isolates show varying efficacy depending on the antagonist isolate and the pathogens isolates ((FS, FO and RS). T4 showed the maximum antifungal activity against FO (Fig.2).Microscopic observation showed that, the mycelium of *Trichoderma* convolved the hyphae of FO, FS and RS, denaturing and inhibiting their mycelium. *Trichoderma* hooked around the hyphae of the pathogen before penetration. Three days after incubation, overlap of *Trichoderma* hyphae and pathogen started (Figure 3).

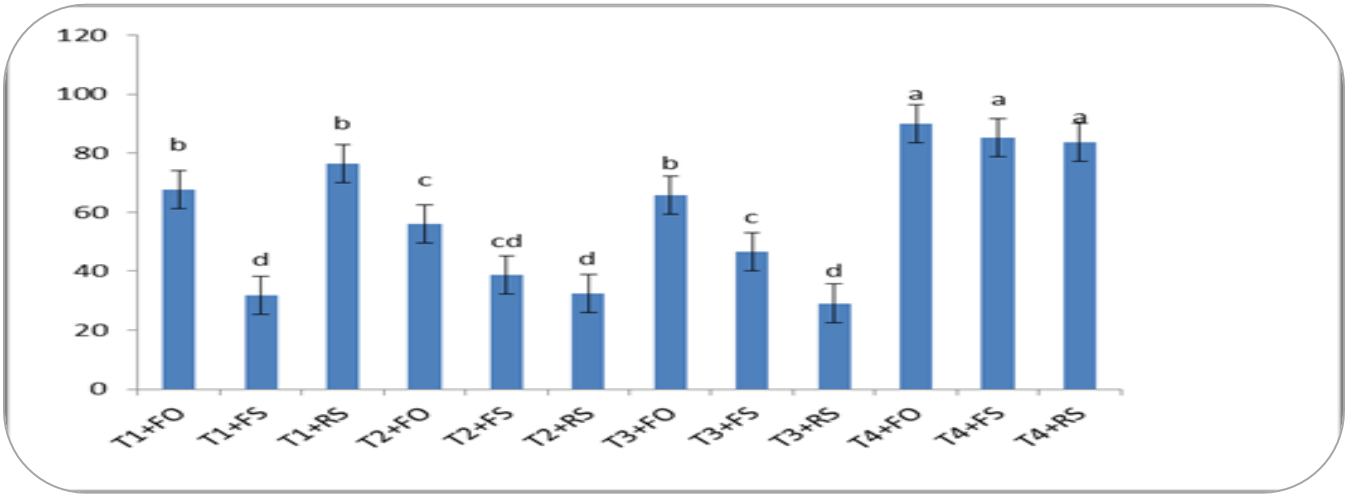

Figure 2. The percentage of mycelial growth inhibition in *vitro test*.
Two results read on the same column followed by two different letters, differ significantly at the 5% threshold.

Biocontrol activity of T4 isolate against the pathogens isolates (FS, FO and RS) in the greenhouse experiment:

The percentages of average disease incidence vary between pathogenic species with 80% for *Rhizoctonia solani*, *Fusarium solani* (70%) and *F. oxysporum* (66.67%).It was found that *in vivo* experiments T4 isolates of *T. harzianum* showed biocontrol activity against FO, FS and RS under greenhouse conditions. The results in Figure 4 showed that the T4 reduces the incidence of dieback disease of olive seedlings. The reduction in the incidence of dieback

diseases is 80%, 58% and 50% for FO, RS and FS respectively compared to the uninoculated control.

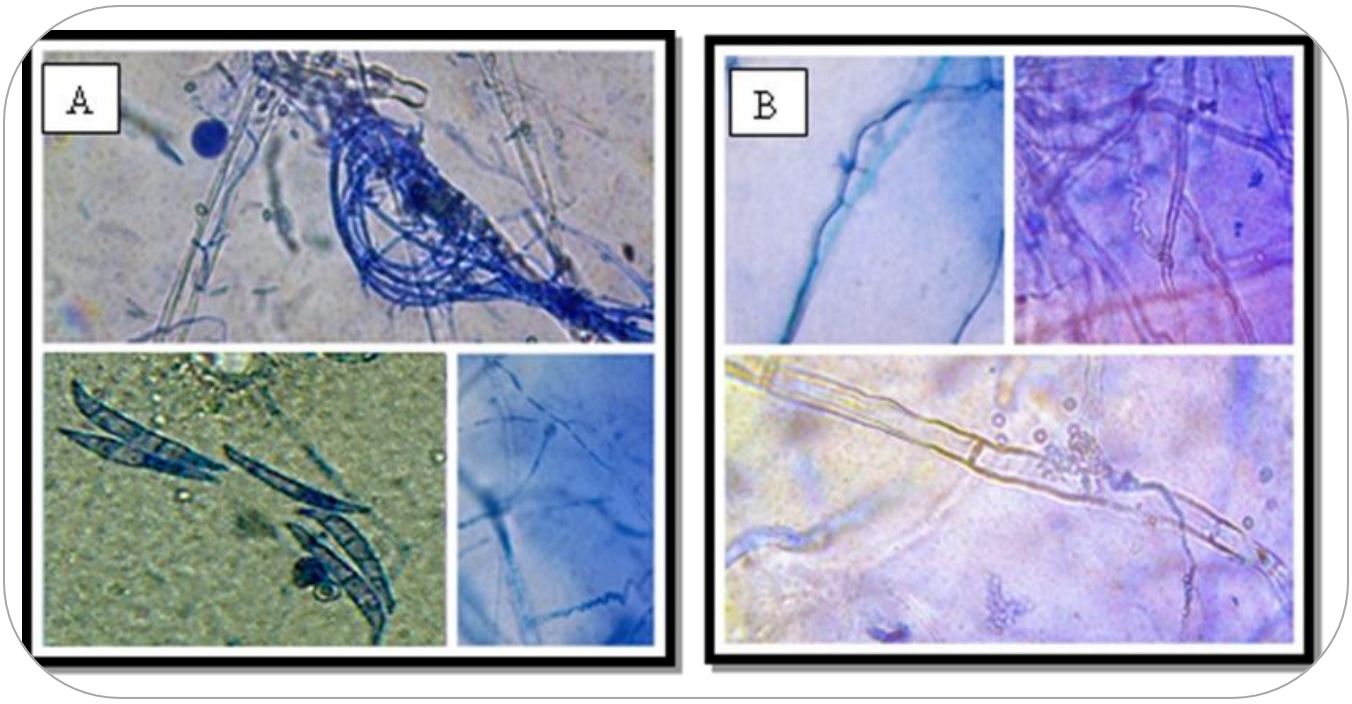

Figure 3. Morphological abnormalities of hyphae and spores of plant pathogenic fungi detected from the interaction zone of dual-culture plate on PDA medium.

A- Coiling of hyphae *F.oxysporum* by *Trichoderma* isolates, spore alterations with increased size and coagulation of the cytoplasm, Lysis of hyphae,

B- Hyphal contact and coiling of hyphae *Rhizoctonia solani* by *Trichoderma harzianum*, lysis and loss of cytoplasmic integrity.

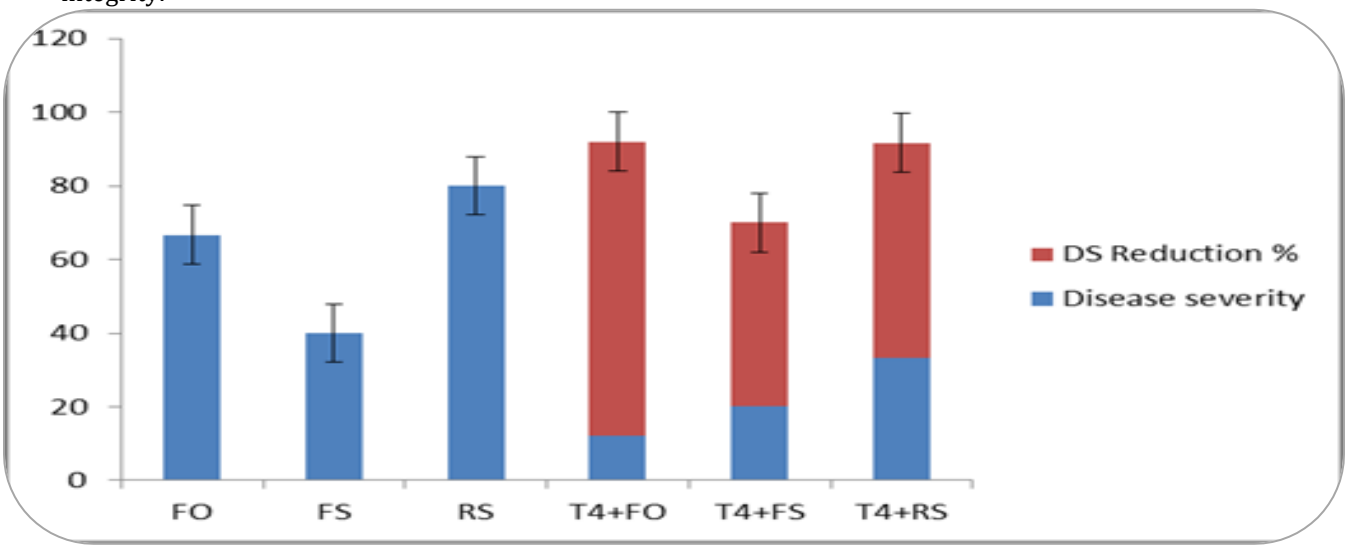

Figure 4. The percentage of growth inhibition

***In vivo* efficacy of some isolates of Trichoderma spp. on vegetative development parameters:** Effects of *Trichoderma* strains mixed together on young olive plants on some growth parameters in pots under artificial infections conditions were studied. The results obtained in Figure 5 revealed relatively high percentage of the development parameters: plant height (cm), number of leafy plants -1 (newly formed), stem diameter

(cm) and fresh and dry weight (g of plant -1) in infected plants compared to the control treatment. The evaluation of growth parameters tested are highly significantly with the mixed inoculation of *Trichoderma* spp. isolates compared to the uninoculated control. The differences were statistically significant p = 0.029. Our results show that the four *Trichoderma* isolates mixed were able to stimulate plant growth parameters (Fig.6). This stimulation resulted mainly in better axial growth and a larger biomass. Stimulation of biomass has been observed not only in the aerial parts, but also in the root parts.

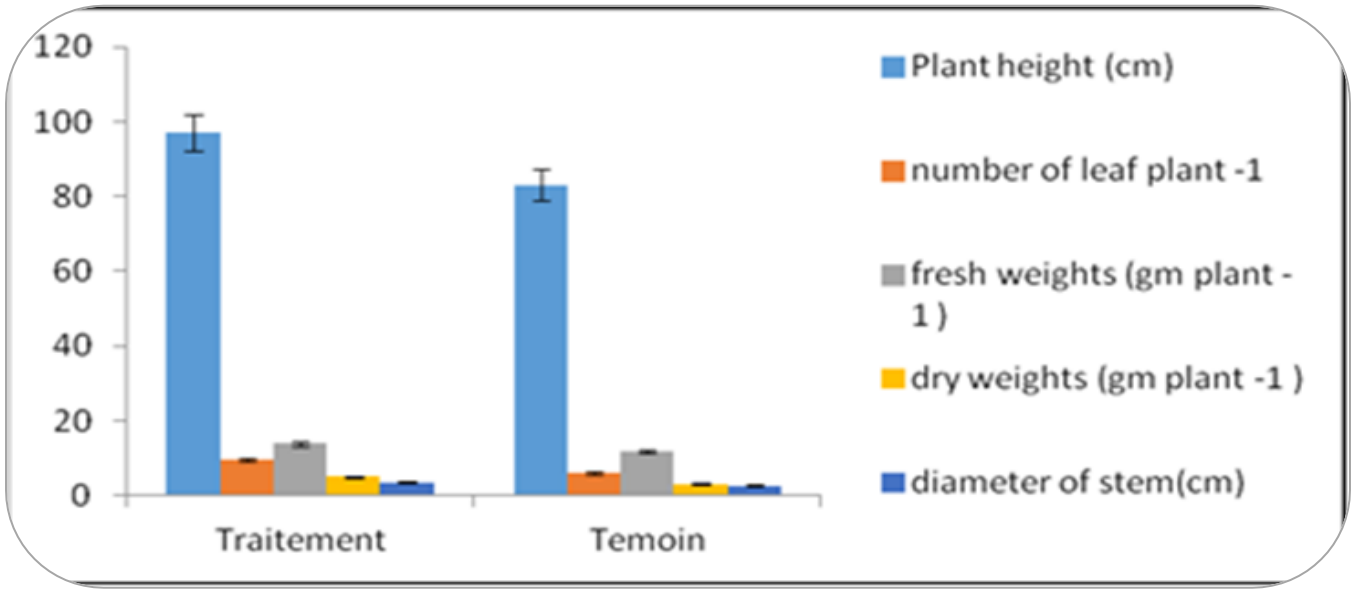

Figure 5. Effects of *Trichoderma spp*. strains mixed together on some growth parameters of olive plants

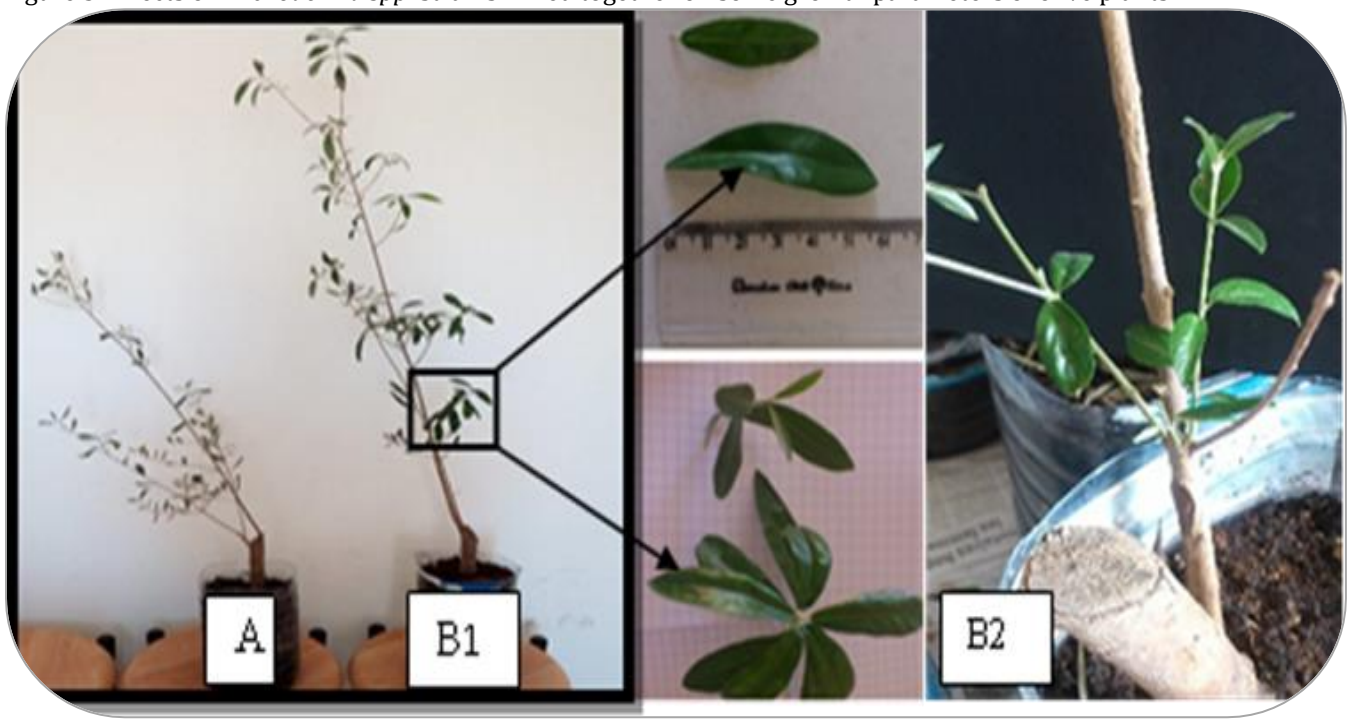

Figure 6. In vivo efficacy of *Trichoderma spp*. on vegetative development parameters
A- Uninoculated control
B1- Inoculated with *Trichoderma spp.*
B2-Emergence of new shoots

DISCUSSION

Surveys carried out in the olive nurseries have revealed a drying out of branches and twigs total or partial, defoliation of branches and twigs and sometimes discolouration of leaves causing drying new shoots. All these symptoms were those of dieback of the olive plants. In the most severe cases, this decline causes death. The dieback of the olive tree is a deadly vascular disease for the olive tree; it is caused by a complex of telluric fungi: *Rhizoctonia bataticola*, *Rhizoctonia solani*, *Armillaria mellea*, *Fusarium solani*, *Corticon rolfsi*, *Fusarium oxysporum*, *Phytophtora megasperma*, *Corticon solani*, *Rosellinea necatrix*. These fungi act synergistically, each taking part in the infection (Boulila, 2011). Mycological analysis of olive trees collected in different olive tree nurseries in northwestern Algeria has shown that several isolated fungal species have been reported around the world. Jardak *et al.* (2007) reported that *Verticillium dahliae*, *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani* were the cause of decline of olive trees. In various Mediterranean's states, telluric fungi cause significant damage (Al-Ahmad, 1984; Porras Soriano *et al.,* 2003). All *Trichoderma* isolates tested stopped mycelial growth of pathogens. *T. harzianum* (T4) exhibited the most rapid antagonistic effect by inhibiting the growth of pathogens after only 2 days of incubation. The mechanism of inhibition may be to concurrence for nutritious and space. *Trichoderma* isolates grow rapidly and are able to invade the culture medium after 4 days of incubation. They have a faster growth rate than pathogens. The invasion of *Trichoderma* was rapid and intense. Sharma. (2011); Fotoohiyan *et al*. (2017); Amer and Osama. (2018) made the same observations. During the interaction between pathogens and *Trichoderma spp*., winding and a lysis of hyphae of pathogens were observed. The winding process is linked with internal mycoparasitism. The same results were obtained with Trichoderma capable of rolling on the *Rhizoctonia solani* mycelium causing the disintegration of the cytoplasm of the pathogen**.** The degradation of the mycelial walls, noticed following the winding phenomenon, is doneto the presence of enzymes as chitinases, proteases β (1, 3), glucanases and sometimes cellulases **(**Kamala and Indira, 2012). Mycoparasitism of *Trichoderma* isolates has been implicated in a number of phytopathogenic fungi. Indeed, each of the antagonist species has a particular ability to eliminate the pathogen. Different modes of action, antibiosis and parasitism can be involved, simultaneously or sequentially (Howell, 2003). In addition, the effectiveness of a biocontrol agent is based an association of various process of action (Alabouvette *et al.,* 1993). Having a wide range of different action against pathogens is one of the fundamental criteria that characterizes the ideal antagonist.

The results of the greenhouse study were very encouraging and showed an important reduction in disease index by the T4 isolate on the three pathogens tested. Many studies have explained bio protection and stimulation of plant development following the application of *T. harzianum* by pathogen inhibition and activation of the plant's defence system by inducing systemic or localized resistance in these plants (Yedidia, 2000; Howell, 2003; Brunner *et al.*2005; Tran 2010**).** The improvement of plant vegetative growth by *Trichoderma spp.* was observed by Windham *et al.* (1986) showed that the application of two Trichoderma species to the sterilized growing medium could improve the dry weight of the roots and aerial parts of tomatoes and tobacco. The use of different Trichoderma spp. has stemmed in a substantial reduction of various soil fungi like: *Rhizoctonia* sp., *Sclerotinia* sp, *Fusarium* sp., *Pythium* sp., *Phytophthora* sp.(Jijakli ,2003).

The work of Mouria *et al.* (2008) demonstrated the ability of *Trichoderma* species to stimulate plant growth independently of any pathogen, which is also confirmed in our study. This stimulation is due to the influence of *Trichoderma* strains on the metabolism and enzymatic activity of plants and not only on the defence systems (Greval *et al.,* 2007). Trichoderma can produce phytohormones such as AIA (indole acetic acid) that can act directly on improving plant growth. These phytohormones act by reinforcing or accelerating the development of both the root system and the aerial part of the plants (Salas-Marina *et al.*, 2011). These phytohormones act by reinforcing or accelerating the growth of root system and aerial part of the plants.The use of *T. harzianum* (T-203) as a treatment of cucumber plants in the soil showed significant increases in root area, leaf area, shoot length and dry weight compared to the untreated control (Yedidia *et al,* 2000**;** Howell, 2003; Rahman *et al.*,

2018).*Trichoderma* isolates are applied for soil application, seed bio-priming, seed coating, and foliar pulverization. For the command of plant diseases, use of Trichoderma preparations with strain induce better than individual strains (Kumar *et al.,* 2016).The importance of the biological control procedure depends on the strains of Trichoderma spp., pathogen, plant to be treated, environmental conditions, pH, temperature and availability of iron and nutrients in the soil (Benítez *el al.*, 2004).

CONCLUSION

The study showed that the use of *Trichoderma spp*. is easier when the work is done under controlled conditions, such as greenhouses, their application can be combined with a chemical treatment at reduced dose and frequency. The *Trichoderma* biopesticide can be incorporated directly into soil or composts for nursery soils as preventive and curative treatments. *Trichoderma* species can be used successfully to stimulate plant growth independently of any pathogen.

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Merzoug Aoumria	:	Conceive idea, conduct research and write manuscript.
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