

Evaluation of *Trichoderma harzianum* as a biocontrol agent against vascular fusariosis of date palm (*Phoenix dactylifera* L.).

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Abstract: Date palm (*Phoenix dactylifera* L.) is qualified as a 'tree' of great ecological and socio-economical importance in desert oases. Unfortunately, this tree suffers from vascular fusariosis commonly named bayoud, caused by *Fusarium oxysporum* f.sp. *albedinis* (*Foa*). Bayoud is the most destructive fungal disease of date palm (*Phoenix dactylifera* L.). The impact of this disease is most severe in North Africa particularly in Morocco where 2/3 of palm trees were destroyed so far (Fernandez *et al.*, 1995; Fernandez *et al.*, 1995). In this work, *Trichoderma harzianum* was tested to determine its effect on the mycelial growth of *Fusarium oxysporum* f. sp. *albedinis* (*Foa*) in dual culture and its control of vascular fusariosis disease in pot-grown date palm sets. In vitro and in vivo antagonistic effect of *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *Albedinis*, tests of direct confrontation, on PDA medium or remote confrontation, between *Fusarium oxysporum* f. sp. *albedinis* and *Trichoderma harzianum*, revealed that the latest has inhibited mycelial growth of the pathogen by more than 65% compared to the control and this after an incubation period of about four days at 25°C. Moreover, beyond this period and after six days, *T. harzianum* invades and also sporulates on *Fusarium oxysporum* f. sp. *albedinis* colonies revealing its high myco-parasitism. Some In pot experiments, where the soil was inoculated with a pathogenic isolate of *Foa* and a biocontrol agent *T.harzianum*. *T. harzianum* inhibited mycelial growth of the pathogen in vitro.... This study suggests the possible role of *T. harzianum* in the induction of antifungal compounds against *F. oxysporum* f. sp. *Albedinis*.

Key words:

INTRODUCTION

Traditional methods used to protect crops from diseases have been largely based on the use of chemical pesticides. Applications of fungicides and fumigants can have drastic effects on the environment and consumer, and are often applied in greater quantities than herbicides and insecticides in agricultural production (Vinale & al., 2008). Chemical methods are not cost effective in the long run because they damage the environment, leave harmful residues, pollute the atmosphere, and can lead to the development of resistant strains among the target organisms with repeated use (Naseby & al., 2000). That's why the reduction of the use of synthetic pesticide in agriculture is highly desirable and one of the most promising means to achieve this goal is by the use of new tools based on biocontrol agents which involves the use of beneficial organisms, their genes, and/or products, such as metabolites, that reduce the negative effects of plant pathogens and promote positive responses by the plant.

In this context, a complementary approach for managing the diseases caused by *F.oxysporum* involves the use of micro-organisms such as bacteria, yeast or saprophytic fungi that have either a significant potential to inhibit the disease agent or the ability to induce defence mechanisms (Benhamou & Nicole, 1999; De Boer & al., 1999; El Hassni & al., 2007; Coskuntuna & N. Ozer, 2007). Among those biological control agents, *Trichoderma spp.* have attracted attention for controlling various soil-borne fungi including

The potential of *Trichoderma* species as biocontrol agents of plant pathogens was first recognized in the early 1930s (Weindling, 1932) and they were subsequently applied successfully as biocontrol agents against several plant diseases in commercial agriculture (Howell, 2003). *Trichoderma* has a considerable efficiency against many pathogenic fungi, e.g. *Fusarium* (Rojo & al., 2007), in a wide range of environmental conditions (Chet, 1987). Preformed antifungal compounds such as water-soluble phenols and flavones appear to constitute an important resistance factor preventing spore germination and penetration of potential fungal pathogens (Link & Walker, 1933). Accumulation of antifungal compounds is one of several biochemical defence responses in plants attacked by pathogens (Nicholson & Hammerschmidt, 1992; Hunt & *et al.*, 1997).

The present investigations evaluated antagonistic effect of *Trichoderma harzianum* against the *Fusarium oxysporum* f.sp. *albedinis* causal agent of vascular fusariosis of date palm *in vitro* and *in vivo*. In these experiments, *Trichoderma harzianum* was applied singly and evaluated for its capacity to control date palm fusariosis.

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MATERIALS AND METHODS

2.1 Biological materials:

a) Plant material:

Seeds of the date palm variety “Boufeggous Gharas” (a very sensitive variety to Bayoud) are disinfected with bleach and are washed several times with tap water then put into water pulp for 24 hours. These seeds, thus treated, were germinated in plastic containers, filled with wet peat and incubated in a greenhouse under a 16h-light-regime and 60 – 70% RH at 25°C for 10 weeks. At the stage of a leave, seedlings were planted in pots of 2 l. containing various substrates (10 pots / substrate).

b) Fungal isolates:

The pathogen:

The isolate of *Fusarium (Foa)* used in this study was isolated from naturally infected date palm plants showing symptoms of fascicular fusariosis and it was maintained on potato dextrose agar (PDA).

The biocontrol agent:

The antagonist agent used to fight against *F. oxysporum* f. sp. *Albedinis* is *Trichoderma harzianum*; it was isolated from a **sample of Northern Moroccan soil** and it was maintained on potato dextrose agar (PDA).

2.2 Working methodology:

2.2.1 Antagonistic activity in vitro of *T. harzianum* against *F. oxysporum* f. sp. *Albedinis* (*Foa*):

The in vitro antagonistic activity of *T. harzianum* was studied by two methods.

1. Dual culture:

➤ **Equidistant confrontation:** this method consists in co-culturing the pathogen and the agent antagonist in the same dish at opposite sides on PDA plates. In this test, Petri dishes (9 cm) containing PDA were inoculated with 0.5 cm diameter mycelial discs of 7-day-old cultures of *Foa* and *T. harzianum* at equal distance from the plate periphery. The incubation is carried out at 25±2°C for 7 days in an incubator. The percentage of inhibition was evaluated by measuring the *Foa* colony radius of the presence of *T.harzianum*. The radial growth of *Foa* was measured after 7 days of incubation. Petri dishes without antagonistic fungi (*T.harzianum*) were used as control. The assay was repeated three times and the experiment was conducted twice. The direct effect of the antagonist agent on the *Foa* mycelia was observed under light microscope.

➤ **Remote confrontation** (distant confrontation): this method consists in the transplanting of the antagonist agent and the pathogen in two separated plates which were connected by superposition: *Trichoderma harzianum* in the bottom and *Foa* on top. The plates were then incubated at 25±2 °C for 7 days. The antagonistic isolate was grown on a sterile cellophane disk lying on PDA in 9 cm Petri dishes for 48 h. The cellophane with the mycelium was removed in the same position in which the microorganism was grown and a mycelial plug was inoculated with a 7 cm-diameter pathogen. The junction between the two plates is provided by parafilm to prevent loss of volatile substances (Daami-Remadi, El Mahjoub, 2001). The diameter growth of the pathogen colonies was determined after 72 h and was compared with that of the plant pathogen grown on PDA without metabolites (control). The control is formed by superposition of two plates, the one on the top containing one disk of *Foa*, whereas the one on the bottom contains ...

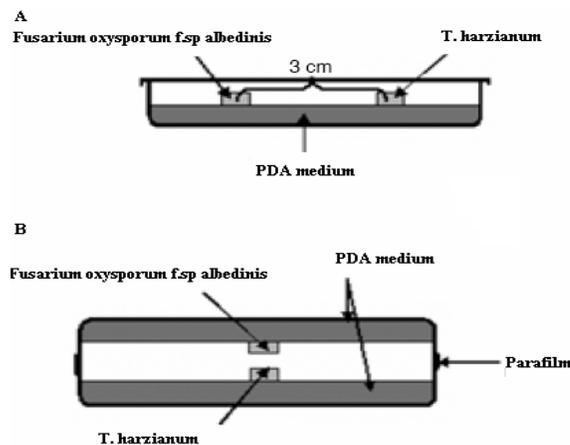


Fig. 1.A: Equidistant confrontation of *F. oxysporum* f. sp. *albedinis* and *T. harzianum*. **B.** Remote confrontation between *F. oxysporum* f. sp. *albedinis* and *T. harzianum*.

The measurement of the average diameter of the colonies is performed when the hyphae reaches the edge of the control dishes. The evaluation of the inhibition exerted by *T.harzianum* is estimated by calculating the percentage of inhibition of the mycelial growth according to the following formula (Hmouni *et al.*, 1996):

$$I (\%) = (1 - C_n/C_o) \times 100$$

Where **C_n** is the average diameter of colonies in the presence of the antagonist and **C_o** the average diameter of the control.

2.2.2 In vivo antagonistic activity of *T. harzianum* against *F. oxysporum* f. sp. *Albedinis* (Foa):

In order to test the antagonistic effect of *T.harzianum* against *F. oxysporum* f. sp. *Albedinis*, an *in vivo* test is carried out.

Preparation of the seedlings:

Seeds of the date palm variety "Boufeggous Gharas" (variety very sensitive to Bayoud) are disinfected with bleach and are washed several times with tap water, then put in water pulp for 24 hours. These seeds, thus treated, are germinated in plastic containers, filled with wet peat and incubated in a greenhouse under a 16h-light-regime and 60 – 70% RH at 25°C for 10 weeks. At the stage of a leave, seedlings were planted in pots of 2 L containing various substrates (10 pots / substrate).

Preparation of the *Fusarium oxysporum* f.sp *albedinis* (Foa) inoculum:

The *Fusarium oxysporum* f.sp *albedinis* (Foa) used in this study was isolated from Bouffaggous Gharas palm rachis infected by the vascular fusariosis. This isolate was preserved in ammonium oxide according to the method of LOCKE and COLHOUNLHOUN (1974). A liquid medium containing 1.5 L of the sterile Czapek medium was also sown with a *Foa* isolated from a date palm infected by the vascular fusariosis. This culture was incubated at 27°C with continuous agitation for 4 weeks. The conidies of this culture were separated from the mycelium filaments by a 2000 tr/min centrifugation and then counted on a Malassez cell under a binocular optical microscope. The conidies suspension of *Foa* thus prepared was used to infect the substrates with an accurate 350,000 UFC/ml mixture of the dry substrate.

Preparation of the *Trichoderma harzianum* inoculum:

The *Trichoderma harzianum* used in this study was isolated from a sample of Northern Moroccan soil and it was maintained on potato dextrose agar (PDA). A liquid medium containing 1.5 L of the sterile Czapek medium was also sown with *T. harzianum*. This culture was incubated at 27°C with continuous agitation for 4 weeks. This culture was centrifuged at 2000 tr/min to separate the filaments from spores. The spores suspension of *T. harzianum* thus prepared was used to inoculate the substrates rightly.

Preparation of the substrates:

Four types of substrates were prepared from the peat mixed with the vermiculite (2/3 peats + 1/3 vermiculite): a control substrate not inoculated (Tt), a substrate inoculated with *T. harzianum* (*Tricho*), a substrate infected by *Foa* (*Foa*) and a fourth substrate inoculated with *T.harzianum* and infected by *Foa* (*Tricho+Foa*).

Estimate of the treatments' effect:

The measurements taken at the end of 12 months of cultures for each substrate are the death rate, the median number of the leaves, the average length of the areal and root parts and their fresh and dry weights for the Boufeggous Gharas seedlings. In order to Isolated of the disease-causing agent starting from the dead seedlings and to confirm the attacks due to *Foa*, the research of the pathogen was carried out on dead seedlings. From three cut palms of each seedling, small pieces of approximately 0.3 cm³ are taken then placed on PDA medium. These cultures were incubated at 27°C for 6 days, then under daylight for 4 to 6 days. Six repetitions were carried out for each seedling.

2.2.3 Chlorophyll:

In this study we used the Laser Induced Chlorophyll Fluorescence (LICF) (Hartmut Lichtenthaler, 1985; Giovanni & al, 1995; Maxwell and Johnson, 2000) to show the important effect of the *T.harzianum* agent to keep the plant healthy and inhibit the effect of *Foa*. For this, we exploited the LICF spectroscopy technique, which is a powerful tool in plant physiology and agriculture, and is also used to track and evaluate stress levels or physiological damages of plants. A LICF setup was applied to investigate the health of three types of palm leaves: a control substrate not inoculated (Tt), a substrate inoculated with *T. harzianum* (*Tricho*) and a substrate inoculated with *T.harzianum* and infected by *Foa* (*Tricho+Foa*). In different chlorophyll fluorescence spectra, we calculated the ratio of the red to far red fluorescence, as this parameter can be considered as a good indicator of stress and chlorophyll content. The plant is healthy when the leaf of date palm is rich in chlorophyll, while it is stressed if not.

2.2.3.1 Fluorescence spectroscopy:

➤ Experimental setup

Spectroscopy measurements of chlorophyll fluorescence of plants were performed by irradiating leaf date palms with a continuum laser He-Ne (632,8 nm) (**figure 2**) as source of excitation which produces an output power of 2 mW. The leaf date palm was placed at a distance of 20 cm from the output laser. The spectrum acquisition was done in reflection mode. An optical fiber (SMA905) with a 400 μ m diameter and 2 m length was used to guide the collected fluorescence light to the spectrometer (AVS-USB2000). In order to collect the maximum fluorescence, the optical fibre was placed at a certain angle with regard to the incident beam of the laser and in the same position for all the samples.

The spectrometer displaying the fluorescence spectrum in wavelength range from 500 to 110 nm is connected with an electrical cable to the computer's USB port. In order to record the spectrum the computer is equipped by software provided by Avantes Firm. The data are treated and analyzed by the Origin software.

All measurements were made in the dark and at room temperature. In average, 5 spectrum of 200 ms integration time are taken for each sample. In such conditions, the green plants are expected to possess two bands around 690 nm (red) and 735 nm (far red).

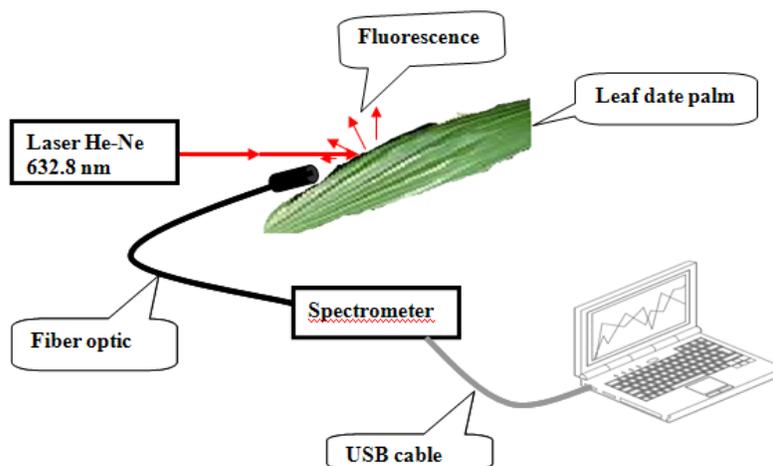


Fig. 2: Chlorophyll fluorescence setup using He-Ne as source of excitation of the chlorophyll molecule in date palm leaf

RESULT AND DISCUSSION

Effect of T. harzianum against Fusarium oxysporum f.sp albedinis causal agent of fascicular fusariosis of date palm:

Dual culture:

Isolates of *Trichoderma spp.* have been demonstrated to be antagonistic toward a number of fungi (Howell, 2003 ; Mark Schubert & al., 2008). This filamentous fungus is one of the most potent agents for the biocontrol of plant pathogens. In the present work, a hierarchical set of assays including dual culture tests on PDA media and in vivo tests were used to identify the antagonistic activity of *Trichoderma harzianum* against *Fusarium oxysporum f.sp albedinis*. In the dual culture tests, hyphal contact between *T.harzianum* and the pathogenic agent *Fusarium oxysporum f.sp albedinis* was observed. In the present work, not only directed growth but also an induced hyphal branching of *T.harzianum* was observed and the equidistant confrontation showed a faster growth of *T. harzianum* isolates than of *Foa* (phtograph 1 & 2) and the ratio of inhibition was 67% (figure 11). Previously, in vitro studies have shown that, due to chemotropism *T.harzianum*, hyphae grew and branched directly towards their host (Chet, 1987).

Pot experiment:

The effect of *T.harzianum* on the growth was measured by the determination of the dry weight, the fresh weight of the seedling air parts and root parts (Figure 4, 5, 6 & 7) and also the length of the date palm seedlings root and air parts (Figure 8 & 9). It arises from the results obtained that *T.harzianum* improved the growth of the seedlings by approximately 20% from the control. *T.harzianum* also showed a protective effect against the attacks of *Foa* in pot-experiment. The presence of the disease-causing agent caused a death rate of 100%, whereas the presence of *T.harzianum* causes a drop in this mortality rate of up to 50% (Table1). Different mechanisms have been suggested as being responsible for *T.harzianum* biocontrol activity, which include

competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Haram and al. 1996; Zimand and al. 1996). Also, previous studies have demonstrated that before mycelia of fungi interacted, *T.harzianum* produced low quantities of extracellular exochitinases (Kullnig & al., 2000; Brunner & al., 2003). The diffusion of these enzymes dissolved cell fragments of host cells. These cell fragments in turn induced the production of additional enzymes and triggered a cascade of physiological changes, stimulating a direct and rapid growth of *T.harzianum* (Zeilinger & al., 1999).

The beneficial effect of *T. harzianum* has been reported also by Sivan and al. (1987) who showed that the coating of the tomato seeds using this antagonist reduced by 80% the fusarium attack of root and crown rot of tomato. Similar results were obtained by Hjeljord and al. (2001), who showed that the application of quiescent conidia of *T. harzianum* on Strawberry flowers allowed the reduction of 85% of *Botrytis cinerea* attacks at a temperature of 24 ° C.

In this same sense, Yedidia *et al.* (1999) reported that the application of *T.harzianum* for a hydroponic melon culture resulted in a good development of treated plants compared to plants not treated with *Trichoderma*. This reflects an activation of the plant defence system, an increased activity and a chitinase and peroxidase which increase the enzymatic activity in leaves, which induces a systemic resistance for these plants.

Chlorophyll fluorescence spectra:

All the date palm planted in the soil only with *Foa* fungi were completely dried. The chlorophyll fluorescence results of all other samples are summarized in **figure 3**.

The red spectrum represents the average of all the fluorescence spectra of control samples. The green color is the average of all fluorescence spectra of leaf date palm developed in *Foa* and *T.harzianum* fungus and the black color is the average of leaf's date palm developed in the presence of *T.harzianum* fungus.

The spectra exhibit the typical double peak curve, with maxima at about 690 nm and 735 nm.

A slight difference between the peak amplitudes shows that all samples contain chlorophyll and can be considered as physiologically healthy, thanks to the *T.harzianum* fungus inhibiting the *Foa* activity.

The use of *T.harzianum* is the best and cleanest solution to develop the date palm attacked by *Foa* (Bayoud).

Therefore, *T.harzianum* is the best solution to protect palm trees which are attacked by *Foa*. It's also an efficient, simple, non expensive and environmentally friendly means.

We have mentioned that the ratio of the red to far red fluorescence F690/F735 is a good indicator of stress and chlorophyll content (G. Agati and al,1995; G. Agati and al, 1995; A. A. Gitelson an al, 1998; Zsolt Csintalan and al, 1998; Doreen Thoren and al, 2010). The results of this ratio for the three spectra are indicated in table 2.

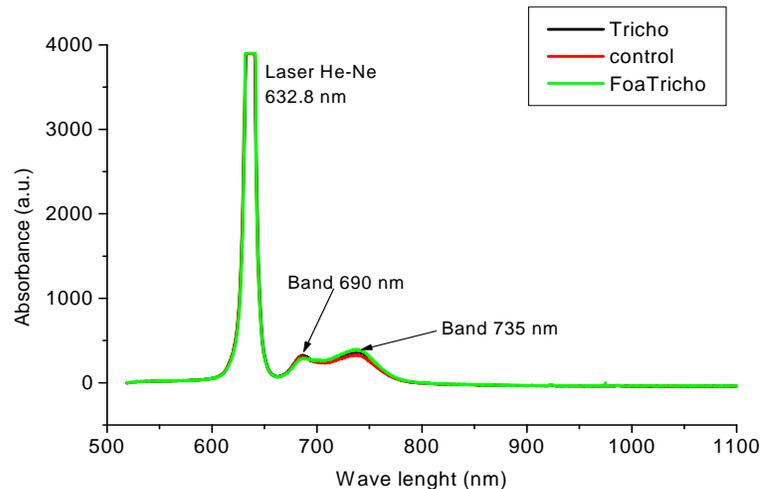
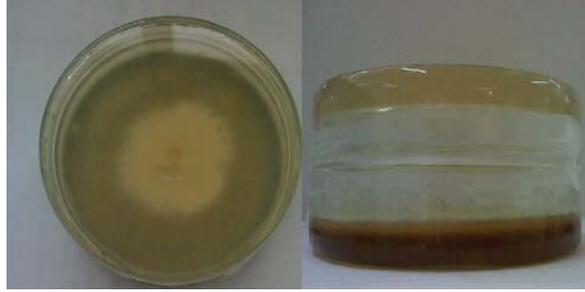


Fig. 3: Chlorophyll fluorescence spectrum of date palm leaves, excited by He-Ne laser at 632.8 nm



Photograph 1: Remote confrontation between *F. oxysporum f. sp. albedinis* and *T. harzianum*.



Photograph 2: Equidistant confrontation test of *F. oxysporum f. sp. albedinis* and *T. harzianum*.

We can notice that the values are almost similar; the small difference between the ratio of red to far red band may be due to a re-absorption of fluorescence by chlorophyll (Giovanni Agati and al, 1993). The shape of the chlorophyll fluorescence spectra and the value of the ratio of fluorescence intensities at the two maxima are related to the chlorophyll content of the leaf. The ratio is not strongly influenced by using *T.harzianum* alone or by *T.harzianum* and *Foa* together. Eventually, we concluded that *T.harzianum* played the role of a fertilizer and, at the same time, as an assailant of *Foa* in order to keep the plant healthy.

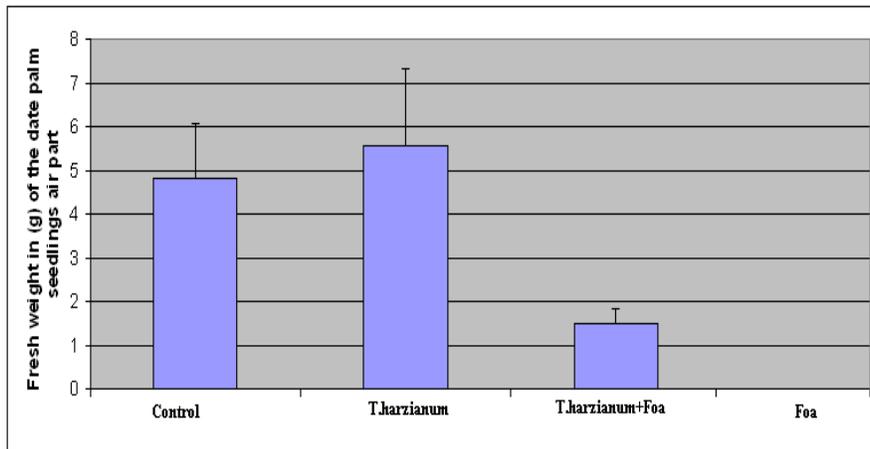


Fig. 4: Fresh weight in (g) of the date palm seedlings air part cultivated under different treatments. Control: Peat without any treatment (pilot); T.harzianum: Peat inoculated by the biocontrol agent *Tricherma harzianum*; T.harzianum+Foa: Peat inoculated by Foa and the biocontrol agent *Tricherma harzianum*; Foa: Peat inoculated by Foa.

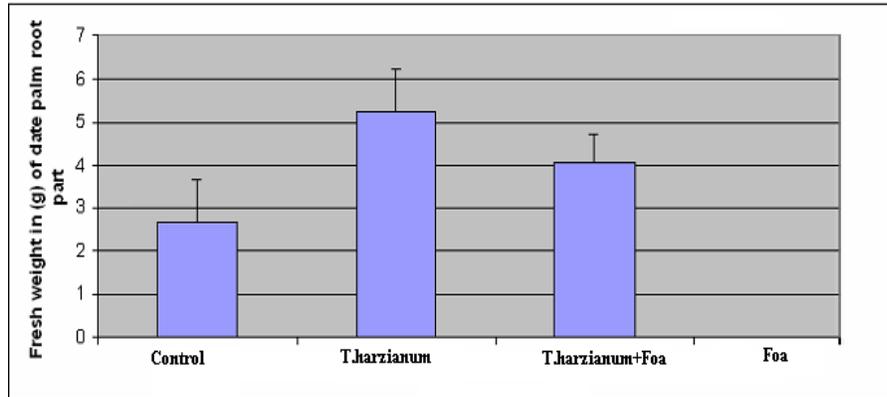


Fig. 5: Fresh weight in (g) of the date palm seedlings root part cultivated under different treatments. Control: Peat without any treatment (pilot); T.harzianum: Peat inoculated by the biocontrol agent *Tricherma harzianum*; T.harzianum+Foa: Peat inoculated by Foa and the biocontrol agent *Tricherma harzianum*; Foa: Peat inoculated by Foa.

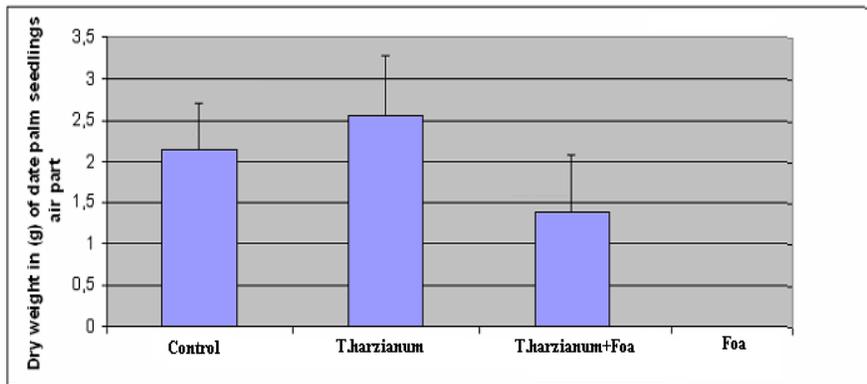


Fig. 6: Dry weight in (g) of the date palm seedlings air part cultivated under different treatments. Control: Peat without any treatment (pilot); T.harzianum: Peat inoculated by the biocontrol agent *Tricherma harzianum*; T.harzianum+Foa: Peat inoculated by Foa and the biocontrol agent *Tricherma harzianum*; Foa: Peat inoculated by Foa.

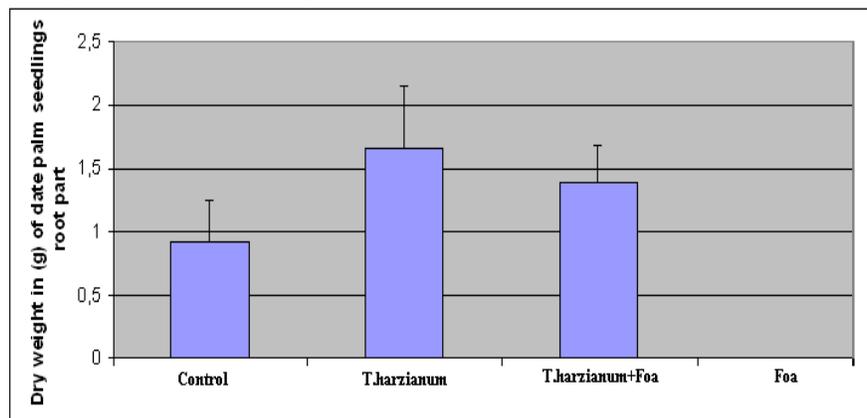


Fig. 7: Dry weight in (g) of the date palm seedlings root part cultivated under different treatments. Control: Peat without any treatment (pilot); T.harzianum: Peat inoculated by the biocontrol agent *Tricherma harzianum*; T.harzianum+Foa: Peat inoculated by Foa and the biocontrol agent *Tricherma harzianum*; Foa: Peat inoculated by Foa.

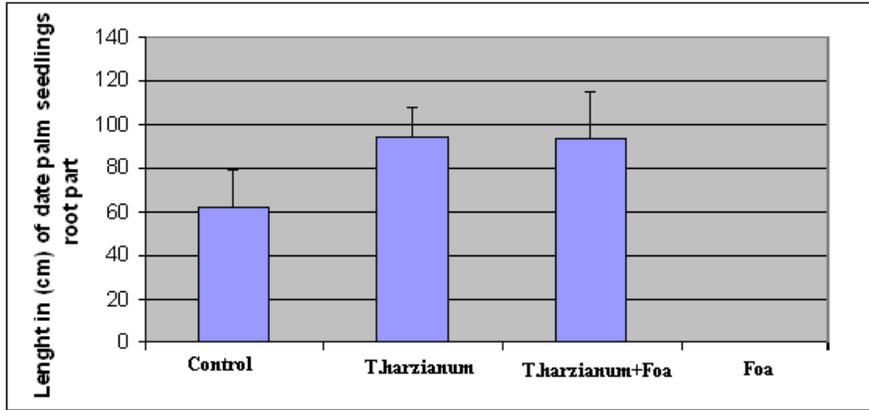


Fig. 8: Length in (cm) of the date palm seedlings root part cultivated under different treatments. Control: Peat without any treatment (pilot); T.harzianum: Peat inoculated by the biocontrol agent *Tricherma harzianum*; T.harzianum+Foa: Peat inoculated by Foa and the biocontrol agent *Tricherma harzianum*; Foa: Peat inoculated by Foa.

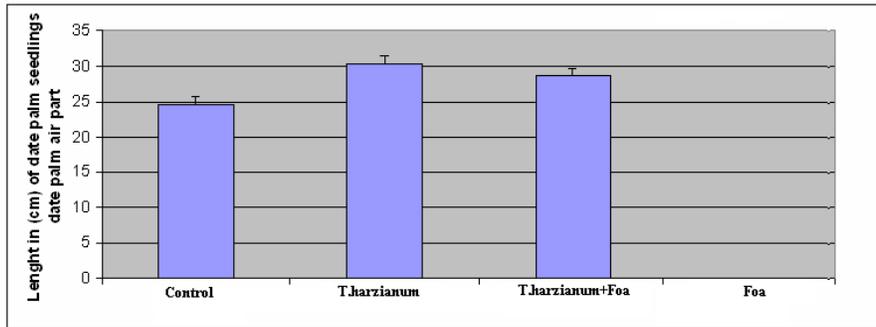


Fig. 9: Length in (cm) of the date palm seedlings air part cultivated under different treatments. Control: Peat without any treatment (pilot); T.harzianum: Peat inoculated by the biocontrol agent *Tricherma harzianum*; T.harzianum+Foa: Peat inoculated by Foa and the biocontrol agent *Tricherma harzianum*; Foa: Peat inoculated by Foa.

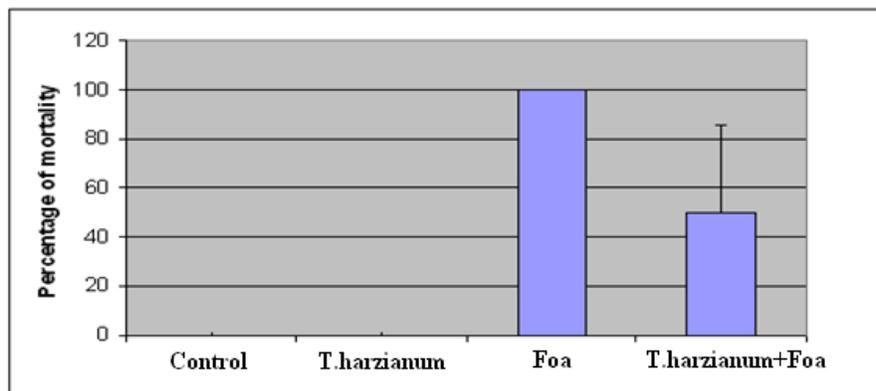


Fig. 10: Death rate of date palm seedlings cultivated under different treatments. Control: Peat without any treatment (pilot); T.harzianum: Peat inoculated by the biocontrol agent *Tricherma harzianum*; T.harzianum+Foa: Peat inoculated by Foa and the biocontrol agent *Tricherma harzianum*; Foa: Peat inoculated by Foa.

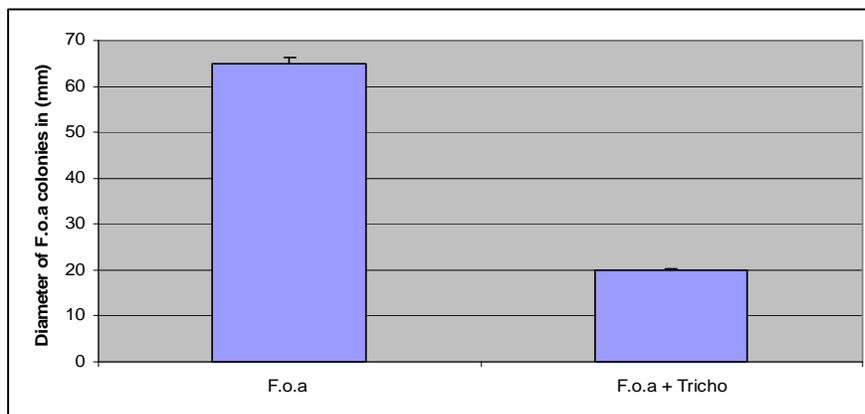


Fig. 11: Diameter of *F. oxysporum f. sp. albedinis* colonies in the presence of *T. harzianum* at six days after incubation at 25°C comparatively with the untreated control.

Table 1: Rate of mortality of date palm infected by *F.o.a*

	Rate of mortality (%)
Témoin	0
Tricho	0
F.o.a	100
Tricho+ f.o.a	50

Table 2: that the ratio of the red to far red fluorescence F690/F735

Samples	Control	Leaves with <i>Tricho</i>	Leaves with <i>Foa</i> and <i>Tricho</i>
F690/F735	0.8	0.9	0.7

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