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Effect of Two *Trichoderma* species on Severity of Potato Tuber Dry Rot Caused by Tunisian *Fusarium* Complex*

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Abstract: *Trichoderma harzianum* and *T. viride* were evaluated for their antagonistic activity against *F. oxysporum* f.sp. *tuberosi*, *F. solani*, *F. graminearum* and *F. sambucinum* causing potato dry rot in Tunisia. *In vitro* dual culture experiments, observed after incubation at 25°C for 6 days, showed that both tested *Trichoderma* species significantly reduced mycelial growth of *Fusarium* spp. comparatively to untreated controls and that a significant interaction was noted between both fixed factors (at $p \leq 0.05$). Light microscopic studies of antagonists × *Fusarium* spp. *in vitro* interactions showed lesser mycelium density, severe lysis, lesser pathogen sporulation, mycelial cords formation and early chlamydospores induction which were observed only at the confrontation zone of both microorganisms. Potato tubers, cv. Spunta, individually treated, at inoculation sites by 100 µL of *Trichoderma* spp. suspensions (10^8 spores mL^{-1}), 24 h prior inoculation by *Fusarium* species, showed reduction in dry rot development, after 21 days of incubation at 25-27°C, comparatively to untreated controls.

Key words: Biocontrol, *Trichoderma*, *Fusarium* spp., interaction, *Solanum tuberosum* L

Introduction

Increasing expectations are emerging in the area of plant disease management for new strategies that have the potential to be efficient, reliable and safe for the environment. *Trichoderma* spp. have been reported to control soil-borne plant pathogens such as *Rhizoctonia solani* Khun., *Sclerotium sclerotiorum* (Sacc.) Curzi., *Pythium* sp., *Stereum purpureum*, *Botrytis cinerea*, *Phomopsis viticola* and *Fusarium* spp. (Ponchet, 1982; Cooney and Lauren, 1998; Escande *et al*., 2002; Thangavelu *et al*., 2004).

Fungi of the genus *Trichoderma* shown antagonism against several plant pathogens by antibiosis, competition for nutrients and hyperparasitism. *T. viride*, *T. harzianum*, *T. hamatum*, *T. pseudokoningii* are the most common species (Ponchet, 1982; Beagle-Ristaino and Papavizas, 1985).

In Tunisia, the absence of resistant potato cultivars to *Fusarium* and the other tuber rot agents and the absence of registered fungicides for control of these post-harvest problems (Anonymous, 2003; Daami-Remadi and El Mahjoub, 1996; Priou *et al*., 1997) together with the introduction of resistant isolates of *F. sambucinum* via contaminated seeds (Daami-Remadi and El Mahjoub, 2006) and pathogen's soil borne origin, justify necessity of searching for other alternatives for tuber protection.

Some benzimidazole fungicides, used in other countries for *Fusarium* dry rot control (Leach and Nielsen, 1975; Carnegie *et al*., 1990; Bang, 1992; Carnegie *et al*., 1998), showed varying interaction with Tunisian *Fusarium* spp. All studies dealing with biological control of potato tuber dry rot concerned in their majority one or two *Fusarium* species (Schisler *et al*., 2000). However, in Tunisia,

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F. solani, F. oxysporum f.sp. *tuberosi, F. sambucinum* and *F. graminearum* are the causal agents of this disease and they are frequently present as mixed infections (Daami-Remadi and El Mahjoub, 2004, 2006).

As *Trichoderma* species are known to inhibit several plant pathogens, two species are tested in the present study for their *in vitro* and *in vivo* antagonistic activity against the *Fusarium* complex causing potato dry rot in Tunisia.

Materials and Methods

Pathogens

F. solani, F. graminearum, F. sambucinum and *F. oxysporum* f.sp. *tuberosi* are isolated from tubers of cv. Spunta showing typical symptoms of dry rot. These *Fusarium* spp. are grown at 25°C on PDA for one week. They are stored at -20°C in 20% glycerol solution for long term preservation.

Potato Cultivars

Tubers cv. Spunta, the most cultivated in Tunisia, are used in this current study. They are obtained from the Technical Center of Potato of Tunisia, stored in darkness at 6°C and bought to room temperature three hours before use.

Trichoderma spp.

T. harzianum and *T. viride*, isolated from Tunisian soils, are tested for their antagonistic activity against *Fusarium* sp. They are cultured on PDA (Potato Dextrose agar), at 25°C, for one week. *T. harzianum* isolate showed efficacy against potato tuber leak agents *Pythium aphanidermatum* and *P. ultimum* (Daami-Remadi, 2001b) and also *Phytophthora erythroseptica* causing pink rot (Triki *et al*.,1996; Triki and Priou, 1997).

In vitro Antagonistic Activity of Trichoderma spp. Against Fusarium spp.

The antagonist×pathogen confrontation method is the dual culture of both microorganisms on PDA (containing streptomycin sulphate at 300 mg L^{-1}). Agar discs of 6 mm in diameter colonized by the pathogen or the antagonist are placed at 2 cm apart from the edge of the petri plate and equidistant of 5 cm. In control plates, pathogen agar discs are placed at the center. The incubation of plates is realized at 25°C and the mean diameter of the pathogen radial growth is noted after 6 days.

Mycelial growth and alteration of pathogen colony compared to the untreated control are also noted. The state of control and treated mycelium removed from the confrontation zone are observed under light microscope. Every elementary treatment is replicated eight times.

Statistical analysis are performed following a completely randomised factorial design where treatments (both *Trichoderma* species and untreated control) and *Fusarium* species are the fixed factors. Means are separated using Fisher's protected LSD test ($p \le 0.05$).

In vivo Antagonistic Activity of Trichoderma spp. Against Fusarium spp.

Tubers are superficially disinfected with a solution of 10% sodium hypochlorite, for 5 min and then rinsed abundantly with sterile distilled water. After air-drying, tubers are dipped in an alcohol solution (at 70%) then briefly blazed for elimination of surface pathogens (*Rhizoctonia solani* and others).

Container and alveolus plaques used for inoculated tubers incubation, are washed before use, dipped for 24 h in sodium hypochlorite solution and then rinsed with sterile distilled water.

As *Fusarium* spp. are wound tuber pathogens, *Trichoderma* spp. are applied by injecting 100 μL of a conidial suspension (10^8 conidia mL^{-1}) at sites of inoculation 24 h before pathogen application. Control tubers are treated similarly by sterile distilled water. Dimension of inoculation sites is of 6 mm diameter and depth. Inoculation technique consists of deposing an agar disc (6 mm

diameter) colonized by pathogen at occasioned wounds. Tuber incubation is realized at 25-27°C for 21 days at high relative humidity. Every elementary treatment is repeated twenty times (ten tubers × two wounds).

After incubation period, tubers were cut longitudinally via sites of inoculation. Parameters of dry rot induced (maximal width (w) and depth (d)) are noted. The pathogen penetration within tubers, is calculated following formula of Lapwood *et al*. (1984) where:

$$\text{Penetration (mm)} = (w/2 + (d - 6))/2$$

Statistical analyses (ANOVA) are performed following a completely randomised factorial design where treatments (both *Trichoderma* species and untreated control) and *Fusarium* spp. are both fixed factors. Means are separated using Fisher's protected LSD test ($p \leq 0.05$).

Results

For potato *Fusarium* dry rot biocontrol, two species of *Trichoderma* are tested *in vitro* and *in vivo* for their antagonistic activity against fungal complex actually responsible of this disease in Tunisia.

Effect of Trichoderma spp. on Mycelial Growth of Fusarium spp.

Growth of *F. solani*, *F. sambucinum*, *F. graminearum* and *F. oxysporum* f.sp. *tuberosi* is followed after dual culture with indigenous *Trichoderma* species. Mean diameter of *Fusarium* spp. colonies, formed after 6 days of incubation at 25°C, depends on tested treatments and *Fusarium* species; a significant interaction was observed between both factors at $p \leq 0.05$. Figure 1 showed that both *Trichoderma* species reduced, by more than 70%, mycelial growth of tested pathogens comparatively to untreated control.

Trichoderma spp. activity was traduced not only by reduction of pathogen radial growth expressed by a significant competition (Fig. 2), but also by mycelium disruption.

In fact, light microscopic studies showed that mycelium of tested *Fusarium* species, removed from the confrontation zone of both microorganisms, was severely altered by the action of *Trichoderma*. Treated *Fusarium* mycelium showed strong lysis, cytoplasm vacuolization, early chlamydospores formation (Fig. 4) and induction of mycelial cords via anastomosis between hyphal filaments (Fig. 3). Mycelium density and sporulation of *Fusarium* spp. at the confrontation zone are reduced, comparatively to controls.

Furthermore, light microscopic studies of *Fusarium* spp. × *Trichoderma* spp. *in vitro* interactions also showed rolling up of antagonist filaments around those of the pathogen, traducing typical mycoparasitism exerted by *Trichoderma* on *Fusarium* spp.

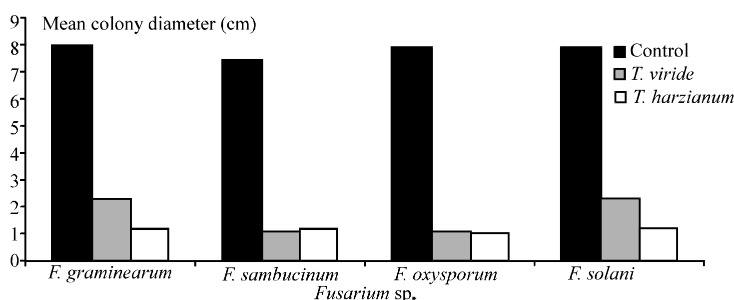

Fig. 1: Effect of *Trichoderma* spp. on mycelial growth of *Fusarium* spp. on PDA after incubation at 25°C for 6 days (eight replicates per elementary treatment). LSD (Treatments × *Fusarium* spp.) = 0.168 cm at $p \leq 0.05$

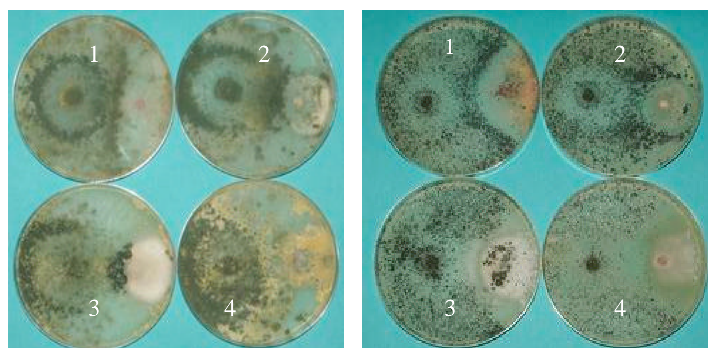

Fig. 2: Dual culture of *T. viride* (in the left) and *T. harzianum* (in the right) with *Fusarium* sp. on PDA observed after 10 days of incubation at 25°C 1: *F. graminearum*, 2: *F. sambucinum* , 3: *F. solani*, 4: *F. oxysporum* f. sp. *tuberosi*

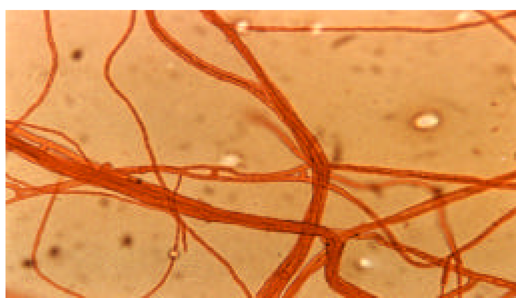

Fig. 3: Anastomosis between *Fusarium* sp. hyphae prior pre-formation of mycelial cords, during its *in vitro* confrontation with *Trichoderma* sp., observed on PDA after 6 days of incubation at 25°C

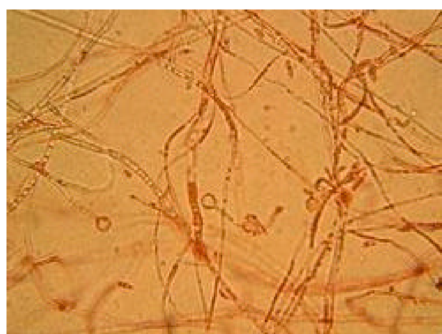

Fig. 4: Lysis and early chlamydospores formation occasioned by *T . harzianum* on *F. oxysporum* f. sp. *tuberosi* at the confrontation zone (observed after 6 days of incubation at 25°C)

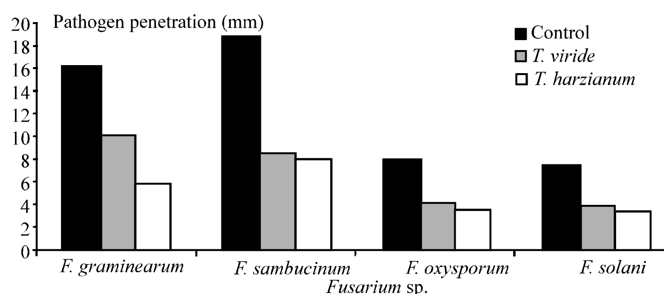

Fig. 5: Effect of a treatment by *Trichoderma* spp. suspension on dry rot incidence on potato tubers (cv. Spunta) inoculated by *Fusarium* species observed after 21 days of incubation at 25-27°C. LSD (Treatments × *Fusarium* spp.) = 2.138 mm (at p≤ 5%)

Fig. 6: Effect of *T. harzianum* on dry rot development occasioned by *F. graminearum* on potato tubers cv. Spunta observed after incubation for 21 days at 25-27°C

Fig. 7: Effect of *T. viride* on dry rot development occasioned by *F. solani* on potato tubers cv. Spunta observed after incubation for 21 days at 25-27°C

A discoloration of pathogen colonies at the confrontation zone was also observed (Fig. 2), it is probably induced by substances elaborated by the antagonist during parasitism of *Fusarium* spp. It is also to note presence of a zone of antibiosis between colonies of both confronted microorganisms and a total recovering of pathogen colony occurred after 10 days after confrontation (Fig. 2).

Fig. 8: Colonization of a tuber inoculation site by *T. harzianum* after inhibition of *in vivo* *Fusarium* spp. development observed after incubation at 25-27°C during 21 days

Effect of Trichoderma spp. on In vivo Growth of Fusarium spp.

Effect of tuber treatment, 24 h prior inoculation, with a *Trichoderma* spp. conidial suspension was assessed on development of dry rot occasioned by four *Fusarium* spp.

Mean pathogen penetration into inoculated tubers noted, after 21 days of incubation at 25-27°C (Fig. 5), varied among treatments and *Fusarium* spp. used for inoculation. An interaction was noted between both studied factors (at $p \leq 5\%$).

Figure 5 showed that tuber cv. Spunta treatment, at wounds, prior inoculation by condial suspensions of *T. harzianum* or *T. viride* significantly reduced dry development by more than 50% (Fig. 6 and 7) for the majority of tested *Fusarium* spp.

Antagonist colonization of inoculation site was traduced by reduction of dry rot development. This phenomenon was not observed on controls treated with sterile distilled water.

Development of mycelium and sporulation of both *Trichoderma* spp. were also observed at treated sites of inoculation, inoculated by *Fusarium* spp. and showing lesser dry rot development (Fig. 8).

Both indigenous *Trichoderma* spp. inhibited *in vivo* development of the entire fungal complex causing dry rot in Tunisia. Furthermore, *F. sambucinum* resistant to benzimidazoles, seems to be susceptible to this biological treatments and was inhibited by *T. viride* and *T. harzianum* respectively by 54 and 58%.

Discussion

Potato-*Fusarium* pathosystem is suitable, according to Slininger *et al*. (2003), for use of biocontrol agents because it is characterized by an exploitable pathogen etiology, its existence in an environment favourable to antagonist development, its resistance to several traditional strategies of control due to pathogen soilborne origin and its capacity of causing serious losses. Furthermore, according to Tunisian conditions, involvement of a *Fusarium* complex in disease development, absence of resistant cultivars and appearance of *F. sambucinum* isolates resistant to benzimidazoles are also of consideration (Daami-Remadi and El Mahjoub, 1996, 2004, 2006; Daami-Remadi *et al*., 2006).

The present study on biocontrol of the *Fusarium* spp. complex, causing tuber dry rot, is original because the interaction of *T. harzianum* and *T. viride* especially with *F. graminearum*, *F. solani* and *F. oxysporum* f.sp. *tuberosi* has never been investigated in Tunisia.

T. harzianum and *T. viride* tested for their antagonistic activities, against *Fusarium* spp. have limited radial mycelial growth of tested pathogens, induced morphological disruption and

mycoparasitism of the pathogen. Damage observed via light microscopic studies, was traduced by an important lysis, induction of mycelial cords and early formation of chlamydospores. Mycelium density reduction, significant competition and discoloration of *Fusarium* colonies at the confrontation zone are the other modes of action observed. Similar modes of action of *Trichoderma* are reported by Lewis and Papavizas (1987) who attributed this phenomenon to several enzymes and antibiotic substances, naturally formed or synthesized by antagonist and affecting pathogen cell permeability. Reduction of mycelium weight, increase in protein losses, reduction in glucose maintenance and pathogen hyphae morphological disruptions are also observed. This last mechanisms justify the reduction of mycelium density noted in this study. Furthermore, several antibiotic substances, such as alamethicin, paracelsin, trichotoxin or gliotoxin, produced by *Trichoderma* and *Gliocladium* isolates are also able of affecting pathogen wall permeability (Roberts and Lumsden, 1990; Rousseau *et al.*, 1996; Benhamou and Chet, 1996). Cell pathogen wall degradation is also due to hydrolytic enzymes such as chitinases and β-1,3-glucanases synthesized by *Trichoderma* isolates (Elad *et al.*, 1983; Chet and Elad, 1983; Singh *et al.*, 1999; Limon *et al.*,1999; Howell, 2003). Chérif and Benhamou (1990) found that these substances diffused by *Trichoderma,* in culture medium, induced reduction in *F. oxysporum* f.sp. *radicis-lycopersici* colony growth observed even before hyphal contact and that an alteration of chitin macromolecules of mycelium precedes wall destruction and cytoplasm loss. Olivier and Germain (1984) reported that, in addition to hydrolytic enzymes, *Trichoderma* mycelium synthetizes *in vitro* a volatile antifungal compound. A similar effect was also observed for the same *T. harzianum* isolate tested in the present study in the case of *Pythium aphanidermatum* and *P. ultimum* (Daami-Remadi, 2001b) and *F. oxysporum f.sp. radicis-lycopersici* (Hibar *et al*., 2005).

However, relative importance of both mechanisms (hydrolytic enzymes and antibiotics) in the antagonistic process seems to be specific of the antagonist × pathogen interaction (Howell and Stipanovic, 1995) and consequently can explain the interaction noted between *Trichoderma* spp. and *Fusarium* species tested in the present study. Haran *et al*. (1996) added that the degree of inhibition induced by *T. harzianum* depends upon host cell and precisely proportionally to the chitin content at the cell wall.

It is also to note that all *in vivo* biocontrol essays are conducted on entire tubers and present results reflected interactions between tuber defense, antagonists and pathogens; this method permits, according to Schisler *et al*. (1998), simulations of natural wounds and screening of antagonist microorganisms able of surviving in potato stores. Furthermore, applied 24 h prior tuber inoculation, as conidial suspension, *Trichoderma* spp. tested in the present study seem to colonize the site of inoculation and consequently, inhibit dry rot development. This result joins those of Daami-Remadi (2001a) where inhibition percentages were superior to those obtained by a thiabendazole treatment. Howell (2003) explained that *Trichoderma* spp. are able to penetrate skin and cortical infested tissues for inhibiting pathogen without damaging plant tissues. This *in vivo* result joins, in part, that obtained by Chérif *et al*. (2001) in the case of *F. sambucinum*×*T. harzianum* interaction and by Chérif *et al*. (2002) in the case of *T. sambucinum*×*Bacillus* sp. interaction and Slininger *et al*. (2003) found that metabolites produced by different antagonists have a direct effect on potato dry rot development.

This same isolate of *T. harzianum* was tested against *Pythium aphanidermatum* (Triki and Priou, 1997; Daami-Remadi, 2001b) and *P. ultimum* (Daami-Remadi, 2001b) causing potato leak and *Phytophthora erythroseptica* causal agent of pink rot (Triki *et al*., 1996); a covering of pathogen colony, a strong mycelium lysis and inhibition of rot development are also observed.

T. viride, inhibited dry rot development induced by all tested *Fusarium* species including *F. oxysporum* f.sp. *tuberosi*. This result joins that obtained by Ayed *et al*. (2006) which found that three tested *Trichoderma* species have limited incidence of *Fusarium* wilt occasioned by isolates of *F. oxysporum* f.sp. *tuberosi*.

Furthermore, *Trichoderma* spp. tested in this study reduced incidence of tuber dry rot occasioned by *Fusarium* species susceptible to benzimidazoles (such as *F. oxysporum*, *F. solani* and

F. graminearum) but also that induced by *F. sambucinum* resistant to these fungicides. This result joins in part, those obtained by Schisler *et al.* (1998) for bacterial antagonists able of controlling 10 strains of *Gibberella pulicaris* (*F. sambucinum*) including those resistant to Thiabendazole.

Against other potato pathogens, *T. harzianum* T39 and *T. virens* DAR 74290, applied in co-inoculation with *Phytophthora erythroseptica*, showed efficacy against potato pink rot development. Furthermore, incidence or root rot was reduced by isolates of this both tested antagonists (Etebarian *et al*., 2000). Consequently, the present study showed for the first time in Tunisia that the tested *Trichoderma* spp. have an antagonistic activity against the entire *Fusarium* complex causing potato dry rot, in addition to their activity to the other pathogens of potato tubers and they can be included in an integrated pest management of potato post-harvest problems.

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