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Potato Vascular *Fusarium* wilt in Tunisia: Incidence and Biocontrol by *Trichoderma* spp.

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Abstract: Pathogen isolations from potato tubers showing dry rot symptoms revealed the presence of *Fusarium oxysporum* f. sp. *tuberosi* in different Tunisian regions. Pathogenicity tests of different isolates were realized on potato plants. Typical symptoms of vascular wilt disease were observed and noted. After wilting, inoculated plants were totally damaged. *Trichoderma* spp. were evaluated for their antagonistic potential against *F. oxysporum* f. sp. *tuberosi* in *vitro* and *in vivo*. *Trichoderma harzianum*, *T. viride* and *T. virens* inhibited the mycelial growth of *F. oxysporum* f. sp. *tuberosi*. The antagonism included lysis and dissolution of the host cytoplasm and/or transformation into cords and/or coiling around pathogen hyphae. Moreover, substrate application of *Trichoderma* species (10^8 spores per mL) before inoculation by *F. oxysporum* f. sp. *tuberosi* controlled *Fusarium* wilt of potato plants compared with non-inoculated plants and untreated-inoculated plants. This approach may be beneficial for biological control in *F. oxysporum* f. sp. *tuberosi* and could allow protecting plants from this pathogen.

Key words: Antagonists, *F. oxysporum* f. sp. *tuberosi*, *Solanum tuberosum* L.

INTRODUCTION

Among the most serious potato diseases, after late blight, are vascular wilts. The most common pathogens causing potato wilt in Tunisia are *Verticillium dahliae* and to a lesser extent *V. alboatrum* and *V. tricorpus*[1,2]. However, these last years, another soilborne fungus, identified as *Fusarium oxysporum* f. sp. *tuberosi*, was frequently isolated from potato dry rot and wilted plants. This emergent pathogen, causing typical vascular wilt and stunting on potato plant, was detected in major Tunisian potato areas (Sousse, Monastir, Mahdia, Nabeul, Bizerte, Jendouba, Sfax and Kasserine) singly or in association with other secondary pathogenic fungi such as *F. solani*, *F. graminearum* and *Colletotrichum coccodes*[3-5]. These pathogens are transmitted with varying degrees of effectiveness from inoculum within and on seed tubers[6]. They can survive in the soil for many years and infect potato plants by direct penetration and/or via wounds on roots and tubers[4,6-9].

In the world, several strategies are used to control *Fusarium* wilt. Preplant soil fumigation with compounds such as methyl bromide and chloropicrin reduces seedling losses[10]. However, fumigant use is being reduced due to undesirable effects on the environment and human health[11]. Chemical control is erratic and expensive, thus control has generally been restricted to the use of long-term rotations (3-5 years), solarization and Brassicaceae tissues having toxic effects on *F. oxysporum* pathogens[12,13,9]. Therefore, the development of biological approaches for managing *Fusarium* wilt of potato, such as search for antagonistic microorganisms with high activity, is necessary[14,15]. *Trichoderma* spp., in particular, have been reported to control soil-borne plant pathogens such as *Rhizoctonia solani* Khun., *Sclerotium sclerotiorum* (Sacc.) Curzi., *Pythium* spp., *Stereum purpureum*, *Botrytis cinerea*, *Phomopsis viticola* and *Fusarium* spp.[16-20].

The major aims of this study were to test the pathogenicity of some *F. oxysporum* f. sp. *tuberosi* isolates on potato plants and to evaluate the *in vitro* and *in vivo* effects of three *Trichoderma* species in reducing *F. oxysporum* f. sp. *tuberosi* incidence.

MATERIALS AND METHODS

Isolation: During 2004, potato tubers showing dry rot symptoms were collected from major regions practicing traditional potato-storage in Cap Bon (Hamam Ghezaz, Hawaria, Korba, Menzil Temim, Dar Alouch, ...).

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Fragments (05 cm) of diseased tubers were washed under a fine spray of tap water, surface-disinfested (3 min in 10% sodium hypochlorite), rinsed three times in distilled water and air-dried on a laminar flow bench. Each fragment was placed on Potato Dextrose Agar (PDA) and incubated at 25°C for 4-5 days in the dark. Single conidial isolates of *F. oxysporum* f. sp. *tuberosi* were selected and identified according to Tivoli[21]. For plant inoculation, mycelium taken from the edge colony of each isolate was transferred to 150 mL of Potato Dextrose Broth (PDL) and incubated at 25°C for 5 days in a rotary incubator (120 rpm). The liquid culture was filtered and the conidial suspension was adjusted to 10^7 spores mL^{-1} by a Malassez cystometer.

Pathogenicity tests for *Fusarium* wilt: Potato tubers, cultivar 'Spunta', were planted in plastic pots (6.74 l) containing an autoclaved mixture of perlite and peat (1:3) and kept at 2-22°C (minimum and maximum temperatures, respectively). Two weeks after emergence, plant inoculation was done by irrigation. Five plants for each isolate (F_1-F_{13}) were inoculated with 100 mL of conidial suspension. Non-inoculated control plants were inoculated with 100 mL of sterile distilled water. Plants were irrigated regularly and fertilized with a nutrient solution (20 N : 20 K_2O : 20 P_2O_5)[22].

Pathogenicity was assessed through disease severity and plant height. A scale of 0-4 was used to assess disease severity weekly: 0 = asymptomatic leaf, 1 = leaf wilted, 2 = leaf with hemiplegic yellowing, 3 = leaf with necrosis, 4 = dead leaf. Isolates pathogenicity were expressed as an index of leaf damage (ILD) which is calculated per potato plant[23]:

$$ILD = \sum notes/max$$

ILD : Index of Leaf Damage.
$\sum$ notes : Total notes.
Max : 4 times of developed-leaves number.

Plant height was recorded two months after inoculation. Pathogen isolations were done at the end of the bioassay.

Data are arranged by completely randomized design where treatments (plants inoculated by each of 13 *Fusarium* isolates and non-inoculated) are the only fixed factor. Mean comparisons are done following the LSD method.

Biocontrol of *F. oxysporum* f. sp. *tuberosi*: *Trichoderma* species were isolated from Tunisian soil and identified as *Trichoderma harzianum*, *T. viride* and *T. virens* (= *Gliocladium virens*).

***In vitro* assays of *F. oxysporum* f. sp. *tuberosi* inhibition by *Trichoderma* spp:** *In vitro* bioassay of *F. oxysporum* f. sp. *tuberosi* biocontrol was realized by dual culture of pathogen and antagonist on PDA medium amended with streptomycin sulfate at 200 g L^{-1}. Three *Trichoderma* species are tested against five *F. oxysporum* f. sp. *tuberosi* isolates (F_3, F_6, F_7, F_{10} and F_{12}). Two mycelial discs (6 mm), one of each antagonist and another of the pathogen, were placed equidistant of 3 cm in diametrical axis. For untreated plates, a disc of a *F. oxysporum* f. sp. *tuberosi* isolate is placed at the center. The fungal radial growth colonies of all culture plates were measured after four days at 25°C. Eight plates were made per elementary treatment.

Inhibition of *Fusarium* wilt with three *Trichoderma* species: *Trichoderma* suspensions were prepared in sterile water using the surface growth of a solid culture of the antagonists. 2.5 L of spore suspensions adjusted to 10^8 spores per mL were used to humidify 6.74 dm^3 of the autoclaved substrate, volume necessary to fill 10 pots. This treatment has been done for each of the three *Trichoderma* species.

After emergence, the most aggressive *F. oxysporum* f. sp. *tuberosi* isolate was used for potato plants inoculation. Disease severity assessment is determined as described above.

Statistical analysis: Data were analyzed using SPSS. They were subjected to analysis of variance and Fisher's Least Significant Difference test (LSD) arranged in completely randomized factorial design. Mean comparison was made with LSD Test ($p<0.05$).

RESULTS

Pathogenicity tests of *F. oxysporum* f. sp. *tuberosi*: Typical symptoms of a *Fusarium* wilt disease were caused by all *F. oxysporum* f. sp. *tuberosi* isolates. These symptoms appeared at 2-22°C (minimum and maximum temperatures, respectively) within 27 days after inoculation. Inoculated potato plants showed hemiplegic yellowing of the lower leaves, browning in the vascular region especially in the stem and ascending wilt symptoms. Inoculated plants completely wilted three months after planting (Fig. 1).

Stunting can occur on inoculated plants. In fact, height of infected plants was usually smaller than that of non-inoculated plants. This reduction of plant growth reached more than 40% for the majority of isolates with a maximum of 44.6% induced by isolate F_6. Analysis of variance showed that plant height was significantly

Table 1: Weekly evolution (L_1-L_8) of Index of Leaf Damage (ILD) for potato plants, cv. Spunta, inoculated by *F. oxysporum* f. sp. *tuberosi* isolates observed 27 days after inoculation (L_1). (NI: Non-inoculated plants; F_1-F_{13}: *F. oxysporum* f. sp. *tuberosi* isolates)

	Isolates														
Time	NI	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	F_{11}	F_{12}	F_{13}	LSD (5%)
L_1	0	0	0	0.02	0.01	0.01	0.02	0.04	0	0.03	0	0	0	0	0.0136
L_2	0	0.03	0.03	0.03	0.02	0.02	0.06	0.07	0.07	0.03	0.02	0.06	0.06	0	0.0215
L_3	0	0.12	0.09	0.19	0.09	0.07	0.06	0.15	0.11	0.06	0.08	0.13	0.14	0.09	0.0486
L_4	0	0.21	0.11	0.3	0.12	0.14	0.09	0.26	0.25	0.1	0.16	0.14	0.17	0.28	0.0914
L_5	0	0.39	0.12	0.35	0.21	0.18	0.16	0.41	0.46	0.19	0.24	0.26	0.36	0.29	0.1652
L_6	0	0.39	0.25	0.4	0.26	0.25	0.19	0.66	0.56	0.25	0.46	0.39	0.44	0.32	0.2931
L_7	0.05	0.62	0.47	0.62	0.34	0.42	0.3	0.98	0.67	0.3	0.84	0.91	0.63	0.52	0.4334
L_8	0.11	0.62	1.13	0.74	0.49	0.55	0.34	1.36	1.22	0.59	1.13	1.23	1.26	0.68	0.7603

Table 2: Diameters of *F. oxysporum* f. sp. *tuberosi* colonies in the presence of *T. harzianum*, *T. viride* and *T. virens* (PDA, four days after incubation at 25°C) comparatively with the untreated control.

	Diameter of *F. oxysporum* f. sp. *tuberosi* colonies (cm)			
Treatments Isolates	Untreated Control	*T. harzianum*	*T. viride*	*T. virens*
F_3	7.65	4.52	3.39	3.37
F_6	7.14	4.04	3.5	3.49
F_7	7.35	4.64	3.89	3.61
F_{10}	7.22	4.19	3.97	3.9
F_{12}	6.74	3.82	3.71	3.57

(F_3, F_6, F_7, F_{10}, F_{12}: Isolates of *F. oxysporum* f. sp. *tuberosi*), LSD at 5% (Isolates×Treatments) = 0.48 cm

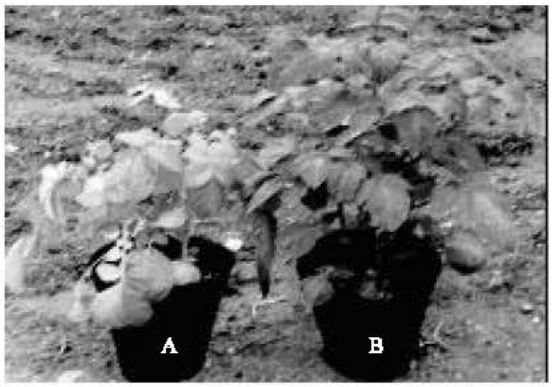

Fig. 1: Comparison between a healthy potato plant, cv. Spunta, (B) and inoculated potato plant by *F. oxysporum* f. sp. *tuberosi* (A) 27 days after inoculation

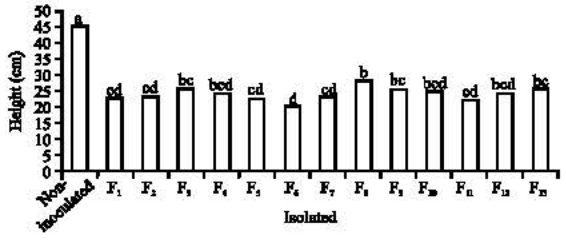

Fig. 2: Incidence of different *F. oxysporum* f. sp. *tuberosi* isolates on height of potato plants (cv. Spunta, three months after inoculation). (F_1-F_{13}: *Fusarium oxysporum* f. sp. *tuberosi* isolates)

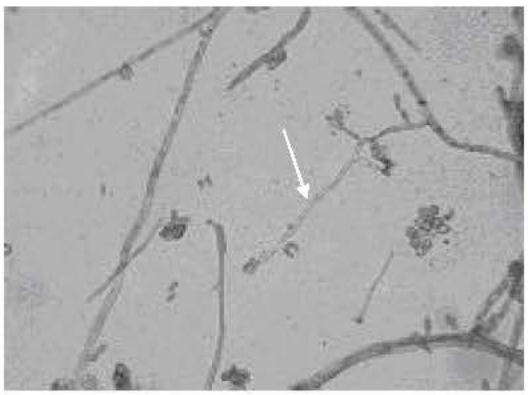

Fig. 3: Lysis on *F. oxysporum* f. sp. *tuberosi* mycelium in the presence of *T. virens* (PDA, 4 days at 25°C)

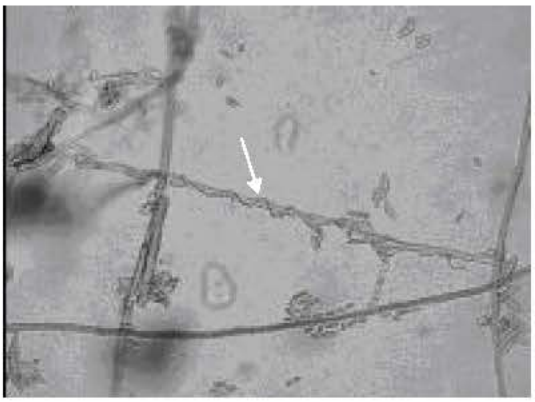

Fig. 4: Coiling of *T. harzianum* mycelium on that of *F. oxysporum* f. sp. *tuberosi* (PDA, 4 days at 25°C)

different between inoculated and non inoculated plants and between isolates (Fig. 2).

At the beginning of this bioassay, fewer plants developed disease symptoms that still increase slowly especially during the three first weeks. However, all *F. oxysporum* f. sp. *tuberosi* isolates were able to cause wilt symptoms in Spunta potato plants (Table 1). Isolate F_7 is the most virulent showing higher disease severity noted at different evaluation periods. Isolates F_8, F_{11} and F_{12} also caused important wilt symptoms. But isolates F_4

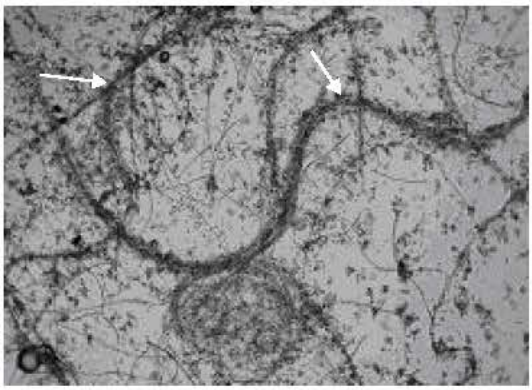

Fig. 5: A transformation into cords of *F. oxysporum* f. sp. *tuberosi* mycelium in the presence of *T. viride* (PDA, 4 days at 25°C)

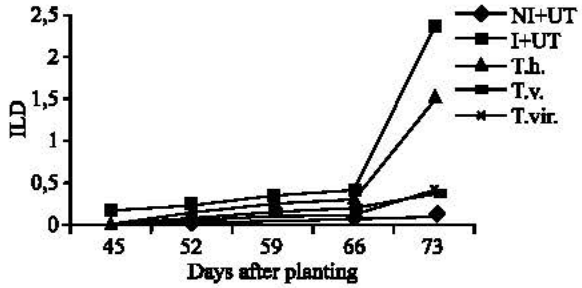

Fig. 6: Effect of *T. harzianum* (T.h.), *T. viride* (T.v.) and *T. virens* (T.vir.) on disease incidence comparatively with healthy (NI+UT) and inoculated (I+UT) plants (cv. Spunta) during the bioassay. LSD (5%) at 45 days after planting = 0.07; LSD (5%) at 52 days after planting = 0.0689; LSD (5%) at 59 days after planting = 0.0598; LSD (5%) at 66 days after planting = 0.0516 and LSD (5%) at 73 days after planting = 0.278

and F_6 were weaker wilt pathogens than the other wilt isolates and showed a lower disease severity (Table 1). The reisolement showed development of *F. oxysporum* f. sp. *tuberosi* colonies in all inoculated plants and their absence in the non-inoculated control.

Inhibition of *F. oxysporum* f. sp. *tuberosi*

Antagonism *in vitro*: Pathogen colony diameters were compared among treatments with *T. harzianum*, *T. viride* and *T. virens* in order to determine the effect of these antagonists on mycelial growth of *F. oxysporum* f. sp. *tuberosi*. Table 2 showed that *Trichoderma* species significantly reduced mycelial growth of each *F. oxysporum* f. sp. *tuberosi* isolate tested. All *Trichoderma* are effective reducing mycelial growth more

Fig. 7: Comparison between Non-inoculated (NI) and inoculated (I) potato plants and inoculated plants treated by *T. harzianum* (T.h.), *T. viride* (T.v.) and *T. virens* (T. vir.) (cv. Spunta, 73 days after inoculation)

than 40% compared to the untreated control. Inhibition by *T. harzianum* varied between 36 and 43% but it was less than that induced by *T. viride* and *T. virens* (46-56%).

Microscopic observations of sections from the edge of *F. oxysporum* f. sp. *tuberosi* colonies in contact with the inhibition zones caused by each of *Trichoderma* species showed lysis on mycelium (Fig. 3). In fact, cells appeared damaged: cytoplasm was disorganized and protoplasm was lost. A transformation into cords of *F. oxysporum* f. sp. *tuberosi* mycelium and a coiling of antagonists mycelium around pathogen were also observed (Fig. 4 and 5). These phenomena was more frequent for *T. viride* and *T. virens*. After one week, *Trichoderma* species invades *F. oxysporum* f. sp. *tuberosi* colonies and sporulates on revealing its high mycoparasitism.

Effects of *Trichoderma* species on *Fusarium* wilt development: Significant decreases in the Index of Leaf Damage (ILD.) of potato plants treated with *Trichoderma* spp. were noted compared to untreated-inoculated plants (Fig. 6 and 7).

Trichoderma spp., amended to the substrate of culture in the same time of plantation, reduced significantly the severity of *Fusarium* wilt (Fig. 6 and 7). The I.L.D. of potato plants treated with *Trichoderma* species was significantly lesser than that noted on untreated inoculated plants. In fact, at the end of the end of bioassay, this index was, respectively 0.4 and 0.36 for *T. viride* and *T. virens* and 2.4 for the untreated-inoculated plants. *T. harzianum* was also effective; however its effectiveness was weaker than the two others species.

DISCUSSION

F. oxysporum f. sp. *tuberosi* is one of the most frequent pathogen causing potato wilt and vascular discolouration in Tunisia[5]. The pathogenicity of all *F. oxysporum* isolates from dry rot tubers confirmed the results obtained by Daami-Remadi and El Mahjoub[4] showing that *F. oxysporum* f. sp. *tuberosi* isolates from dry rot tubers caused *Fusarium* wilt symptoms. Furthermore, Tivoli[21] indicated inability of his *F. oxysporum* isolates, causing dry rot, to cause *Fusarium* wilt. A study carried out by Venter *et al.*[24] dealing with the relationship between vegetative compatibility and pathogenicity of this pathogen isolated from potato, showed that isolates obtained from rotted tubers were only able to cause weak wilt symptoms in some cases. This could explain the development of weak symptoms noted for some of Tunisian *F. oxysporum* isolates. Other studies showed that pathogenicity of *Fusarium* isolates could be influenced by temperature with an optimum of 28°C for *F. oxysporum* f. sp. *tuberosi*[6,7,25]. The maximum temperature recorded during this study is about 22°C, than this could explain the weak virulence of some isolates.

The current study showed efficiency of *Trichoderma* species to inhibit *Fusarium* growth and to reduce symptom development on the potato plants.

Microscopic observations showed a transformation into cords and a mycoparasitism of *Fusarium* hyphae. This included coiling around pathogen hyphae, penetration and subsequent dissolution of the host cytoplasm. It occurred regardless of the supply of external nutrients to the host. The same phenomenon was observed for *F. oxysporum* f. sp. *radicis-lycopersici*, *F. solani* var. *coeruleum*, *F. roseum* var. *sambucinum*, *F. roseum* var. *graminearum*, *Verticillium dahliae*, *Verticillium alboatrum* and *Pythium* spp. with the same isolate of *Trichoderma harzianum*[1,26-28]. Similar phenomen on *Rhizoctonia solani* and *Sclerotinia rolfsii* mycelium was observed by Howell[29] and Chet and Elad[30].

Lorito *et al.*[31] and Howell[32] reported that enzymes such as chitinases, β-1,3-glucanases and cellulases produced by the biocontrol agent acted by breaking down the polysaccharides, chitin and β-glucans that are responsible for rigidity fungal cells walls, thereby destroying cell wall integrity and causing a partial lysis. Furthermore, volatile antibiotics and antifungal metabolites such as viridin, gliotoxin, glioviridin produced by *Trichoderma virens*, 6PAP (6-*n*-pentyl-*2H*-pyran-2-one) by *Trichoderma harzianum*, alkyl pyrones, isonitriles, sesquiterpenes, steroids,... are responsible of mycelial growth inhibition[17,29,33]. Daami-Remadi[27] and Hibar *et al.*[28] observed an away effect of *T. harzianum* on pathogen mycelial development.

Inhibition of *Fusarium* wilt by *T. viride* and *T. virens* during this bioassay is explained. Howell[32] signalled similar inhibition effects of *Trichoderma* species as follows: Hyphae of the biocontrol agent penetrated into infected epidermal and root cortical tissue to destroy the pathogen hyphae, with little or no damage to uninfected plant tissue. Some *Trichoderma* species like *T. lignorum* produced a "lethal principle" that was excreted into the surrounding medium, allowing parasitic activity by the biocontrol agent[32]. Windham *et al.*[34] reported that *Trichoderma* spp. produced a growth-regulating factor inducing plant growth.

Camporata[35] reported a protection of *Trichoderma* incorporation on the soil against *F. oxysporum*. In the same way, Thangavelu *et al.*[20] found that soil application of *T. harzianum* effectively controlled *Fusarium* wilt of banana caused by *F. oxysporum* f. sp. *cubense*. Moreover, the use of *Trichoderma* formulation was also successful in reducing disease incidence in environments highly conducive to head rot caused by *Sclerotinia sclerotiorum*[19].

However reducing disease incidence is faced to some problems. First, *Trichoderma* species that appear to be involved in biocontrol are strongly influenced by the rhizosphere. In fact, they cannot compete for space and nutrients if they are unable to grow in the rhizosphere[36]. Second, temperature also has a notable effect on the production and activities of enzymes and antibiotics associated with biocontrol by these antagonists. Besides, a presence of other members of the soil microflora also may influence biocontrol activity by inhibiting the growth and development of *Trichoderma* species or by metabolizing its enzymatic and antibiotic products[32]. For this reason, the level of antagonism towards *F. oxysporum* f. sp. *tuberosi in vitro* was more important than that observed *in vivo*.

CONCLUSIONS

Trichoderma species were effective in controlling potato *Fusarium* wilt induced by *F. oxysporum* f. sp. *tuberosi*. The reduction of disease severity by applying these antagonists on autoclaved substrate, especially *Trichoderma viride* and *T. virens*, should be investigated and considered as an interesting tool for the integrated disease management of emergent *Fusarium* wilt. Mixtures of microbial antagonists can be used successfully to

increase the level of their biological control above that achieved with individual strains of the mixture. Biological products, being cost effective, having a long shelf-life, supporting high propagules density and readily adopted by farmers must be formulated. Moreover, some fungicide can be used with *Trichoderma* spp. and improved their effects.

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