The African Journal of Plant Science and Biotechnology ©2009 Global Science Books

Biological Control of Tomato Verticillium Wilt by Using Indigenous *Trichoderma* spp.

Hayfa Jabnoun-Khiareddine[1] • Mejda Daami-Remadi[2*] • Fakher Ayed[3] • Mohamed El Mahjoub[1]

[1] Institut Supérieur Agronomique de Chott-Mariem, 4042, Chott-Mariem, Sousse, Tunisia
[2] Centre Régional des Recherches en Horticulture et Agriculture Biologique, 4042, Chott-Mariem, Sousse, Tunisia
[3] Centre Technique de l'Agriculture Biologique de Chott-Mariem, 4042, Chott-Mariem, Sousse, Tunisia

Corresponding author: * **daami_rm@yahoo.fr**

ABSTRACT

Three endogenous *Trichoderma* species were tested *in vitro*, *in vivo* and *in situ* for their antagonistic activity against *Verticillium* spp. causing tomato vascular wilt in Tunisia. *Trichoderma harzianum*, *T. viride* and *T. virens* isolates reduced the radial growth of *V. dahliae*, *V. albo-atrum* and *V. tricorpus* in comparison to the untreated controls. Antagonistic potential of *Trichoderma* spp. against tested wilt agents showed intra- and inter-specific variations. Additionally to the sclerotinization inhibitory activity and to the reduced abundance of resting structures of *Verticillium* spp. observed, comparatively to untreated controls, *Trichoderma* spp. isolates caused profound alterations of *Verticillium* spp. mycelium at the confrontation zone. The germination of *V. dahliae* microsclerotia, exposed for 30 min to liquid cultures of antagonists tested and incubated at 20°C, was completely suppressed compared to the control microsclerotia treated with sterile distilled water. Furthermore, germinating microsclerotia dual cultured with *Trichoderma* spp. became unable to germinate and mature microsclerotia progressively lost their typical dark colour. All tomato cv. 'Ventura' plants, when treated at planting with a *Trichoderma* spp. spore suspension and inoculated with *V. dahliae*, showed after 60 days of culture under growth chamber conditions, a reduced severity of Verticillium wilt in comparison to inoculated and untreated control plants. In addition, plants treated with *Trichoderma* spp. showed increased height and root and stem fresh weights in comparison to the inoculated and untreated control. The discoloration index, noted on tomato plants treated at planting by *T. harzianum*, *T. viride* and *T. virens* and grown under greenhouse conditions, was significantly reduced compared to the untreated control. Plants treated with *Trichoderma* spp. showed, after 90 days of culture, an increase of more than 50% of their roots and stem fresh weights in comparison to the untreated control.

Keywords: disease severity, inhibition, inoculation, *Lycopersicon esculentum* Mill., microsclerotia, mycelial growth, plant growth, sclerogenesis, vascular wilt

INTRODUCTION

Verticillium dahliae, *V. albo-atrum* and *V. tricorpus* are the *Verticillium* species causing vascular wilt on different economically important vegetable crops in Tunisia such as tomato, potato and melon (Jabnoun-Khiareddine *et al.* 2005, 2006, 2007). Economic losses caused by these pathogens can reach 50% especially on tomato cultivars severely infected by *V. dahliae* (Jabnoun-Khiareddine *et al.* 2007). Furthermore, once symptoms have been established, it is difficult to efficiently control the disease.

In Tunisia, as in most countries around the world, tomato Verticillium wilt control is still based on resistant cultivars carrying the *Ve* resistance gene (Bender and Shoemaker 1984; Harrington and Dobinson 2000; Kawchuk *et al.* 2001). However, during the last years, several cases of resistance breakdown were noted on many resistant tomato cultivars such as 'Colibri' and 'Rio Grande'. In fact, the emergence of the race 2 of *V. dahliae* in Tunisia was recently reported and was shown to be involved in the outbreak of Verticillium wilt symptoms on resistant tomato cultivars (Daami-Remadi *et al.* 2006).

As soil fumigation, the most control method used, was shown to have negative effects on health and soil environment (Aghighi *et al.* 2004), several non-chemical alternative methods were tested for their efficiency against the soil-borne pathogens such as steam sterilization, solarization and cultural practices (Concibido *et al.* 1994; Lynch *et al.* 1997; Jansky and Rouse 2000; Pegg and Brady 2002; Jansky *et al.* 2004; Uppal *et al.* 2007). However, the efficiency of these methods was limited by the diversity of the pathogen host plants, the ability to colonize the rhizosphere of non-host plants and the persistence in the soil via several structures of resistance such as microsclerotia, chlamydospores and dark mycelia (Aghighi *et al.* 2004). Thus, the microbial interactions at the rhizosphere zone between soil-borne pathogens and their antagonists were shown to be of crucial importance for disease development and severity. In fact, the suppression of *Verticillium* was achieved, as shown in several studies worldwide, by the use of rhizosphere-colonizing fungi of the genera *Talaromyces*, *Trichoderma*, *Penicillium*, *Fusarium* and *Gliocladium* in several studies in different countries (Matta and Garibaldi 1977; Hall and Scheiber 1984; Millar *et al.* 1984; Henni 1987; Kim *et al.* 1988; Berg *et al.* 1999; El Aissami and Lahlou 1999; D'Ercole *et al.* 2000; Larena *et al.* 2003; Aghighi *et al.* 2004).

The biocontrol efficiency of soil-borne pathogens, and especially *Verticillium* species, depends mainly on the antagonist establishment and development in the rhizosphere and on its ability to colonize the subterranean plant tissues for avoiding infection. Thus, the present study focused on the use of three endogenous *Trichoderma* species, colonizing naturally solanaceous roots, for the biocontrol of the tomato Verticillium wilt in a growth chamber and under greenhouse conditions. The effect of these antagonists on pathogen mycelial growth and viability of resting structures was also investigated.

Received: 13 July, 2009. Accepted: 3 November, 2009.

Original Research Paper

MATERIALS AND METHODS

Plant material

Verticillium susceptible (*ve*) tomato seeds (*Lycopersicon esculentum* Mill. cv. 'Ventura') used were gratefully provided by the laboratory of seeds and plants control of the General Direction of the Protection and Control of the Agricultural Product Quality, Tunisia.

Tomato seeds were superficially disinfected by immersion in absolute ethanol for 2 min, followed by extensive rinsing in sterile distilled water (SDW). Seeds were sown in alveolus plates filled with previously sterilised peat. Seedlings were grown in a growth chamber at 24-26°C with 12-h photoperiod and 70% humidity. They were watered daily and fertilized twice a week with a standard nutrient solution according to Pharand *et al.* (2002). Experiments were performed with 4-week-old tomato plants.

Pathogens

Three *Verticillium* species, *V. dahliae*, *V. albo-atrum* and *V. tricorpus*, were tested in the present study. They were isolated from different host plants (**Table 1**) showing wilt symptoms and vascular discoloration. *Verticillium* spp. isolates were cultured at 20°C on PDA (potato dextrose agar) medium added with 300 mg/l of streptomycin sulphate (Pharmadrug Production Gmbh, Hamburg, Germany). Liquid cultures used for substrate inoculation were prepared on PDB (potato dextrose broth) and incubated at 20°C under continuous agitation at 150 rpm during 4 to 5 days. The spore suspension concentration used was adjusted to 10^7 spores/ml by a Malassez cystometer. For their long term preservation, pathogen isolates were stored at -20°C in a 25% glycerol solution.

To assess and qualify the effects of *Trichoderma* spp. on microsclerotia, *V. dahliae* inoculum was prepared on perlite contained in Roux culture flasks (volume 800 ml) and autoclaved twice during 30 min within 24 h. After the second sterilization, the perlite was inoculated with a mixture of liquid cultures (100 ml/ flask) of *V. dahliae* isolates (**Table 1**). Flasks were maintained at room temperature during three months for abundant microsclerotia formation.

Table 1 *Verticillium* spp. isolates tested and their origins.

Verticillium species	Isolates	Original plant hosts	Origins
V. dahliae	Vd13	Tomato	Chott Mariem
	Vd66	Tomato	Téboulba
	Vd95	Tomato	Chott Mariem
	Vd14	Potato	Nabeul
	Vd20	Potato	Chott Mariem
	Vd28, Vd29	Potato	Sidi Bou Ali
	Vd82	Potato	Nabeul
	Vd64	Melon	Chott-Mariem
	Vd8	Eggplant	Téboulba
V. tricorpus	Vt1	Tomato	Chott-Mariem
	Vt2	Potato	Sidi Bouali
	Vt15	Melon	Mahdia
V. albo-atrum	Vaa1, Vaa3	Tomato	Chott-Mariem
	Vaa2	Potato	Chott-Mariem

Trichoderma spp.

T. harzianum, *T. viride* and *T. virens* were isolated from roots of several Solanaceous plants (tomato, eggplant and potato) and the monoconidial cultures were identified according to the Kubicek and Harman (1998) key. *Trichoderma* spp. isolates (three isolates per *Trichoderma* species) were cultured at 25°C on PDA and stored at -20°C in a 10% glycerol solution until use. Liquid cultures were prepared on PDB and the spore concentration used was adjusted to 10^7 spores/ml.

Effect of *Trichoderma* spp. on mycelial growth of *Verticillium* spp.

The *Verticillium* spp. × *Trichoderma* spp. *in vitro* interactions were studied following the dual culture method on PDA added with streptomycin sulphate (300 mg/l). Furthermore, as *Verticillium* spp. mycelial growth was relatively slow compared to *Trichoderma* spp., *Verticillium* isolates were plated on PDA three days in advance. In fact, three agar plugs (diameter 6 mm) colonized by the pathogen were plated at 2 cm from the edge of the Petri dish (diameter 9 cm) and equidistantly spaced from each other by 3 cm. *Trichoderma* isolates were placed at the centre of the Petri dish. Untreated control plates were plated with pathogen plugs only.

The mean diameter of the pathogen developing colonies was noted after 6 days of incubation at 20°C. These *in vitro Trichoderma* spp. × *Verticillium* spp. interaction studies were completed by several macroscopic and microscopic observations of colonies confronted with antagonists for elucidating and qualifying the damages occasioned on pathogen mycelium in comparison to untreated controls.

Statistical analyses were performed following a completely randomised factorial design where treatments (*Trichoderma* spp. isolates and untreated controls) and *Verticillium* spp. isolates were the fixed factors. Six replicates were used per elementary treatment and means were separated using Fisher's protected LSD test (at $p \leq 0.05$).

Effect of *Trichoderma* spp. on *V. dahliae* microsclerotia

For a qualitative assessment of the capacity of antagonists tested to reduce the *V. dahliae* inoculum released into the soil or the viability of the pre-existent inoculum, two essays were conducted.

Effects on microsclerotia germination

The ability of *Trichoderma* spp. tested to inactivate the microsclerotia germination was studied by using a perlite previously colonized by the pathogen. In fact, 3 g of colonized perlite wrapped in a sterile cloth were soaked for 30 min in the liquid culture of each antagonist tested (concentration adjusted to 10^7 spores/ml). The treated perlite was washed with SDW, air dried at room temperature and ground in mortar with 10 ml of SDW. The microsclerotia suspension obtained was sprayed on 2% agar medium (1 ml per Petri dish). Colonized perlite soaked in SDW only served as untreated control.

After 10 days of incubation at 20°C, the different cultures were observed for eventual *V. dahliae* growth emerging from plated microsclerotia. The microsclerotia viability was estimated via the formation or not of secondary microsclerotia (Nagtzaam *et al.* 1998).

Effects on germinating microsclerotia

The effect of antagonists tested was also assessed against *V. dahliae* germinating microsclerotia by dual culture of *Trichoderma* spp. isolates with microsclerotia in the same Petri dish. In fact, 1 g of colonized perlite, prepared as previously described, was ground in a mortar with 5 ml of SDW. The microsclerotia suspension obtained was filtered, for elimination of perlite grains, and sprayed (200 µl) at the surface of the Petri dishes containing PDA medium. After incubation at 20°C during 7 days, sufficient period for germination of viable microsclerotia and formation of secondary microsclerotia, agar plugs (diameter 6 mm) of the tested *Trichoderma* spp. isolates were plated at the centre of the colonized Petri dishes.

The antagonist × germinating microsclerotia interaction was qualified, after 20 at 35 days of incubation at 20°C, via some macroscopic and microscopic notations.

Effect of *Trichoderma* spp. on Verticillium wilt development and severity under growth chamber conditions

V. dahliae was the only species used in the *in vivo* essays as it is the predominant and the most virulent *Verticillium* species in Tunisia. In fact, a mixture of 10 isolates obtained from different hosts and regions (**Table 1**) was used for tomato inoculation. Furthermore, due to the presence of intra-specific variations of the anta-

gonist potential recorded *in vitro*, a mixture of isolates of each *Trichoderma* species was tested *in vivo* in comparison to untreated and inoculated or non controls.

Antagonists tested were applied to the culture substrate in alveolus plates. In fact, each alveolus, containing autoclaved peat (25 ml per alveolus), was watered with 3 ml of mixed liquid culture (adjusted to 10^7 spores/ml) of each tested *Trichoderma* species. Control peat was watered by a similar quantity of SDW. Seeds were sowed in these treated alveolus plates and placed in a growth chamber during three weeks with a 12-h photoperiod at 12-25±2°C and 70-90% relative humidity.

Before final transplanting, roots were soaked for 30 min in the similar mixed antagonist suspension (used for peat treatment i.e. first treatment in nursery), prepared as previously described.

Treated plants were separately placed in pots (diameter 10 cm) containing a mixture of peat and perlite infested with *V. dahliae* microsclerotia (final concentration of 50 microsclerotia/g of mixed substrate). The number of microsclerotia/g of perlite was estimated as follows: 1 g of colonized perlite was crushed in a mortar with 10 ml of SDW and the concentration of suspension filtered was determined by using the classical Malassez cell method.

Ten plants were used per elementary antagonistic treatment. Ten non-treated plants soaked in SDW were transplanted in sterile substrate or in substrate infested with *V. dahliae* served as un-inoculated untreated (NIC) and inoculated untreated (IC) controls, respectively.

Plants were maintained in a growth chamber at 15-30°C during 60 days and regularly watered and fertilized with a nutritious solution (N: 150 ppm, P: 50 ppm, K: 150 ppm, Ca: 150 ppm, Mg: 30 ppm, Fe: 3 ppm, Mn: 1.5 ppm, Zn: 0.2 ppm, B: 0.4 ppm, Cu: 0.1 ppm, Mo: 0.05 ppm and H_2O: qsp 1l) (Pharand *et al*. 2002).

Verticillium wilt severity was estimated, 60 days post planting, via the leaf damage index and according to 0-4 scale depending on symptom severity on leaves as previously described by Beye and Lafay (1985) and used in Jabnoun-Khiareddine *et al.* (2007).

Plant height and the root and stem fresh weights were noted for all tomato plants. Furthermore, the presence of pathogen in the stem was also verified by re-isolation on PDA.

Statistical analyses were performed, for all parameters measured, following a completely randomised design where treatments (*Trichoderma* spp. and untreated but inoculated or non controls) were the sole fixed factor. Ten replicates were used per elementary treatment and means were separated using Fisher's protected LSD test (at $p \leq 0.05$). The whole experiment was repeated twice but only the data of one essay is presented in the present study.

Effect of *Trichoderma* spp. on Verticillium wilt development and severity under greenhouse conditions

For a large-scale assessment of *Trichoderma* spp. efficiency against *V. dahliae*, the essay was conducted, during the 2006-2007 agricultural campaign, in a field known with severe Verticillium wilts history on tomato cv. 'Colibri' for three consecutive years ago at the domain of the Higher Agronomic Institute of Chott-Mariem. The infectious potential of this naturally infested field was estimated at 34 microsclerotia/g of soil, according to the method of Nadakavukaren and Horner (1959) previously described in Jabnoun-Khiareddine *et al.* (2007).

Tomato plants cv. 'Ventura' susceptible to *V. dahliae* race 1 were prepared as previously described in the *in vivo* essays. In fact, tomato seeds were sown in culture substrate treated or non with *Trichoderma* spp. Furthermore, plants at the 3-4 leaf true stage were soaked just before transplanting in a conidial suspension of the tested antagonists. Control plants were soaked in SDW. Plants were transplanted directly in the soil under a plastic greenhouse following six lines 80 cm apart with an inter-plant spacing of 40 cm. Plants were irrigated via a drip irrigation system. Fertilization and other cultural practices were the most commonly used for tomato farming in the region.

Disease severity was assessed 90 days post-transplanting via three parameters (root and stem fresh weights and Verticillium wilt severity) used for comparison between tested treatments. In fact, wilt severity was visually estimated via the progression of vascular discoloration in longitudinal roots, collar and stem sections of each plant. A vascular discoloration index was calculated based on a 0-4 scale where 0 = absence of vascular discoloration; 1 = vascular discoloration occurred on roots only; 2 = vascular discoloration occurred on roots and collar; 3 = vascular discoloration occurred at the stem base (3 cm from the collar); 4 = vascular discoloration reached the middle of the stem; 5 = vascular discoloration reached the stem end.

Stem segments showing or not vascular discoloration symptoms were used for fungal isolation on PDA at 20°C.

Statistical analyses were performed, for all parameters measured, following a randomised complete block design with 3 blocks and 7 replicates (plants) per block and per elementary treatment. Treatments (*Trichoderma* spp. and untreated control) were randomly arranged into blocks. Means were separated using Fisher's protected LSD test (at $p \leq 0.05$). The whole experiment was repeated during two years (2006 and 2007) and only the data of the second year was considered.

RESULTS

Three endogenous *Trichoderma* species were tested *in vitro*, *in vivo* and *in situ* for their antagonistic activity against *Verticillium* spp. causing tomato vascular wilt in Tunisia.

Effect of *Trichoderma* spp. on mycelial growth of *Verticillium* spp. *in vitro*

Mean diameter of *Verticillium* spp. colonies, formed after six days of incubation at 20°C, depended on antagonistic treatments tested and pathogen isolates; a significant interaction was observed between both fixed factors at $p \leq 0.05$. In fact, all *Trichoderma* spp. isolates reduced the radial growth of the Verticillium wilt agents in comparison to their relative untreated controls (**Table 2**). The most important mycelial growth reduction, of about 72.3% compared to the untreated control, was recorded in the case of the isolate Vaa1 of *V. albo-atrum* treated with *T. virens*.

The importance of the mycelial growth reduction of *Verticillium* spp. isolates depended on the antagonistic species tested and within a *Trichoderma* species between isolates used; thus, intra- and inter-specific variations were noted within these endogenous fungi regarding their antagonistic potential against the target pathogens. In fact, for the same *Verticillium* isolate, as is the case of *V. dahliae* Vd82, reduction varied from 25.97 (treatment with *T. harzianum* Th1) to 62.15% (treatment with *T. virens* Tvs1). Furthermore, for the same antagonistic treatment, as is the case of *T. viride* Tv3, mycelial growth inhibition, in comparison to the untreated control, varied from 16.41 (*V. albo-atrum* Vaa 3 isolate) to 58.84% (*V. dahliae* Vd82 isolate).

Verticillium spp. colonies, observed 6 days after dual culture (**Fig. 1**), were invaded by *Trichoderma* spp. isolates; this phenomenon showed the important competitive potential of the antagonists used. Furthermore, as no antibiosis zone was observed, this competition for nutrients seemed to be involved in the registered mycelial growth reduction.

Fig. 1 illustrates not only the *Trichoderma* spp.'s competitive potential but also the significant reduction abundance in resting structures of *Verticillium* spp. isolates compared to untreated controls. Furthermore, 15-20 days after the dual culture *Verticillium*-*Trichoderma*, a dark typical colour (of melanin) of the resting structures, microsclerotia, dark mycelium and chlamydospores disappeared progressively and turned light brown. This phenomenon was not observed on untreated controls. Additionally to this sclerotinization inhibitory activity, *Trichoderma* spp. isolates caused profound alterations of *Verticillium* spp. mycelium at the confrontation zone. In fact, the mycelium showed lysis and deformation. Furthermore, several mycelial cords were formed via anastomosis mechanism and the mycelium density and sporulation were reduced compared to the untreated controls.

Table 2 Effect of some *Trichoderma* spp. isolates on the mean colony diameter (cm) of *Verticillium* spp. isolates observed after 6 days of incubation at 20°C in comparison to the untreated controls.

Verticillium	Co.	*T. harzianum*			*T. viride*			*T. virens*			Mean[a*]
isolates		Th1	Th2	Th3	Tv1	Tv2	Tv3	Tvs1	Tvs2	Tvs3	
V. dahliae											
Vd82	3.02	2.23	1.29	1.23	2.08	1.52	1.24	1.14	1.24	1.31	1.63 d
Vd13	3.25	1.43	1.55	1.53	2.04	1.77	1.85	1.32	1.3	1.38	1.74 d
Vd66	3.03	1.58	1.4	1.57	2.41	1.83	1.61	1.08	1.18	1.18	1.69 d
V. tricorpus											
Vt1	3.95	2.33	2.21	2.25	2.59	2.52	2.28	1.68	1.73	1.83	2.34 a
Vt3	4.18	2.05	2.56	2.46	2.85	2.68	3.04	1.3	1.43	1.18	2.37 a
Vt15	3.89	1.88	1.93	2.05	2.51	2.26	1.92	1.54	1.63	1.63	2.12 b
V. albo-atrum											
Vaa1	4.24	2.41	2.22	2.38	2.9	2.26	2.33	1.18	1.44	1.43	2.28 a
Vaa2	3.95	1.73	1.48	1.89	2.16	2.07	1.71	1.84	1.76	1.58	2.02 c
Vaa3	2.74	1.89	1.65	1.93	1.24	1.25	2.29	0.98	1.36	0.98	1.63 d
Mean[b*]	3.58 a	1.95 c	1.81 d	1.92 c	2.31 b	2.02 c	2.03 c	1.34 e	1.45 e	1.39 e	

LSD (*Verticillium* spp. isolates x Treatments tested) = 0.28 cm at $p \leq 0.05$.
[a] Mean colony diameters per *Verticillium* spp. isolates independently of antagonistic treatments tested
[b] Mean colony diameters of *Verticillium* spp. (independently of isolates used) per antagonistic treatment tested.
[*] For *Verticillium* spp. isolates and antagonistic treatments tested, values (means) affected with the same letter are not significantly different at $p \leq 0.05$.
Co: untreated control; Th1, Th2 and Th3: *T. harzianum* isolates; Tv1, Tv2 and Tv3: *T. viride* isolates; Tvs1, Tvs2 and Tvs3: *T. virens* isolates; Vd82, Vd13 and Vd66: *V. dahliae* isolates; Vt1, Vt3 and Vt15: *V. tricorpus* isolates; Vaa1, Vaa2 and Vaa3: *V. albo-atrum* isolates.

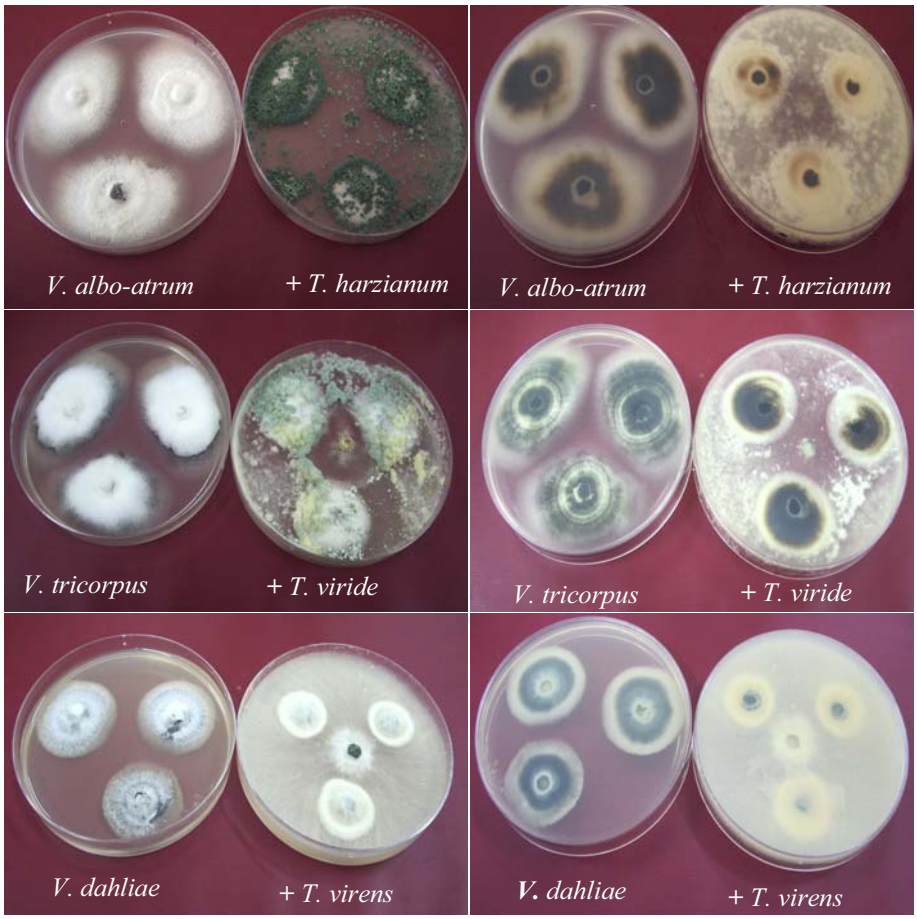

Fig. 1 Competitive potential of three *Trichoderma* species against *Verticillium* spp. observed after 6 days of dual culture on PDA at 20°C in comparison to the untreated controls.

Effects of *Trichoderma* spp. on *V. dahliae* microsclerotia

1. Microsclerotia germination

The germination of *V. dahliae* microsclerotia, exposed for 30 min to liquid cultures of antagonists tested and incubated at 20°C, was completely suppressed compared to the control microsclerotia treated with SDW only. The germination of microsclerotia in 2% agar was verified by the formation of secondary microsclerotia as noted on control plates (**Fig. 2**). In fact, after 10-30 days of incubation at 20°C, *V. dahliae* microsclerotia confronted with *Trichoderma* spp. lost their germinative capacity and consequently their viability.

2. Germinating microsclerotia

Direct confrontation of *Trichoderma* spp. with mature and viable *V. dahliae* microsclerotia, on PDA at 20°C, revealed an important space colonizing potential of *Trichoderma* spp. isolates tested. In fact, for colonizing a media already occupied by *V. dahliae*, *Trichoderma* spp. isolates caused several alterations of *V. dahliae* microsclerotia expressed by:

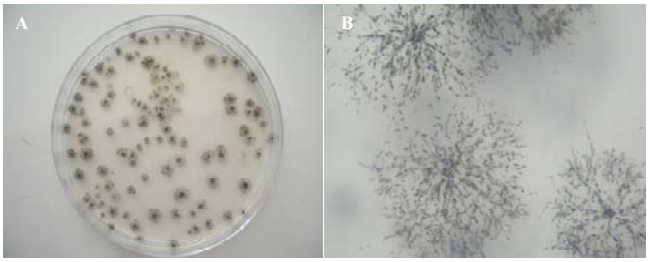

Fig. 2 Germination of *V. dahliae* microsclerotia after 10 days of incubation at 20°C on agar 2%. (**A**) Control *V. dahliae* plate; (**B**) Secondary microsclerotia formed after germination of untreated microsclerotia (G X40).

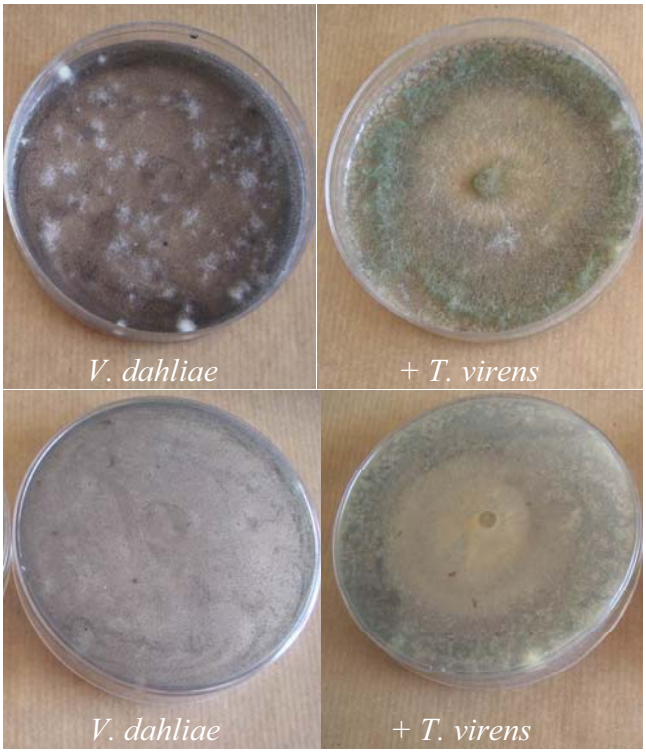

Fig. 3 Melanin bleaching of *V. dahliae* colonies treated with *T. virens* compared to untreated control observed after 20 days of incubation at 20°C.

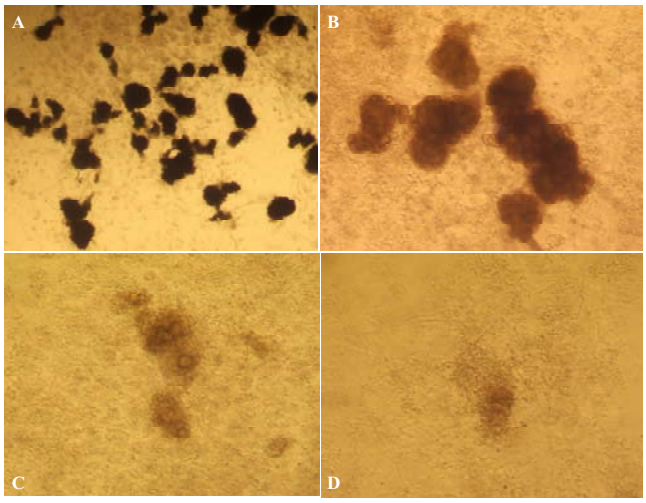

Fig. 4 Progressive degradation of melanin of *V. dahliae* microsclerotia treated with *Trichoderma* spp. compared to the melanization degree noted on untreated control microsclerotia. (**A**) Control microsclerotia; (**B**) Treated microsclerotia observed after 20 days, (**C**) Treated microsclerotia observed after 23 days, (**D**) Treated microsclerotia observed after 27 days; incubation at 20°C (G X40).

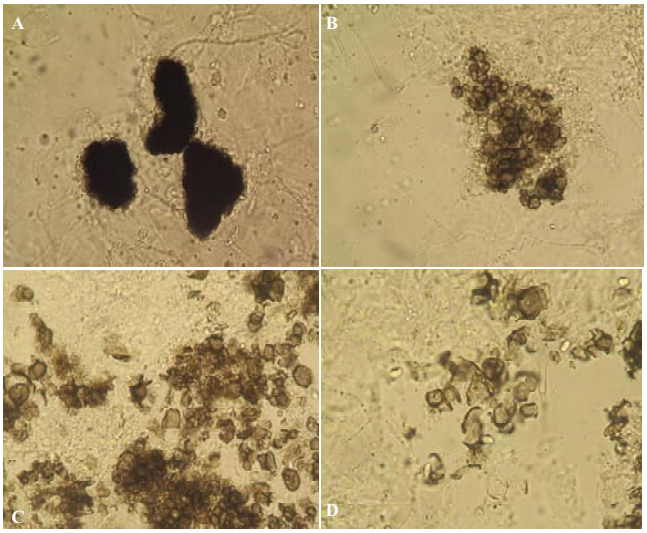

Fig. 5 Cell wall degradation and progressive disintegration of *V. dahliae* microsclerotia treated with *Trichoderma* spp. (**A**) Untreated microsclerotia; (**B**) Microsclerotia treated and observed after 20 days, (**C**) Microsclerotia treated and observed after 25 days, (**D**) Microsclerotia treated and observed after 30 days; incubation at 20°C (G X40).

❖ Inhibition of sclerogenesis: treated germinating microsclerotia became incapable of forming secondary microsclerotia (**Fig. 3A**) due to its lysis, breaking of its cellular membrane, and to the probable inhibition of melanin synthesis, estimated via the absence of the typical dark colour of cell walls, both observed microscopically (**Fig. 3B**).

❖ Melanin degradation of mature microsclerotia: mature microsclerotia treated progressively lost their typical dark colour and turned to brown and finally to light (**Fig. 3**). The typical melanin pigment disappeared after 20-35 days of confrontation; microsclerotia became invisible and could not be distinguished from agar particles (**Fig. 4**). Untreated control microsclerotia were not altered.

❖ Microsclerotia cell wall degradation: *Trichoderma* spp. were also able to degrade the cell wall microsclerotia which lost their typical mass form and became totally disintegrated (**Fig. 5**).

Effects of *Trichoderma* spp. on tomato Verticillium wilt development under growth chamber conditions

The efficiency of *Trichoderma* spp. against *V. dahliae in vivo* was evaluated on wilt severity and plant growth in comparison to untreated but inoculated or non controls (IC and NIC, respectively).

Leaf damage index

All tomato plants, treated at planting with *Trichoderma* spp. spore suspensions and transplanted in culture substrate infested with *V. dahliae*, showed typical Verticillium wilt symptoms but uninoculated and untreated (NIC) plants were symptomless. However, disease severity, estimated via the leaf damage index (LDI), depended significantly on treatments tested. In fact, the LDI, recorded 60 days post-planting on 'Ventura' tomato plants treated with *T. harzianum*, *T. viride* and *T. virens*, was significantly comparable to that noted on uninoculated and untreated control (NIC) plants and it was reduced by more than 60% in comparison to the inoculated and untreated control (IC) plants (**Fig. 6**).

Plant height

Tomato plant height, noted 60 days post-planting (**Fig. 7**),

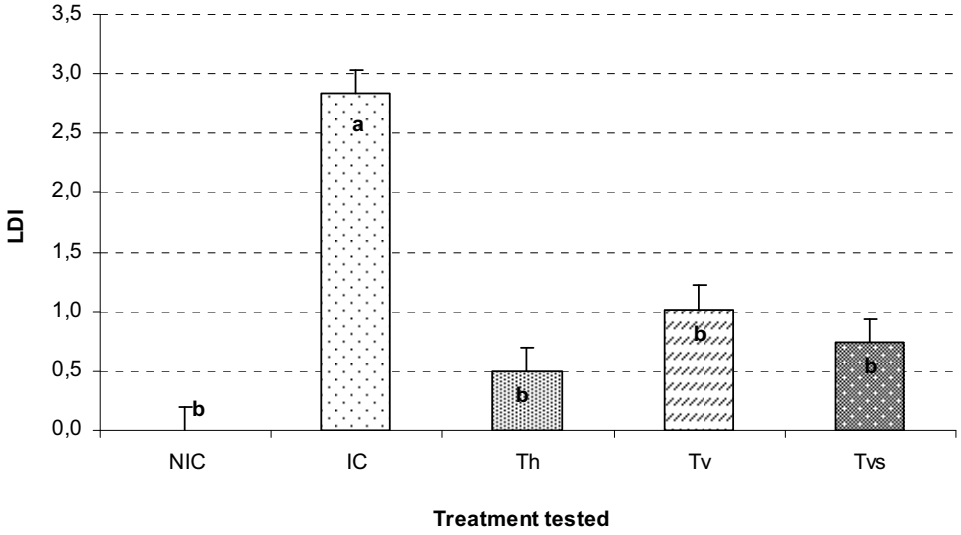

Fig. 6 Effect of treatments at planting with *Trichoderma* species on Verticillium wilt severity observed on tomato cv 'Ventura' plants 60 days after their transplanting in a culture substrate infested with *V. dahliae* in comparison to the controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test (p≤0.05). LDI: Leaf damage index, NIC: untreated and uninoculated control, IC: untreated and inoculated control, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, 15 <T< 30°C.

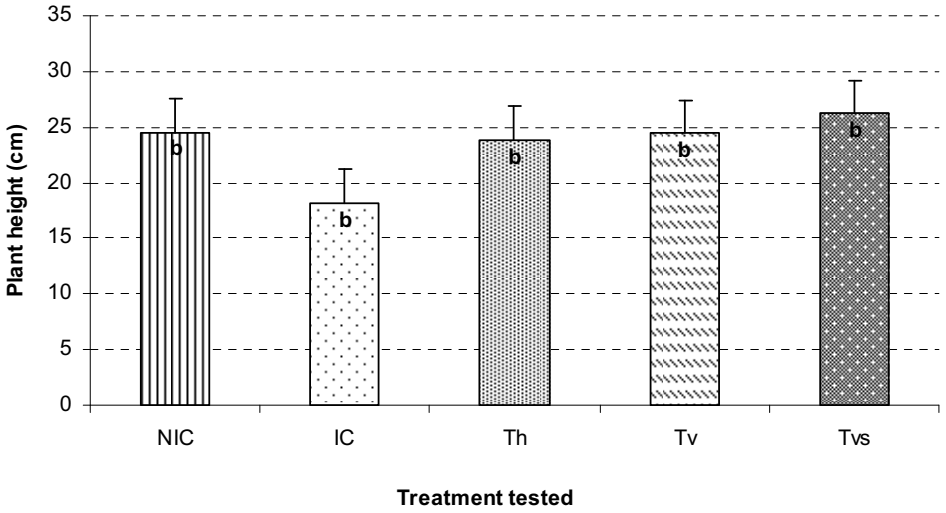

Fig. 7 Effect of treatments at planting with *Trichoderma* species on tomato cv. 'Ventura' plant height noted 60 days after transplanting in a culture substrate infested with *V. dahliae* in comparison to the controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test (p≤0.05). NIC: untreated and uninoculated control, IC: untreated and inoculated control, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, 15 <T< 30°C.

did not significantly depend on treatments tested. However, plants treated with *T. harzianum*, *T. viride* and *T. virens* showed a slight increase of about 24%, even statistically insignificant, of their height in comparison to the inoculated and untreated control plants (IC).

Root fresh weight

Tomato root fresh weight, noted 60 days post-planting, depended significantly on treatments tested. In fact, plants treated with *T. viride* and *T. virens* showed increased root fresh weight, of about 34 and 40% respectively, in comparison to the inoculated and untreated control (IC), which was significantly comparable to the untreated and uninoculated control (NIC). Plants treated with *T. harzianum* showed a root fresh weight significantly similar to the untreated but inoculated or non controls, IC and NIC, respectively (**Fig. 8**).

Stem fresh weight

Tomato stem fresh weight, noted 60 days post-planting, depended significantly on treatments tested. In fact, plants treated with *T. virens* showed increased stem fresh weight, of about 40% in comparison to the inoculated and untreated control (IC), which was significantly comparable to the untreated and uninoculated control (NIC). Plants treated with *T. harzianum* and *T. viride* showed a stem fresh weight significantly similar to that noted on inoculated and untreated (IC) control plants (**Fig. 9**).

For all treatments tested, excepted the untreated and uninoculated control (NIC), a vascular discoloration was observed on roots, collar and the stem base and the pathogen presence was confirmed after re-isolation on PDA from collar and stem.

Effects of *Trichoderma* spp. on tomato Verticillium wilt development under greenhouse conditions

The efficiency of *Trichoderma* spp. against *V. dahliae*, previously tested *in vitro* and *in vivo*, was also evaluated *in situ*, in a naturally infested soil (infectious potential estimated at 34 microsclerotia/g), on wilt severity and plant growth in comparison to the untreated control.

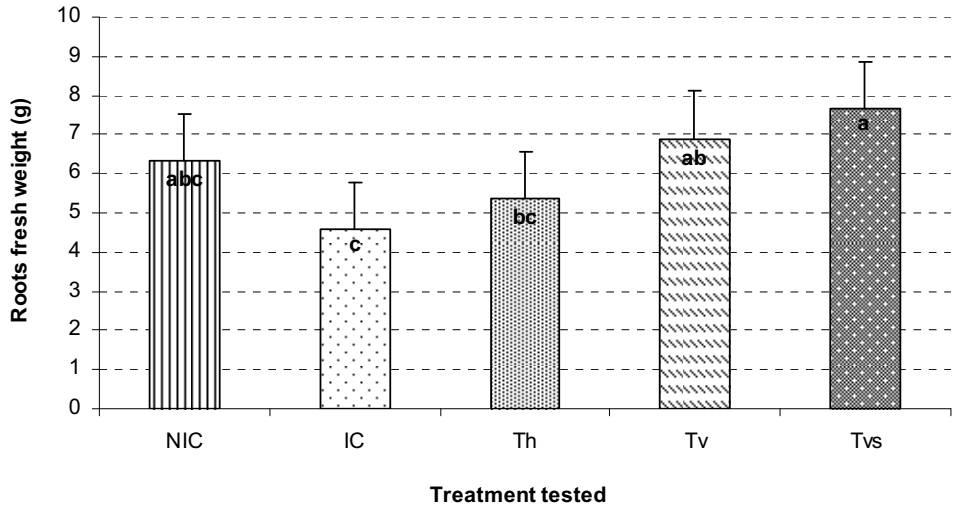

Fig. 8 Effect of treatments at planting with *Trichoderma* species on tomato cv. 'Ventura' root fresh weight noted 60 days after transplanting in a culture substrate infested with *V. dahliae* in comparison to the controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test (p≤0.05). NIC: untreated and uninoculated control, IC: untreated and inoculated control, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, 15 <T< 30°C.

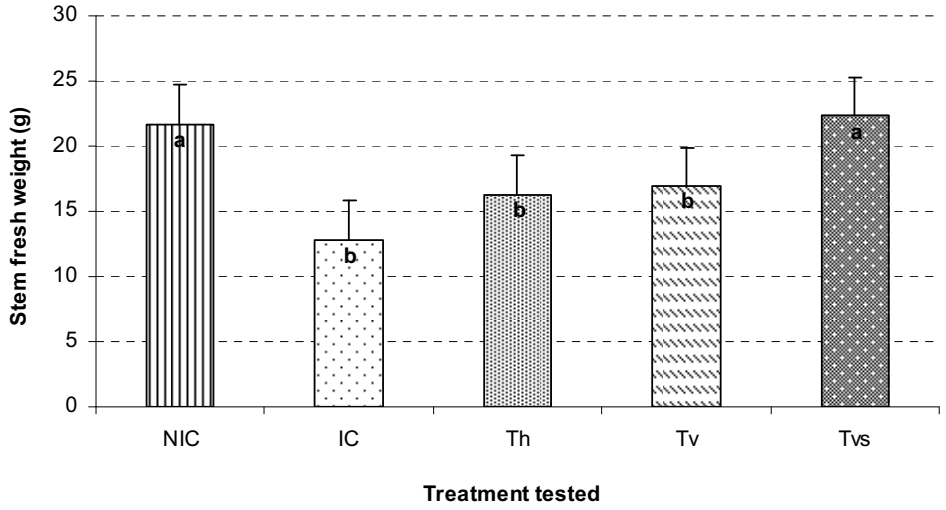

Fig. 9 Effect of treatments at planting with *Trichoderma* species on tomato cv. 'Ventura' stem fresh weight noted 60 days after transplanting in a culture substrate infested with *V. dahliae* in comparison to the controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test (p≤0.05). NIC: untreated and uninoculated control, IC: untreated and inoculated control, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, 15 <T< 30°C.

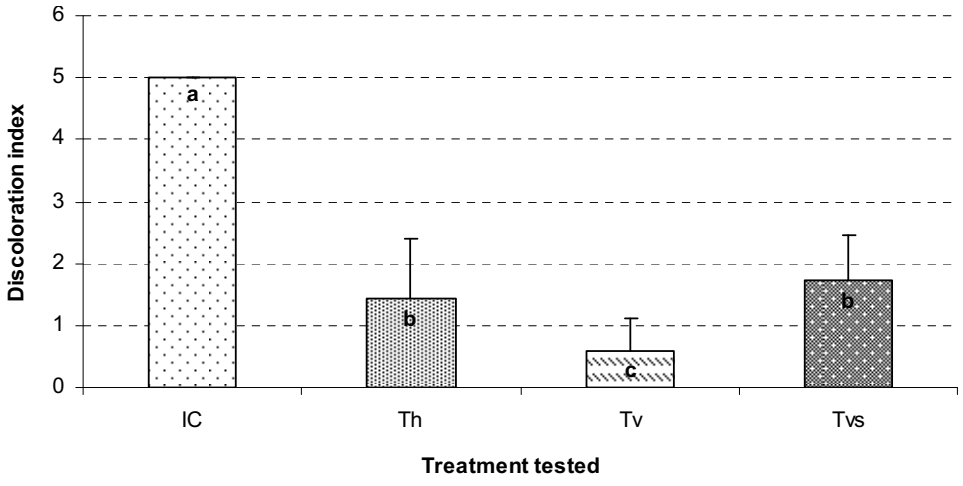

Fig. 10 Effect of treatments of tomato plants cv. 'Ventura' at planting with *Trichoderma* species on the discoloration index noted 90 days after their transplanting in a naturally infested soil with *V. dahliae* in comparison to the untreated control. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test (p≤0.05). IC: infested and untreated control, Th: infested and treated with *T. harzianum*, Tv: infested and treated with *T. viride*, Tvs: infested and treated with *T. virens*.

Discoloration index

The Verticillium wilt severity, estimated via a discoloration index, noted 90 days after transplanting, depended significantly on treatments tested. In fact, the discoloration index noted on tomato plants, treated at planting by *T. harzianum*, *T. viride* and *T. virens*, was significantly reduced compared to the untreated and infested control (IC) (**Fig. 10**). The most important reduction of this parameter, of about 88% in comparison to the untreated control, was recorded on tomato plants treated with *T. viride*.

Root fresh weight

Tomato root fresh weight, noted 90 days post-planting (**Fig. 11**), did not significantly depend on treatments tested. However, plants treated with *T. harzianum*, *T. viride* and *T. virens* showed an increase of more than 50%, even statistically insignificant, of their root fresh weight in comparison to the untreated and infested control plants (IC).

Stem fresh weight

Tomato stem fresh weight, noted 90 days post-planting (**Fig. 12**), did not significantly depend on treatments tested. However, plants treated with *T. harzianum*, *T. viride* and *T. virens* showed an increase of more than 50%, even statistically insignificant, of their stem fresh weight in comparison to the untreated and infested control.

DISCUSSION

Endogenous fungi: ecological interest and microbial interactions in the rhizosphere

Biocontrol of soil-borne pathogens and particularly *V. dahliae* was widely investigated in several countries around the world (Berg *et al.* 2001; Pegg and Brady 2002) but this is the first study in Tunisia. The originality of the present study resides in the indigenous nature of the antagonists tested and in the multiple criteria used for elucidating their effects on pathogen and, consequently, on Verticillium wilt severity. In fact, as *V. dahliae* infection occurs generally at root tips (Huisman 1988) and as colonization of this area by antagonists is considered strategic for wilt pathogens inhibition (Alstrom 2000; Narisawaa *et al.* 2000), endogenous *Trichoderma* spp., tested in the present study, were originally isolated from healthy solanaceous roots.

Endogenous agents were considered ecologically more adaptable and able to protect the plant environment from soil-borne pathogens infections (Narisawaa *et al.* 2000). In fact, in the colza-*V. dahliae* pathosystem, Alstrom (2000)

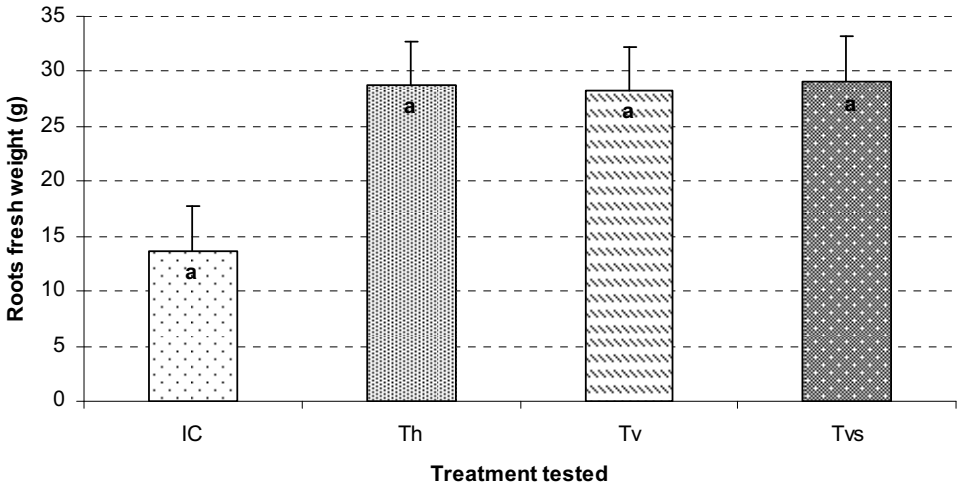

Fig. 11 Effect of treatments of tomato plants cv. 'Ventura' at planting with *Trichoderma* species on the roots fresh weight noted 90 days after their transplanting in a naturally infested soil with *V. dahliae* in comparison to the untreated control. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test ($p \leq 0.05$). IC: infested and untreated control, Th: infested and treated with *T. harzianum*, Tv: infested and treated with *T. viride*, Tvs: infested and treated with *T. virens*.

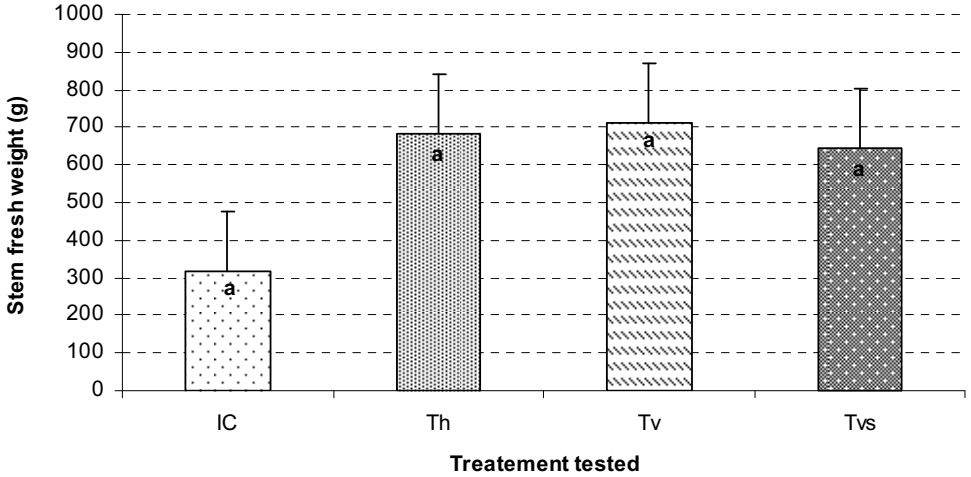

Fig. 12 Effect of treatments of tomato plants cv. 'Ventura' at planting with *Trichoderma* species on the stem fresh weight noted 90 days after their transplanting in a naturally infested soil with *V. dahliae* in comparison to the untreated control. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test ($p \leq 0.05$). IC: infested and untreated control, Th: infested and treated with *T. harzianum*, Tv: infested and treated with *T. viride*, Tvs: infested and treated with *T. virens*.

isolated and identified *Mortierella*, proved to be capable of producing a metabolite with an antifungal activity, and also isolated *Trichoderma* and *Gliocladium*, universally known as biocontrol agents. They are able to mineralize nutrients, present at the root system zone, and to affect the competitive ability of soil-borne microorganisms such as *V. dahliae.* In the same way, Narisawa *et al.* (2002) isolated 123 fungal isolates from 225 root segments of eggplant, melon, tomato, strawberry and Chinese cabbage, obtained from different fields in Shizuoka region in Japan. Amongst these isolates, 11 inoculated to eggplant plants suppressed the effect of a virulent *V. dahliae* strain. These isolates belonged to *Heteroconium chaetospira*, *Phialocephara fortinii* and to unidentified species of *Fusarium*, *Penicillium* and *Trichoderma*. Stinson *et al.* (2003) obtained an endophytic isolate of *Gliocladium* sp. from *Eucryphia cordifolia* capable to produce several organic and volatile compounds lethal for *Pythium ultimum* and *V. dahliae*.

Endogenous *Trichoderma* spp.: *in vitro* inhibition of the pathogen mycelial growth

The present results showed that all *Trichoderma* spp. Isolates reduced the radial growth of the three *Verticillium* species in comparison to the untreated controls. Similar results were reported in the *in vitro* studies of Henni (1987), D'Ercole *et al*. (2000), El Rafai *et al.* (2003) and Regragui (2005) concerning the antagonistic activity of *T. harzianum* against *V. dahliae*, and those of El Rafai *et al.* (2003) concerning *T. hamatum* and *T. harzianum* against some tomato pathogens. In fact, *T. hamatum* and *T. harzianum* culture filtrates were shown to significantly reduce sporulation, germination of conidia and length of the germinating tube of *V. dahliae* and other tomato pathogens such as *F. oxysporum* f. sp. *lycopersici* and *Alternaria solani*.

The mycelial growth reduction, noted in the present study, was mainly due to the important competitive potential of the antagonists used and to the reduction of resting structures abundance of *Verticillium* spp. isolates compared to untreated controls. Additionally to this sclerogenesis inhibitory activity, *Trichoderma* spp. isolates caused profound alterations of *Verticillium* spp. mycelium at the confrontation zone. In fact, the mycelium showed lysis and deformation. Furthermore, several mycelial cords were formed via an anastomosis mechanism and the mycelium density and sporulation were reduced compared to untreated controls. Similar modes of action of *Trichoderma* were reported by Lewis and Papavizas (1987) who attributed this phenomenon to the action of several enzymes and antibiotic substances, naturally formed or synthesized by the antagonist, affecting pathogen cell permeability. Reduction of mycelium weight, increase in protein losses, reduction in glucose maintenance, and morphological disruptions of pathogen hyphae were also observed. Similar effects on mycelial growth and microsclerotia formation of *V. dahliae* were obtained with mycelial culture or culture filtrates of *Stemphylium* sp. and the active component involved, precipitated from culture filtrates by ethanol or $(NH_4)_2SO_4$, was heatlabile (100°C for 10 min) and a non-protein secondary metabolite (Tian *et al.* 1998). In the same way, Al-Rawahi and Hancock (1998) also found that *Pythium oligandrum* parasitized, in dual culture, *V. dahliae* and affected negatively its *in vitro* growth and ability to form microsclerotia.

Endogenous *Trichoderma* spp.: inhibition of the sclerogenesis process

Besides the mycelial growth reduction and the drastic mycelium alterations, antagonistic activity of *Trichoderma* spp. tested was also expressed by a delay in the sclerogenesis process. In fact, the present study showed that germinating microsclerotia treated with *Trichoderma* spp. became incapable of forming secondary microsclerotia due to the lysis and to the probable inhibition of melanin synthesis. Furthermore, mature microsclerotia treated progressively lost their typical dark colour and turned brown and finally light. Thus, endogenous *Trichoderma* spp. isolates, used in the present study, were also able to degrade the cell wall of microsclerotia, which lost their typical mass form and became totally disintegrated. Similar sclerogenesis inhibition was also reported in several studies where *V. dahliae* was confronted with different antagonists such as *Streptomyces plicatus* (Aghighi *et al.* 2004), *T. harzianum* (Regragui 2005) and rhizobacteria (Alstrom 2001). Regragui (2005) attributed the *T. harzianum* effect on sclerogenesis to production of volatile antifungal metabolites. Furthermore, according to Alstrom (2001), this delay in microsclerotia production may be also caused by gaseous metabolites produced by bacterial strains via direct dual culture *in vitro***.**

The inhibition of sclerogenesis is an important mechanism in *Verticillium* biocontrol as soil inoculum could be reduced by these indigenous antagonists. Furthermore, as in monocyclic diseases, such as Verticillium wilts, disease severity was correlated with initial soil inoculum, reduced microsclerotia viability could, consequently, decrease significantly wilt incidence (Keinath *et al.* 1991; Fahima and Henis 1995; Nagtzaam *et al.* 1998; Alstrom 2000). In the same way, Tjamos (2000) reported that any biocontrol agent able to interact with microsclerotia formation, to affect their survival and to delay or prevent pathogen establishment in plant, was considered efficient as these resting structures are important for pathogen survival and infection process initiation.

Endogenous *Trichoderma* spp. isolates, tested on colonized perlite in the present study, were also able to inactivate and to reduce germination of microsclerotia dipped during 30 min in antagonist spore suspension. Microsclerotia were considered non-viable when they did not germinate after their rinsing with SDW and their viability was evaluated by the formation of secondary microsclerotia (Nagtzaam *et al.* 1998). Population analysis of *T. harzianum* in California soils showed that densities of *V. nubilum* were negatively correlated with increases in *T. harzianum* populations which were highest during wet winter months and had a second peak in early summer (Eastburn and Butler 1988). Lost of reserve compounds such as lipids, necessary for microsclerotia germination, was shown to be involved in this reduced germination ability as also induced by permeabilizing activity of biosurfactants (Kim *et al.* 2004). However, *V. dahliae* microsclerotia reduced viability was due, in the case of *Talaromyces flavus,* to production of an extra cellular glucose oxidase enzyme (Marois *et al*. 1982; Fravel and Roberts 1991). Similarly, Stosz *et al*. (1996) found that inhibition of microsclerotia germination was correlated with glucose oxidase production.

Endogenous *Trichoderma* spp.: reduction of Verticillium wilt severity *in vivo* and *in situ*

The present study revealed that all tomato plants, grown under growth chamber conditions and treated at planting with *Trichoderma* spp. spore suspensions and transplanted in culture substrate infested with *V. dahliae*, showed reduced Verticillium wilt severity in comparison to the inoculated and untreated control plants. In the same way, the discoloration index, noted on tomato plants, grown under natural greenhouse conditions and treated at planting by *T. harzianum*, *T. viride* and *T. virens*, was significantly reduced compared to the untreated and infested control. Verticillium disease intensity was also reduced when roots of susceptible tomato varieties were injected with both *Verticillium* and *Trichoderma* compared to an injection with *Verticillium* alone (Pegg and Brady 2002). *T. viride* was able to reduce the severity of Verticillium wilt (Sportelli *et al.* 1983). Furthermore, two strains of *T. virens* were found to significantly reduce the disease severity ratings in cotton plants of the two cultivars 'Rowden' and 'Deltapine' 50, inoculated with *V. dahliae* (Hanson 2000). In the same way, D'Ercole *et al.* (1984) isolated from soil *T. viride* (29 strains), *T. harzianum* (14 strains), *T. koningii* (6 strains), *T. hamatum* (2

strains), *T. polysporum* (2 strains) and *T. pseudokoningii* (one strain), all active against *V. dahliae*. D'Ercole and Nipoti (1986) claimed successful control of tomato wilt using the first three *Trichoderma* species. Similarly, the eggplant Verticillium wilt was reduced when *Trichoderma* mycelium was directly incorporated into soil (D'Ercole *et al.* 1997). Marois *et al.* (1982) tested 34 fungal soil isolates, in a glasshouse experiment, for their activity against *V. dahliae* from eggplant; the most active ones were *Aspergillus alutaceus*, *Gliocladium virens*, *Paecilomyces lilacinus*, *Talaromyces flavus* and *T. viride*. These fungal isolates reduced the Verticillium wilt by 0–20% compared with 90% in the controls, when they are introduced into a *V. dahliae*-infested soil. Furthermore, Khalimov and Ynusov (1980) found that a *T. viride*–lignin mixture applied at 120 kg/ha reduced cotton wilt by half; at 2000 kg/ha, wilt was reduced by two-thirds. In laboratory experiments, Wilderspin and Heale (1984) obtained a 50% reduction in *V. albo-atrum* infection in *Antirrhinum*. Czaplinska (1973), using *Trichoderma* to control wilts also reported, via small-scale experiments, inhibition of *V. albo-atrum* on lucerne roots.

Similar reduced Verticillium wilt severity was obtained by antagonists other then *Trichoderma* species. In fact, Larena *et al.* (2003) found that *Penicillium oxalicum*, applied by watering of tomato plants by spore suspension, 7 days prior transplanting, decreased vascular wilts caused by *V. dahliae* and *Fusarium oxysporum* f. sp. *lycopersici* under greenhouse and field conditions. Comparable decreases in Verticillium wilt severity, estimated via a disease index, were also noted on cotton plants inoculated with *Stemphylium* pre- or post-*V. dahliae* inoculation (Tian *et al.* 1998). Furthermore, two weeks after incorporation of *Talaromyces flavus* into infested soil, germination of *V. dahliae* microsclerotia decreased by 32-41% in comparison to uninfested soil. Reduction of *V. dahliae* microsclerotia viability and increase of *T. flavus* population in the rhizosphere were shown to be involved in the consequent decrease of Verticillium wilt severity on eggplant, cotton and potato (Marois *et al.* 1982).

Verticillium wilt suppression, under controlled and field conditions, was also achieved by treatment of cotton plants with species of the genus *Gliocladium*; *G. roseum* isolates were also capable to reduce viability of *V. dahliae* microsclerotia (Pegg and Brady 2002). Muromtsev (1980) also showed, in both laboratory and field experiments, that *Gliocladium* spp. isolates were able to suppress cotton wilt. Globus and Muromtsev (1990) alleviated *V. dahliae* wilt of cotton by 12–34% by a powder containing 2–3 × 10^8 propagules of *G. roseum*/g at a rate of 5–10 kg/ha. In the same way, Zeise (1997) and Solarska (1997), working with tomato, oilseed rape and hops, noted greater disease control by inoculating soil with *Talaromyces flavus* before sowing or planting.

Endogenous *Trichoderma* spp.: enhancement of plant growth

Tomato plants treated with *T. harzianum*, *T. viride* and *T. virens* showed a slight increase of about 24% of their height in comparison to the inoculated and untreated control. Furthermore, treated with *T. virens*, tomato plants showed increased root and stem fresh weights, of about 40% in comparison to the inoculated and untreated control. However, under greenhouse conditions, roots and stem fresh weights of tomato plants treated with *T. harzianum*, *T. viride* and *T. virens* were enhanced by more than 50% in comparison to the untreated and infested control. Similar growth promoting effects were induced by *Trichoderma* sp., *Coniothyrium* sp. and *Penicillium* sp. In fact, Jordan and Tarr (1978) obtained a significant increase in plant size after treatment of strawberry roots before planting in *V. dahliae*-infested soil, compared with untreated controls. Application of *T. harzianum* into sowing rows resulted in disease incidence reduction and yield increase in potato (Ordentlich *et al.* 1990). Furthermore, soil inoculation and seed coating with *T. hamatum* spores completely controlled Verticillium wilt caused by *V. dahliae* in tomato and improved yield (El Rafai *et al.* 2003).

In greenhouse studies, growth of pepper and fruit yield were higher in *V. dahliae*-infested soil in the presence of *Pythium oligandrum* compared with *Pythium*-free controls. However, in the absence of *Verticillium*, plants grown in the presence of *P. oligandrum* were 40–50% taller, suggesting a growth-promoting role similar to the fluorescent pseudomonads effect (Al-Rawahi and Hancock 1998). Cotton wilt, reduced in grey semi-desert soils by 22–28%, was associated with increased yield by 0.3–0.5 T/ha (Azimkhodzbayeva and Ramasanova 1990). In the same way, using isolate Tf1 (*Talaromyces flavus*) ascospore pre-plant drenches in soil, Marois *et al.* (1982) suppressed *V. dahliae* wilt of eggplant, with a concomitant yield increase comparable with chemical control.

Finally, the effect of these biological treatments on tomato yield will be evaluated in future experiments conducted in different geographical sites where mainly the soil infectious potential, the Verticillium wilt incidence and the consequent impact on tomato yield will be used as indicators of treatments efficacy. Furthermore, as biocontrol agent's efficacy and consequent effect on plant growth and yield was shown to vary depending on plant species and cultivars (Handelsman and Stabb 1996) and certain plants were more attractive and supported diverse antagonistic communities of pathogen (Azad *et al.* 1985), these *Trichoderma* spp. isolates will be also tested against potato Verticillium wilt because this crop is the most rotated with tomato in the major agricultural fields in Tunisia.

ACKNOWLEDGEMENTS

We thank the Regional Center of Research in Horticulture and Organic Agriculture (CRRHAB) and the Higher Agronomic Institute of Chott-Mariem (ISA-CM) for financial contribution. We also thank Mouna Gueddess-Chahed and Aymen Youssef for technical assistance.

REFERENCES

Aghighi S, Shahidi Bonjar GH, Saadoun I (2004) First report of antifungal properties of a new strain of *Streptomyces plicatus* (strain101) against four Iranian phytopathogenic isolates of *Verticillium dahliae*, a new horizon in biocontrol agents. *Biotechnology* **3**, 90-97

Al-Rawahi AK, Hancock JG (1998) Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. *Plant Disease* **82**, 1100-1106

Alstrom S (2000) Root colonizing fungi from oilseed rape and their inhibition of *Verticillium dahliae*. *Journal of Phytopathology* **148**, 417-423

Alstrom S (2001) Characteristics of bacteria from oilseed rape in relation to their biocontrol activity against *Verticillium dahliae*. *Journal of Phytopathology* **149**, 57-64

Azad HR, Davis JR, Kado CI (1985) Relationships between rhizoplane and rhizosphere bacteria and Verticillium wilt resistance in potato. *Archives of Microbiology* **140**, 347-351

Azimkhodzbayeva NN, Ramasanova SS (1990) Application of organomineral fertilizers with lignin in Verticillium wilt control. *Proceedings of the 5th International Verticillium Symposium*, 25th-30th June, Leningrad, USSR, p 88 (Abstract)

Bender CG, Shoemaker PB (1984) Prevalence of Verticillium wilt of tomato and virulence of *Verticillium dahliae* race 1 and race 2 isolates in Western North Carolina. *Plant Disease* **68**, 305-309

Berg G, Frankowski J, Bahl H (1999) Biocontrol of Verticillium wilt in oilseed rape by chitinolytic *Serratia plymuthica*. *Proceedings of 10th International Rapeseed Congress*, 26th-29th September, Canberra, Australia, p 369 (Abstract)

Berg G, Fritze A, Roskot N, Smalla K (2001) Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. *Journal of Applied Microbiology* **91**, 963-971

Béye I, Lafay JF (1985) Etude des critères de sélection pour une résistance générale à la Verticilliose chez la tomate. *Agronomie* **5**, 305-311

Concibido VC, Secor GA, Jansky SH (1994) Evaluation of resistance to Verticillium wilt in diploid, wild potato interspecific hybrids. *Euphytica* **76**, 145-152

Czaplinska S (1973) Studies on the diseases of lucerne caused by the fungi *Verticillium albo-atrum* and *Ascochyta imperfecta*. *Acta Mycologica* **9**, 23-52

Daami-Remadi M, Jabnoun-Khiareddine H, Barbara DJ, Ayed F, El Mah-

joub M (2006) First report of *Verticillium dahliae* race 2 in Tunisia. *Plant Pathology* **55**, 816

D'Ercole N, Nipoti P (1986) Lotta biologica contro fusariosi e verticilliosi del pomodoro in coltura protetta. *Colture Protette* **15**, 55-59

D'Ercole N, Nipoti P, Di Pillo L, Gavina F (1997) *In vitro* and *in vivo* tests of *Trichoderma* spp. as biocontrol agents of *Verticillium dahliae* in eggplants. *Proceedings of the 7th International Verticillium Symposium*, 6th-10th October, Cape Sounion, Athens, Greece, p 68 (Abstract)

D'Ercole N, Nipoti P, Di Pillo L, Gavina F (2000) *In vitro* and *in vivo* tests of *Trichoderma spp*. as a biocontrol agent of *Verticillium dahliae* kleb. in eggplants. In: Tjamos EC, Rowe RC, Heale JB, Fravel DR (Eds) *Advances in Verticillium: Research and Disease Management*, APS Press, St. Paul, MN, USA, pp 260-263

D'Ercole N, Sportelli M, Nipoti P (1984) Differenti tipi di antagonismo di *Trichoderma* sp. nei confronti di miceti fitopatogeni del terreno. *Informatore Fitopatologico* **34**, 43-47

Eastburn DM, Butler EE (1988) Microhabitat characterization of *Trichoderma harzianum* in natural soil: evaluation of factors affecting population density. *Soil Biology and Biochemistry* **20**, 541-545

El Aissami A, Lahlou H (1999) Adaptation progressive à la luzerne d'un isolat de *Verticillium dahliae* originaire de la tomate. *Bulletin OEPP/EPPO* **29**, 191-195

El-Rafai IM, Asswah SMW, Awdalla OA (2003) Biocontrol of some tomato disease using some antagonistic microorganisms. *Pakistan Journal of Biological Science* **6**, 399-406

Fahima T, Henis Y (1995) Quantitative assessment of the interaction between the antagonistic fungus *Talaromyces flavus* and the wilt pathogen *Verticillium dahliae* on eggplant roots. *Plant and Soil* **176**, 129-137

Fravel DR, Roberts DP (1991) *In situ* evidence for the role of glucose oxidase in the biocontrol of Verticillium wilt by *Talaromyces flavus. Biocontrol Science and Technology* **1**, 91-99

Globus GA, Muromtsev GS (1990) The use of *Gliocladium roseum* as antagonist for defence of cotton from phytopathgenic fungi. *Proceedings of the 5th International Verticillium Symposium*, 25th-30th June, Leningrad, USSR, p 90 (Abstract)

Hall TJ, Schreiber LR (1984) Effect of *Bacillus* on Verticillium wilt in silver maple. *Phytopathology* **74**, 805

Handelsman J, Stabb EV (1996) Biocontrol of soilborne plant pathogens. *The Plant Cell* **8**, 1855-1869

Hanson L (2000) Reduction of Verticillium wilt symptoms in cotton following seed treatment with *Trichoderma virens*. *The Journal of Cotton Science* **4**, 224-231

Harrington MA, Dobinson KF (2000) Influences of cropping practices on *Verticillium dahliae* populations in commercial processing tomato fields in Ontario. *Phytopathology* **90**, 1011-1017

Henni JE (1987) Evaluation de l'efficacité de certains champignons antagonistes vis-à-vis de *Verticillium dahliae. Cryptogamie, Mycologie* **8**, 203-207

Huisman OC (1988) Colonization of field-grown cotton roots by pathogenic and saprophytic soilborne fungi. *Phytopathology* **78**, 716-722

Jabnoun-Khiareddine H, Daami-Remadi M, El Mahjoub M (2005) Emergence en Tunisie de nouveaux pathotypes de *Verticillium tricorpus* pouvant attaquer la tomate, l'aubergine et la pomme de terre. *Bulletin EOPP/OEPP* **35**, 497-503

Jabnoun-Khiareddine H, Daami-Remadi M, Hibar K, Ayed F, El Mahjoub M (2006) Pathogenicity of Tunisian isolates of three *Verticillium* species on tomato and eggplant. *Plant Pathology Journal* **5**, 199-207

Jabnoun-Khiareddine H, Daami-Remadi M, Ayed F, Jebari H, El Mahjoub M (2007) Incidence of Verticillium wilt of melon in Tunisia. *The African Journal of Plant Science and Biotechnology* **1**, 10-15

Jansky SH, Rouse DI (2000) Identification of potato interspecific hybrids resistant to Verticillium wilt and determination of criteria for resistance assessment. *Potato Research* **43**, 239-251

Jansky SH, Rouse DI, Kauth PJ (2004) Inheritance of resistance to *Verticillium dahliae* in diploid interspecific potato hybrids. *Plant Disease* **88**, 1075-1078

Jordan VWL, Tarr HS (1978) Inoculum suppression of *Verticillium dahliae*. *Annals of Applied Biology* **89**, 139-141

Kawchuk LM, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, Kokko E, Byers R, Howard RJ, Fischer R, Prüfer D (2001) Tomato *Ve* disease resistance genes encode cell surface-like receptors. *Proceedings of the National Academy of Science USA* **98**, 6511-6515

Keinath AP, Fravel DR, Papavizas GC (1991) Potential of *Gliocladium roseum* for biocontrol of *Verticillium dahliae*. *Phytopathology* **81**, 644-648

Khalimov A, Ynusov M (1980) Lignin in combination with *Trichoderma*. *Khlopkovodst* **5**, 22-23

Kim PI, Bai H, Bai D, Chae H, Chung S, Kim Y, Park R, Chi YT (2004) Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *Journal of Applied Microbiology* **97**, 942-949

Kim KK, Fravel DR, Papavizas GC (1988) Identification of a metabolite produced by *Talaromyces flavus* as glucose oxidase and its role in biocontrol of *Verticillium dahliae. Phytopathology* **78**, 488-492

Larena I, Sabuquillo P, Melgarejo P, De Cal A (2003) Biocontrol of Fusarium and Verticillium wilt of tomato by *Penicillium oxalicum* under greenhouse and field conditions. *Journal of Phytopathology* **151**, 507-512

Lewis JA, Papavizas GC (1987) Permeability changes in hyphae of *Rhizoctonia solani* induced by germling preparations of *Trichoderma* and *Gliocladium*. *Phytopathology* **77**, 699-703

Lynch DR, Kawchuk LM, Hachey J, Bains PS, Howard RJ (1997) Identification of a gene conferring high levels of resistance to Verticillium wilt in *Solanum chacoense*. *Plant Disease* **81**, 1011-1014

Marois JJ, Johnston SA, Dunn MT, Papavizas GC (1982) Biological control of Verticillium wilt of eggplant in field. *Plant Disease* **66**, 1166-1168

Matta A, Garibaldi A (1977) Control of *Verticillium* of tomato by preinoculation with avirulent fungi. *Netherlands Journal of Plant Patholology* **83**, 457-462

Millar RL, Kalb DW, Keinath AP (1984) Biological and chemical control of Verticillium wilt of alfafa. *Phytopathology* **74**, 805

Muromtsev GS (1980) Microbiological aspects of wilt control of cotton. *Zashchita Rastenii* **10**, 35-36

Nadakavukaren MJ, Horner CE (1959) An alcohol agar medium selective for determining *Verticillium* microsclerotia in soil. *Phytopathology* **49**, 527-528

Nagtzaam MPM, Bollen GJ, Termorshuizen AJ (1998) Efficacy of *Talaromyces flavus* alone or in combination with other antagonists in controlling *Verticillium dahliae* in growth chamber experiments. *Journal of Phytopathology* **146**, 165-173

Narisawa K, Kawamata H, Currah RS, Hashiba T (2002) Suppression of Verticillium wilt in eggplant by some fungal root endophytes. *European Journal of Plant Pathology* **108**, 103-109

Narisawaa K, Ohkib KT, Hashibab T (2000) Suppression of clubroot and *Verticillium* yellows in Chinese cabbage in the field by the root endophytic fungus, *Heteroconium chaetospira*. *Plant Pathology* **49**, 141-146

Ordentlich A, Nachmias A, Chet I (1990) Integrated control of *Verticillium dahliae* in potato by *Trichoderma harzianum* and captan. *Crop Protection* **9**, 363-366

Pegg GF, Brady BL (2002) *Verticillium Wilts*, CABI Publishing, Oxford, UK, 541 pp

Pharand B, Carisse O, Benhamou N (2002) Cytological aspects of compost-mediated induced resistance against Fusarium crown and root rot in tomato. *Phytopathology* **92**, 424-438

Regragui A (2005) Contribution à l'étude de l'influence de la salinité sur le couple tomate-*Verticillium*: Conséquences physiologiques et impact sur la bioprotection des tomates contre la Verticilliose. PhD thesis, Université Mohammed V- Agdal Faculté des Sciences, Rabat, Morocco, 231 pp

Solarska E (1997) Antagonistic action of *Talaromyces flavus* and *Trichoderma viride* against *Verticillium albo-atrum* on hops. *Proceedings of the 7th International Verticillium Symposium*, 6th-10th October, Cape Sounion, Athens, Greece, p 62 (Abstract)

Sportelli M, Nipoti P, D'Ercole N (1983) Prove di 'lotta biologica' contro alcune micopatie del pomodoro in coltura protetta. *Informatore Fitopatologico* **33**, 35-38

Stinson M, Ezra D, Hess WM, Sears J, Strobel G (2003) An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. *Plant Science* **165**, 913-922

Stosz SK, Fravel DR, Roberts DP (1996) *In vitro* analysis of the role of glucose oxidase from *Talaromyces flavus* in biocontrol of the plant pathogen *Verticillium dahliae*. *Applied and Environmental Microbiology* **62**, 3183-3186

Tian L, Wang KR, Lu JY (1998) Effect of carbendazim and tricyclazole on microsclerotia and melanin formation of *Verticillium dahliae*. *Acta Phytopathologica Sinica* **28**, 263-268

Tjamos EC (2000) Strategies in developing methods and applying techniques for the biological control of *Verticillium dahliae*. In: Tjamos EC, Rowe RC, Heale JB, Fravel DR (Eds) *Advances in Verticillium: Research and Disease Management*, APS Press, St. Paul, MN, USA, pp 249-252

Uppal AK, El Hadrami A, Adam LR, Tenuta M, Daayf F (2007) Pathogenic variability of *Verticillium dahliae* isolates from potato fields in Manitoba and screening of bacteria for their biocontrol. *Canadian Journal of Plant Pathology* **29**, 141-152

Wilderspin IF, Heale JB (1984) Verticillium wilt (*V. albo-atrum*) of hops. Report of the East Malling Research Station 1983, p 105

Zeise K (1997) The potential of *Talaromyces flavus* (Kloekner) Stolk & Samson in controlling *Verticillium dahliae*. *Proceedings of the 7th International Verticillium Symposium*, 6th-10th October, Cape Sounion, Athens, Greece, p 61 (Abstract)