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Evaluation of Several Indigenous Microorganisms and Some Bio-Fungicides for Biocontrol of Potato Verticillium Wilt

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

Verticillium wilt development was completely suppressed with treatments based on indigenous *Trichoderma harzianum*, *T. virens*, *Gliocladium catenulatum*, *Penicillium* sp. and rhizobacteria incorporated into the culture substrate 15 days prior inoculation with pathogen. The vascular discoloration extent noted on all potato (*Solanum tuberosum* L.) 'Spunta' plants treated biologically was statistically comparable to that recorded on non-inoculated and untreated plants which were symptomless. Potato plants treated with *G. roseum*, *G. catenulatum*, *T. harzianum*, *T. virens*, *Penicillium* sp. and indigenous rhizobacteria showed a significant increase, by more than 55%, of their height in comparison to the untreated and inoculated control. Roots and stem fresh weights increase, compared to the untreated and inoculated control, with some indigenous antagonistic treatments ranging between 49-109 and 28-102%, respectively. Plants treated with *Penicillium* sp. and indigenous rhizobacteria showed an increase of 44-46% of their tuber weight compared to the untreated and inoculated control. The LDI recorded with bio-fungicides based treatments was reduced by more than 65% in comparison to the inoculated and untreated plants. Wilt development was completely suppressed with Biofolar™, based on citric acid and mint oil, which was incorporated into infested culture substrate. All plants treated with bio-fungicides showed a significantly increased height of about 12 to 28% compared to the untreated and inoculated control. With *T. harzianum* (Biocont-T™) and *Pythium oligandrum* (Polyversum®) based bio-fungicides, the roots, stem and tuber fresh weights recorded were 28-86, 48-81 and 28-57% higher than that noted on untreated and inoculated control plants, respectively.

Keywords: antagonistic treatment, disease severity, inoculation, plant growth, *Solanum tuberosum* L., yield

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the third most important food crop in the world after wheat and rice (Wang *et al*. 2008; Schieber and Aranda Saldaña 2009; Visser *et al*. 2009). In Tunisia, Verticillium wilt (VW) is widespread in the major potato producing regions from the North-West to the Centre-East of the country, and favoured by the mild Mediterranean climate. *Verticillium* spp. cause premature senescence resulting into 30-50% yield losses and low quality tubers (Johnson 1988; Rowe and Powelson 2002). *V. dahliae* is both a soil- and seed-borne Deuteromycete fungus (Alström 2001; Rowe and Powelson 2002). Potato tubers represent the more efficient means of disease spread because initial infection is often unnoticed due to pathogen localization in the vascular ring and symptoms developed relatively late during culture. Thus, VW represents a serious constraint to local seed production programs. Furthermore, in a recent study, Daami-Remadi *et al.* (2011) have shown that three *Verticillium* species are involved in potato vascular wilt symptoms and contributing, with variable degrees, to the potato Verticillium wilt complex in Tunisia. In addition, synergistic interactions between *V. dahliae* and certain phytoparasitic nematodes such as *Pratylenchus*, *Globodera*, *Meloidogyne* species lead to increased wilt incidence and severity (Tjamos 1989; Rotenberg *et al*. 2004; Daami-Remadi *et al*. 2009).

V. dahliae has a wide host range, including vegetable and field crops, herbaceous ornamentals, ground cover, shrubs and trees (Bhat and Subbarao 1999; Fradin and Thomma 2006). In Tunisia, *V. dahliae* was isolated from tomato, pepper, eggplant, potato, melon, cucumber, squash, artichoke and olive tree (Jabnoun-Khiareddine *et al.* 2005, 2006; Triki *et al.* 2006; Jabnoun-Khiareddine *et al.* 2007a, 2007b, 2008).

VW is difficult to control due not only to the endogenous growth of the pathogen, and to its ability to infect multiple hosts, but also to the multi-years longevity of its resting structures in the soil and to the inefficacy of fungicides on pathogens once they reach the xylem (Hawke and Lazarovits 1994; Alström 2001; Fradin and Thomma 2006). Thus, VW control is limited to crop rotation (Scholte 1989), fumigation with methyl bromide and other fumigants (Rowe *et al.* 1987), and soil solarization (Tjamos and Paplomatas 1988; Katan and DeVay 1991). However, these strategies are either inefficient in reducing inoculum levels in the soil or harmful to the environment and human health (Uppal *et al.* 2007).

The deploying of resistant cultivars is efficient, economically profitable and environmentally safe for potato VW control. However, the most commercialized potato cultivars are susceptible and breeding lines for Verticillium resistance was undertaken for satisfying disease control (Jadari *et al.* 1992; Wheeler *et al.* 1994; Cristinzio *et al.* 1995; Omer *et al.* 2000; Bae *et al.* 2008). In fact, in Tunisia, most grown potato cultivars exhibit varying degrees of susceptibility to VW, ranging from moderate to high resulting in significant reductions in yield (Daami-Remadi *et al.* 2010).

Other alternative strategies to control Verticillium wilt rely currently on the use of fungal (Daayf *et al.* 2003; El Hassni *et al.* 2004; Jabnoun-Khiareddine *et al.* 2009a, 2009b) or rhizobacterial antagonists (Ongena *et al.* 1999; Narisawa *et al.* 2002; Berg *et al.* 2005; Tjamos *et al.* 2005;

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Uppal *et al.* 2007; Li *et al.* 2008; Zhu *et al.* 2009), endophytes (Alström 2001; Narisawa *et al.* 2004; Shittu *et al.* 2009), mycorrhiza (Karagiannidis *et al.* 2002; Garmendia 2004), nitrogenous and organic amendments including composts (Tenuta and Lazarovits 2002; Bailey and Lazarovits 2003; Termorshuizen *et al.* 2006; Ochiai *et al.* 2008; Giotis *et al.* 2009), biological soil desinfestation (Blok *et al.* 2000; Gould *et al.* 2004) and plant extracts (Uppal *et al.* 2008). In fact, the suppression of *Verticillium* was achieved by 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). *Gliocladium* species were largely used as antagonists of *Verticillium* spp. and particularly *G. roseum* was successfully used for the control of *V. dahliae* and reduction of microsclerotia viability (Marois *et al.* 1984; Keinath *et al.* 1991; Fravel 1996). Larena *et al.* (2003) used *Penicillium oxalicum* for reduction of tomato vascular wilts. In a previous study, the tomato VW severity was significantly reduced, under growth chamber and greenhouse conditions, by the use of endogenous *Trichoderma* spp., *Gliocladium* spp. and *Penicillium* sp., colonizing naturally vegetable roots (Jabnoun-Khiareddine *et al*. 2009a, 2009b).

Rhizobacteria were studied for their plant growth-promoting effects and also for their antagonistic potential against plant diseases (Kloepper and Beauchamp 1992; Liu *et al.* 1995; Silva *et al.* 2004). Endophytic bacteria were defined by Hallmann *et al.* (1997) as bacteria which can be isolated from the surface of disinfected root tissues or extracted from the internal plant tissues visibly undamaged. This root colonization can reduce pathogen action directly via production of antimicrobial substances or competition for space, nutrients or ecological niches or indirectly, via induction of systemic resistance (Kloepper and Beauchamp 1992; Liu *et al.* 1995). Several rhizosphere or endophytic bacteria have been reported as biocontrol agents against *V. dahliae* (Berg *et al.* 2001; Mercado-Blanco *et al.* 2004; Berg *et al.* 2005; Malandraki *et al.* 2008; Uppal *et al.* 2008). *Bacillus subtilis* was shown to be efficient for control of the strawberry VW (Jordan and Tarr 1978). Similarly, rhizospheric strains of *Bacillus*, *Erwinia*, *Flavobacterium*, *Pantoea*, *Pseudomonas*, *Serratia*, *Sphingomonas*, *Stenotrophomonas* and *Streptomyces* isolated from *Brassica* spp., *Capsella* spp. and *Fragaria* spp. produced substances with inhibitory activities against *V. dahliae* and which suppressed strawberry and colza VW (Berg *et al.* 1994; Berg 1996; Berg *et al.* 2000).

The aim of the present work was to investigate the *in vivo* antagonistic potential of some endophytic fungi and bacteria, isolated from vegetable crops and compared with chemical fungicide and a known biocontrol reference agent, for their ability to control the potato VW. Some bio-fungicides based on plant extracts or antagonistic microorganisms are also tested in a separate assay for comparison.

MATERIALS AND METHODS

Plant material

Potato seed tubers (*Solanum tuberosum* L. cv. 'Spunta'), the most cultivated in Tunisia, were used. They were superficially disinfected with a 10% sodium hypochlorite solution for 5 min, rinsed with tap water and air dried. They were placed under environmental conditions favourable for pre-germination (15-20°C, 60-80% relative humidity and natural room light).

Pathogen inoculum

V. dahliae isolates used in the present study were obtained from different hosts including solanaceous and cucurbits plants (**Table 1**) showing wilt symptoms and vascular discoloration. They were cultured at 20°C on PDA (potato dextrose agar) medium added with 300 mg/l of streptomycin sulphate (Pharmadrug production Gmbh-Hambourg, 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 4-5 days. The spore suspension concentration used was adjusted to 10^7 spores/ml by a Malassez cytometer. For their long-term preservation, pathogen isolates were stored up to 12 months at -20°C in a 25% glycerol solution.

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

Isolates	Original plant hosts	Origins
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

Fungal antagonists

Isolates of *Trichoderma harzianum*, *T. viride*, *T. virens*, *Gliocladium catenulatum* (three isolates per species), *G. roseum* and *Penicillium* sp. (two isolates each), used in the present study, were already screened for their ability to control tomato VW via *in vitro*, *in vivo* and *in situ* experiments (Jabnoun-Khiareddine *et al*. 2009a, 2009b).

They were isolated from roots of several vegetables (tomato, eggplant, potato and melon) and the monoconidial cultures were identified according to the Kubicek and Harman (1998) key. They 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.

Bacterial antagonists

Rhizobacteria (*Bacillus* sp.) used were isolated from roots and rhizosphere of healthy and vigorous tomato and pepper plants, collected from regions of Chott Mariem and Bembla (Coastal regions of Tunisia, Governorates of Sousse and Monsatir, respectively).

Isolation of bacteria was made according to the soil dilution method of Waksman (1922). In fact, 100 g of soil, collected with roots, were added to 100 ml of sterile distilled water (SDW) and shaken at 200 rpm during 30 min. Three to four successive dilutions were made and the obtained soil suspensions were cultured on NA (nutrient agar) and KB (King's B agar) media (King *et al.* 1954; Raaijmakers *et al.* 1995) added with chloramphenicol (13 µg/l, 98%, Agros, Organics, New Jersey, USA) and ampicillin (100 µg/l, 100% a.i., Sigma, St. Louis, MO).

A reference strain of *Pseudomonas fluorescens* p 417, with known antagonistic activity and gratefully provided by Dr. Bakker (Department of Plant Ecology and Evolutionary Biology, Section of Plant Pathology, Utrecht University, Utrecht, the Netherlands), was included in the present study for comparison with Tunisian stains.

Liquid bacterial cultures were prepared on KB liquid media and stored at 6°C until use. For their long term preservation, bacterial isolates were stored at –20°C in NA added with 40% of glycerol following the Kim *et al*. (1997) technique.

Chemical and biological fungicides

Three biological products (bio-fungicides) and a fungicide were tested *in vivo* against *V. dahliae*. Active antagonistic agents or chemical ingredients and rates used are presented in **Table 2**. The chemical fungicide was included in the experiment as a positive control.

Table 2 Bio-fungicides tested and doses used against *V. dahliae in vivo*.

Active ingredient/agent	Commercial name	Dose
Trichoderma harzianum	Biocont-T WP™	1 g/l
Pythium oligandrum	Polyversum®	1 g/l
Citric acid + mint oil	Biofolar™	200 cc/hl

Effect of antagonists tested on potato VW development

At the multi-germ stage, tubers were planted in plastic pots (25 cm diameter) containing a peat and perlite mixture (2: 1), previously sterilized at 110°C for 1 h, and humidified with 200 ml of liquid culture of each mixture of fungal or bacterial isolates tested (5 tubers per each fungal or bacterial treatment). Five tubers planted in peat and perlite mixture humidified with a similar volume of SDW only served as untreated controls.

Five tubers planted in uninfected culture substrate watered with 100 ml of oxyquinolin suspension (Cryptonol®, Novartis AG, Basel, Switzerland) used at 3.5 l/hl) were included as positive control (control treated chemically). After emergence, plants were watered every 2 to 3 days depending on environmental conditions and plants' needs.

Potato plants were inoculated with *V. dahliae*, 14 days post-emergence, by watering each pot with 100 ml of pathogen spore suspension (adjusted to 10^7 spores/ml).

Tubers planted in sterile culture substrate and watered with 100 ml of SDW or 100 ml of pathogen spore suspension served as untreated and non-inoculated (NIC) and untreated and inoculated (IC) controls, respectively.

Effect of bio-fungicides on potato VW development

Germinating tubers (5 tubers/elementary treatment) were individually dipped in different bio-fungicides, except for Biofolar™, prepared according to doses mentioned in **Table 2** then planted in colonized peat and perlite prepared as described above. However, Biofolar™, which was a fertilizing, was mixed with the culture substrate just before plantation.

Tubers dipped in SDW and planted in sterile or infested culture substrate served as untreated and non-inoculated (NIC) and untreated and inoculated (IC) controls, respectively.

Plants were placed under greenhouse conditions for 60 days where temperatures ranged between 11 and 35°C (minima and maxima, respectively). They were regularly watered and fertilized with a nutritious solution (Pharand *et al*. 2002).

Evaluation criteria

VW severity was estimated, 60 days post inoculation (DPI), via the leaf damage index (LDI) and according to 0-4 scale depending on symptom severity on leaves as previously described by Béye and Lafay (1985) and used in Jabnoun-Khiareddine *et al.* (2007b). In addition, the vascular discoloration extent was determined after longitudinal sectioning of stems.

Effects of biological and chemical treatments tested were also evaluated via plant growth and production parameters: height and roots, stems and tubers fresh weights.

The presence of pathogen in the stem was also verified by re-isolation on PDA and a percentage of pathogen re-isolation from treated plants was calculated for each treatment tested. In fact, per elementary treatment, 12-15 segments obtained from the base, the medium and the top of the stems were plated on PDA and incubated at 20°C for 7 days. The percentage of pathogen re-isolation was calculated via the number of segments colonized with *V. dahliae* in comparison to the total number of segments placed.

Statistical analyses

Statistical analyses were performed, for all parameters measured, following a completely randomised design where treatments (fungal and bacterial antagonists (or bio-fungicides tested), IC and NIC controls, and/or the chemical treatment) were the sole fixed factor. The chemical fungicide was included in the antagonist's assay only. Five replicates were used per elementary treatment and means were separated using Fisher's protected LSD test (at $P \leq 0.05$).

RESULTS

Suppressive effects of fungal and bacterial antagonists tested

The efficacy of the antagonists tested was evaluated, 60 DPI, via several parameters expressing disease severity (LDI and vascular discoloration extent) and plant growth (height and roots, stems and tuber weights).

Leaf damage index

Wilt severity depended significantly on treatments tested. In fact, the LDI recorded on potato 'Spunta' plants treated biologically or chemically was statistically comparable to that noted on non-inoculated and untreated control plants (NIC)

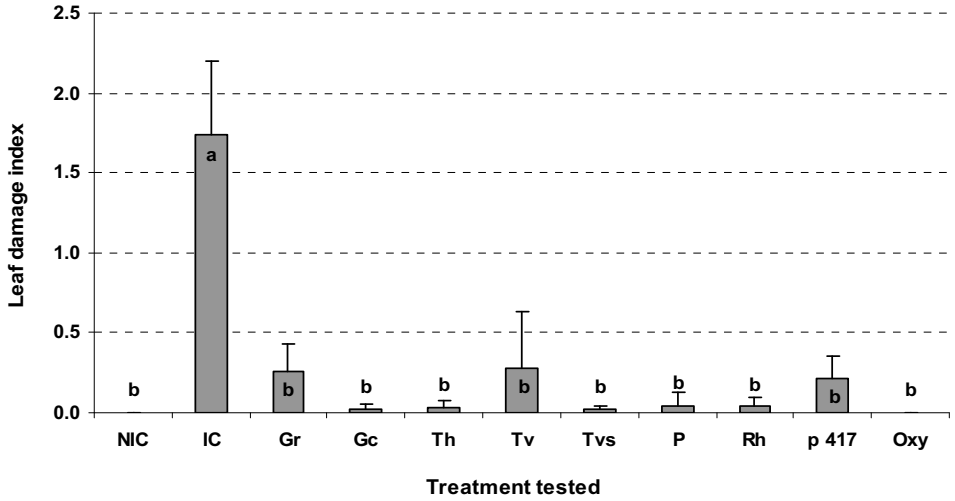

Fig. 1 Effect of antagonists tested on the leaf damage index noted on potato 'Spunta' plants, 60 days post-inoculation with *V. dahliae* in comparison to the different controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test ($P \leq 0.05$). NIC: untreated and non-inoculated control, IC: untreated and inoculated control, Gr: inoculated and treated with *G. roseum*; Gc: inoculated and treated with *G. catenulatum*, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, P: inoculated and treated with *Penicillium* sp., Rh: inoculated and treated with indigenous rhizobacteria; p 417: inoculated and treated with the reference strain of *Pseudomonas fluorescens* p 417; Oxy: inoculated and treated with oxyquinolin, 11 < T < 35°C.

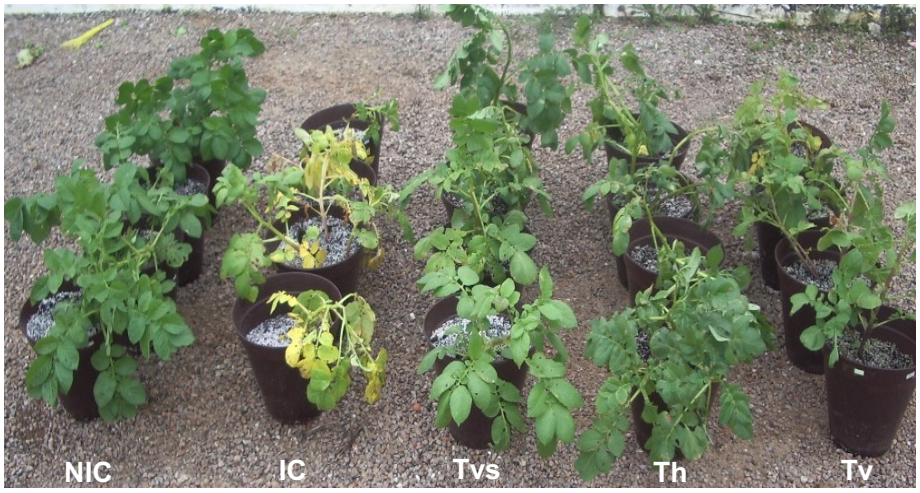

Fig. 2 Potato 'Spunta' plants treated with 3 *Trichoderma* species in comparison to the untreated but inoculated or non controls observed 60 days after their incorporation into the culture substrate and their subsequent inoculation with *V. dahliae*. NIC: untreated and non-inoculated control, IC: untreated and inoculated control, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, 11 < T < 35°C.

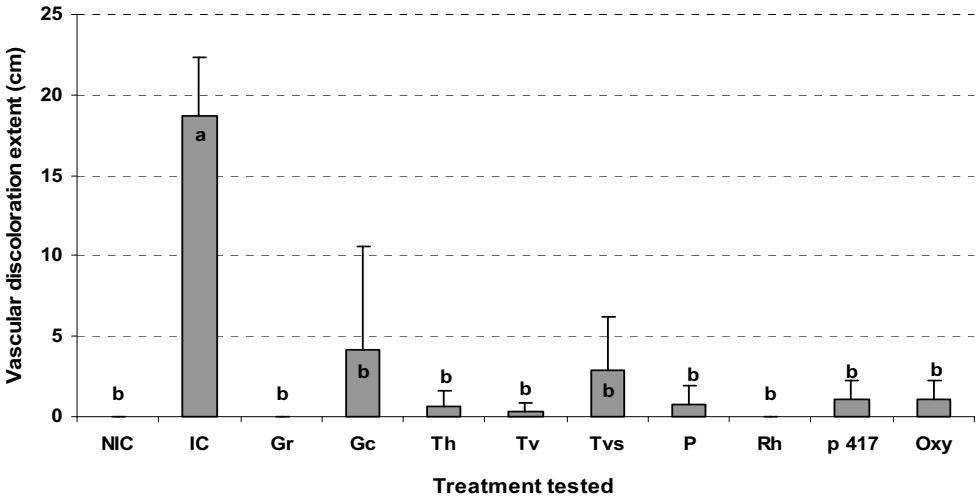

Fig. 3 Effect of antagonists tested on the vascular discoloration extent noted on potato 'Spunta' plants, 60 days post-inoculation with *V. dahliae* in comparison to the different controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test ($P \leq 0.05$). NIC: untreated and non-inoculated control, IC: untreated and inoculated control, Gr: inoculated and treated with *G. roseum*; Gc: inoculated and treated with *G. catenulatum*, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, P: inoculated and treated with *Penicillium* sp., Rh: inoculated and treated with indigenous rhizobacteria; p 417: inoculated and treated with the reference strain of *Pseudomonas fluorescens* p 417; Oxy: inoculated and treated with oxyquinolin, 11 < T < 35°C.

which were symptomless and it was reduced by more than 80% in comparison to the inoculated and untreated plants (IC).

Incorporation of fungal (*T. harzianum*, *T. virens*, *G. catenulatum*, *Penicillium* sp.) and bacterial (indigenous rhizobacteria) antagonists into the culture substrate, 15-21 days prior inoculation by watering potato plants with a mixture of *V. dahliae* isolates, completely prevented the expression of VW symptoms, as also observed with the fungicide-based treatment (oxyquinolin), in comparison to the untreated and inoculated control (**Figs. 1, 2**).

Vascular discoloration extent

Wilt severity, also estimated via the vascular discoloration extent (or discoloration height) depended significantly on treatments tested. In fact, this parameter noted on all potato 'Spunta' plants treated biologically or chemically was statistically comparable to that recorded on non-inoculated and untreated (NIC) plants (**Fig. 3**) as also concluded via the LDI parameter. The most important vascular discoloration extent of about 18.7 cm, representing 78% of plant height, was registered on the untreated and inoculated potato plants.

Plant height

Potato plant height, noted 60 DPI (**Fig. 4**), depended significantly on treatments tested. In fact, plants treated with *G. roseum*, *G. catenulatum*, *T. harzianum*, *T. virens*, *Penicillium* sp. and indigenous rhizobacteria showed a significant increase, by more than 55%, of their height in comparison to the inoculated and untreated control (IC). However, plants treated biologically with *T. viride* and the *P. fluorescens* reference strain p 417 and chemically with oxyquinolin showed statistically comparable height as untreated and inoculated plants.

Plant weight

Potato roots fresh weight, noted 60 DPI (**Table 3**), did not significantly depend on treatments tested. However, plants treated with *G. roseum*, *T. harzianum* and *Penicillium* sp. showed an increase of about 81-109%, even statistically insignificant, of their root fresh weight in comparison to the untreated and inoculated control (IC). Plants treated with indigenous rhizobacteria showed increased roots weight, of about 55%, which was nearly three times higher than that

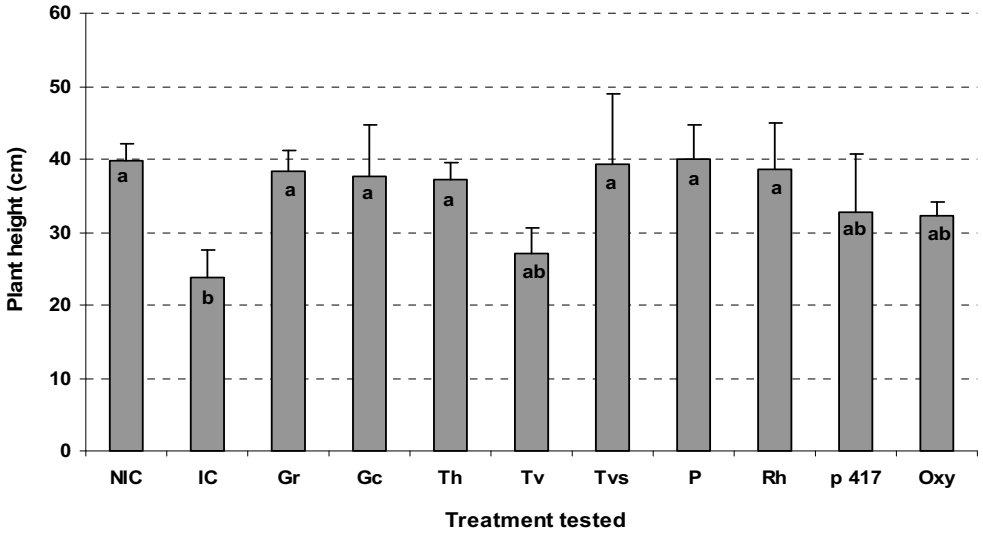

Fig. 4 Effect of antagonists tested on potato 'Spunta' plants height noted, 60 days post-inoculation with *V. dahliae*, in comparison to the different controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test ($P \leq 0.05$). NIC: untreated and non-inoculated control, IC: untreated and inoculated control, Gr: inoculated and treated with *G. roseum*; Gc: inoculated and treated with *G. catenulatum*, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, P: inoculated and treated with *Penicillium* sp., Rh: inoculated and treated with indigenous rhizobacteria; p 417: inoculated and treated with the reference strain of *Pseudomonas fluorescens* p 417; Oxy: inoculated and treated with oxyquinolin, 11 < T < 35°C.

Table 3 Effects of different fungal and bacterial antagonists on roots, stems and tubers weight of potato plants inoculated at planting with *V. dahliae* in comparison to a chemical treatment and to the untreated but inoculated or non controls.

Treatment	Root weight (g)*	Stem weight (g)	Tuber weight (g)
NIC	18.64 a	58.32 bcd	100.74 a
IC	11.36 a	42.05 d	73.82 a
Gr	23.74 a	61.15 bcd	84.64 a
Gc	16.96 a	68.34 b	88.36 a
Th	21.75 a	65.65 b	95.62 a
Tv	15.27 a	48.74 bcd	73.65 a
Tvs	11.80 a	62.48 bc	90.30 a
P	20.61 a	85.17 a	106.28 a
Rh	17.58 a	53.90 bcd	107.89 a
p 417	13.58 a	43.28 cd	90.16 a
Oxy	12.20 a	48.31 bcd	91.96 a

* Different letters within a column indicate significant differences according to Fisher's protected least significant difference LSD test ($P \leq 0.05$). NIC: untreated and non-inoculated control, IC: untreated and inoculated control, Gr: inoculated and treated with *G. roseum*; Gc: inoculated and treated with *G. catenulatum*, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, P: inoculated and treated with *Penicillium* sp., Rh: inoculated and treated with indigenous rhizobacteria; p 417: inoculated and treated with the reference strain of *Pseudomonas fluorescens* p 417, Oxy: inoculated and treated with oxyquinolin, 11 < T < 35°C.

obtained with the reference strain of *P. fluorescens* p 417, compared to the untreated and inoculated control.

Table 3 shows that all treatments tested on potato plants affected significantly the stems fresh weight noted 60 DPI. In fact, the most important stem weight was recorded on plants inoculated and treated with *Penicillium* sp. which was two times higher than that noted on untreated and inoculated plants.

Plants treated at planting with *Gliocladium* and *Trichoderma* species and indigenous rhizobacteria showed significantly comparable stem weight as the untreated and non-inoculated control plants; moreover, their weight was increased by 28 to 62% compared to the inoculated and untreated control.

All treatments tested did not affect significantly the tubers fresh weight noted 60 DPI. However, plants treated with *Penicillium* sp. and indigenous rhizobacteria showed an increase of 44-46% of their tuber weight compared to the untreated and inoculated control; this increase was also nearly two times higher, even statistically insignificant, than that recorded with oxyquinolin and the reference strain *P. fluorescens* p 417.

Pathogen re-isolation

Fig. 5 showed that pathogen re-isolation from potato plants, 60 days after their inoculation varied depending on treatments tested (data not analyzed statistically). In fact, *V. dahliae* was isolated from 66% of segments plated on PDA issued from untreated and inoculated plants. However, pathogen was not recovered from segments removed untreated and non-inoculated plants and those inoculated and treated with *T. harzianum*, *T. virens*, *Penicillium* sp. and the reference stain of *P. fluorescens* p 417.

With the other biological treatments tested, the percentage of pathogen re-isolation ranged between 12 and 33%, which was 50 to 81% less than that noted on untreated and inoculated control plants, compared to 25% recorded with the oxyquinolin-based treatment.

Suppressive effects of bio-fungicides tested

The efficacy of the bio-fungicides tested was evaluated, 60 DPI, via several parameters expressing disease severity (leaf damage index only) and plant growth.

Leaf damage index

The LDI depended significantly on treatments tested on potato 'Spunta' plants (**Fig. 6**). In fact, the LDI recorded with bio-fungicides based treatments was statistically comparable to that noted on non-inoculated and untreated control plants (NIC) and it was reduced by more than 65% in comparison to the inoculated and untreated plants (IC). Moreover, wilt development was completely suppressed, where LDI reduction reached 100% compared to IC treatment, with the bio-fungicide Biofolar^{TM}, based on citric acid and mint oil, incorporated into culture substrate. VW severity was reduced by 65 and 69% with *Pythium oligandrum* and *T. harzianum* based bio-fungicides (Polyversum$^{®}$ and Biocont-T^{TM}, respectively).

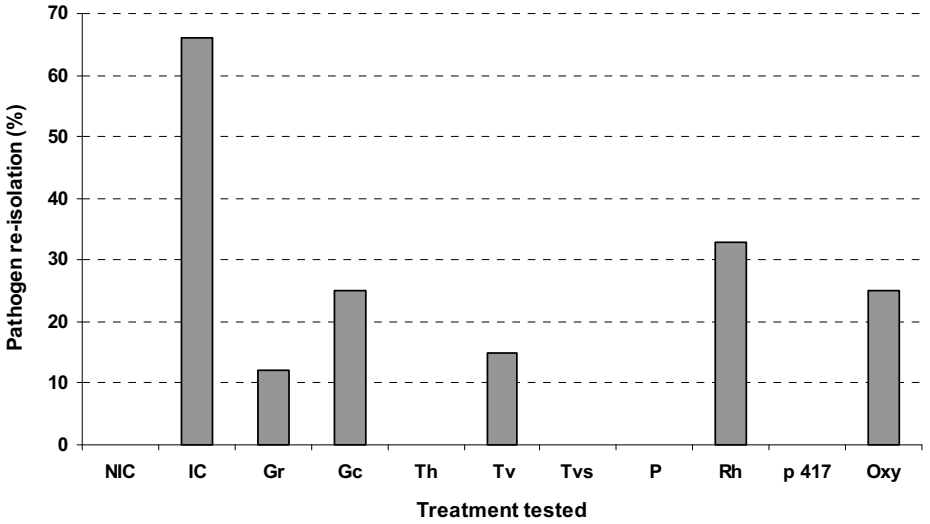

Fig. 5 Percentage of *V. dahliae* re-isolation on PDA from potato 'Spunta' plants depending on treatments tested. NIC: untreated and non-inoculated control, IC: untreated and inoculated control, Gr: inoculated and treated with *G. roseum*; Gc: inoculated and treated with *G. catenulatum*, Th: inoculated and treated with *T. harzianum*, Tv: inoculated and treated with *T. viride*, Tvs: inoculated and treated with *T. virens*, P: inoculated and treated with *Penicillium* sp., Rh: inoculated and treated with indigenous rhizobacteria; p 417: inoculated and treated with the reference strain of *Pseudomonas fluorescens* p 417; Oxy: inoculated and treated with oxyquinolin, 11 < T < 35°C.

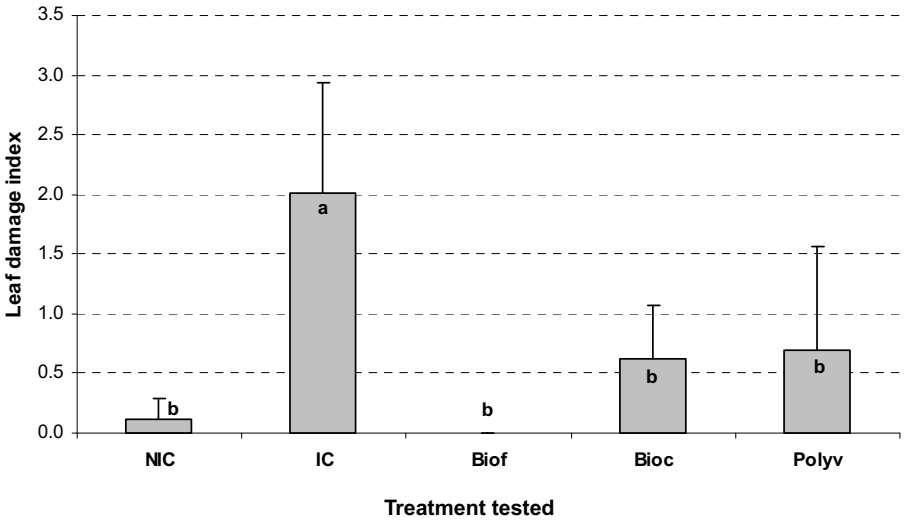

Fig. 6 Effect of some bio-fungicides on the leaf damage index noted on potato 'Spunta' plants, 60 days post-inoculation with *V. dahliae* in comparison to the untreated but inoculated or non controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test ($P \leq 0.05$). NIC: untreated and non-inoculated control, IC: untreated and inoculated control Polyv: inoculated and treated with Polyversum® based on *P. oligandrum*; Bioc: inoculated and treated with Biocont-T™ based on *T. harzianum*, Biof: inoculated and treated with Biofolar™ based on citric acid and mint oil, 11 < T < 35°C.

Plant height

Potato plant height, noted 60 DPI (**Fig. 7**), depended significantly on bio-fungicides and controls used. In fact, all plants treated with bio-fungicides showed a significantly increased height of about 12 to 28% compared to the untreated and inoculated control. Moreover, plants treated with Biocont-T™, based on *T. harzianum*, showed a significantly comparable height as untreated and non-inoculated controls.

Plant weight

Potato roots fresh weights, noted 60 DPI (**Table 4**), did not significantly depend on bio-fungicides and treatments tested. However, plants treated with Biocont-T™, based on *T. harzianum*, and Polyversum®, composed of *Pythium oligandrum*, showed an increase of about 86 and 28% respectively, even statistically insignificant, of their root fresh weight in comparison to the untreated control (IC).

Table 4 showed that all treatments tested on potato plants did not affect significantly the stems fresh weight noted 60 DPI. However, the most important stem weights were recorded on plants inoculated and treated with Biocont-T™ and Polyversum® which were 81 and 48% higher, respectively, than that noted for untreated and inoculated plants.

All bio-fungicides and treatments used affected significantly the tubers fresh weight. In fact, the least tubers fresh weight was noted on potato plants treated with Biofolar™ composed of citric acid and mint oil. However, plants treated with Biocont-T™ and Polyversum® produced more tubers for which the registered weight was 57 and 28% higher, respectively than that recorded on untreated and inoculated plants. Moreover, this tuber weight increase exceeded by 49 and 22% that noted on untreated and non-inoculated control (NIC) plants for treatments based on Biofolar™ and Polyversum®, respectively.

DISCUSSION

Biocontrol of soil-borne fungi such as *V. dahliae* has been actively explored worldwide (Berg *et al.* 2001; Pegg and

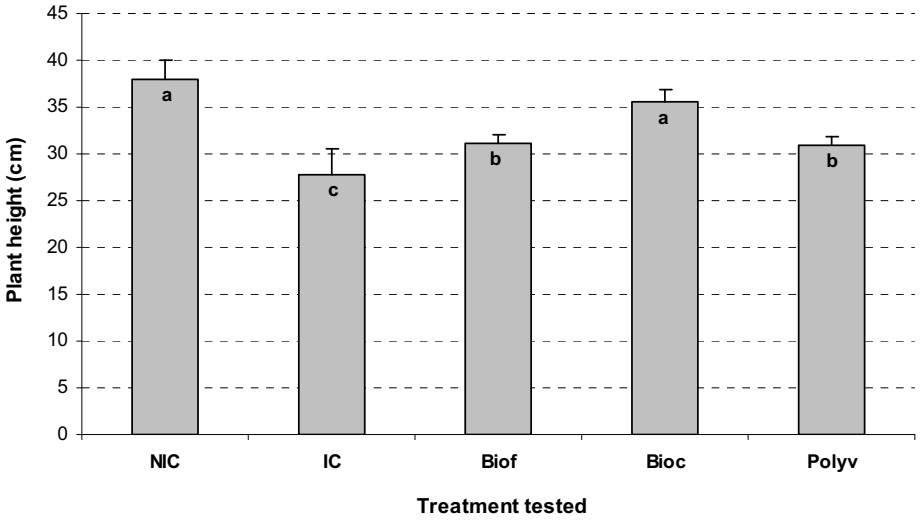

Fig. 7 Effect of some bio-fungicides on potato 'Spunta' plants height noted 60 days post-inoculation with *V. dahliae* in comparison to the untreated but inoculated or non controls. Bars with the same letter are not significantly different according to Fisher's protected least significant difference LSD test ($P \leq 0.05$). NIC: untreated and non-inoculated control; IC: untreated and inoculated control; Polyv: inoculated and treated with Polyversum® based on *P. oligandrum*; Bioc: inoculated and treated with Biocont-T™ based on *T. harzianum*, Biof: inoculated and treated with Biofolar™ based on citric acid and mint oil, 11 < T < 35°C.

Table 4 Effects of different bio-fungicides on roots, stems and tubers weight of potato plants inoculated at planting with *V. dahliae* in comparison to the untreated but inoculated or non controls.

Treatment	Root weight (g)*	Stem weight (g)	Tuber weight (g)
NIC	13.71 a	7.53 a	52.13 ab
IC	4.35 a	5.1 a	49.51 ab
Biof™	4.72 a	5.29 a	31.83 b
Bioc™	8.09 a	9.22 a	77.61 a
Polyv®	5.58 a	7.53 a	63.44 ab

* Different letters within a column indicate significant differences according to Fisher's protected least significant difference LSD test ($P \leq 0.05$). NIC: untreated and non-inoculated control, IC: untreated and inoculated control Polyv: inoculated and treated with Polyversum® based on *P. oligandrum*; Bioc: inoculated and treated with Biocont-T™ based on *T. harzianum*, Biof: inoculated and treated with Biofolar™ based on citric acid and mint oil, 11 < T < 35°C.

Brady 2002) but the present study is the first one dealing with potato VW biocontrol in Tunisia.

Potential biocontrol of VW by indigenous antagonists and some bio-fungicides with regard to pathogen and antagonist inoculum and application conditions

Given that the key element for maximizing chances of selection of biocontrol agents efficient against VW agents is firstly the assessment of genetic variability within *Verticillium* population, the inoculum used is composed of a mixture of virulent isolates of *V. dahliae*, the predominant species in Tunisia, issued from different plants and regions and for which aggressiveness has been previously determined (Jabnoun-Khiareddine *et al*. 2007a, 2007b).

As the rhizosphere is the site of interactions between plants, microbial pathogens, and antagonistic fungi and rhizobacteria (Handelsman and Stabb 1996; Sörensen 1997; Berg *et al.* 2000, 2005), it constitutes an interesting setting for the interactions that lead to disease and biocontrol of disease (Handelsman and Stabb 1996). In fact, all biocontrol agents tested in this study belonged to the indigenous microflora. In fact, *Trichoderma* spp., *Gliocladium* spp. and *Penicillium* sp. isolates as well as rhizobacteria of the genus *Bacillus*, were obtained from soil and roots of different *Verticillium* host plants. These fungal isolates were also already tested for their inhibitory activity against three *Verticillium* species *in vitro* and for their capacity to reduce wilt severity and to promote growth of tomato plants in *in vivo* and *in situ* previous experiments (Jabnoun-Khiareddine *et al*. 2009a, 2009b).

Moreover, as the majority of naturally occurring biological control results likely from mixtures of microorganisms in suppressive soils (Lemanceau and Alabouvette 1991), we used in the present work a mixture of isolates belonging to the different antagonist species tested. Furthermore, rhizobacterial isolates were already evaluated for their *in vitro* inhibitory activity against *Verticillium* spp. and were shown to be antagonistic to this pathogen (Jabnoun-Khiareddine, unpublished data). In fact, according to Spadaro and Gullino (2005), by combining specific strains of microorganisms, multiple traits antagonizing the pathogen can be combined and this may result in a higher level of protection. For example, when *Pseudomonas putida* WCS358 strain, a siderophore-producing bacteria, was combined with *P. putida* RE8 strain, inducing systemic resistance, against *F. oxysporum* f. sp. *raphani*, Fusarium wilt suppression was significantly enhanced (de Boer *et al.* 2003).

The present results revealed that potato bioprotection against VW is possible with some indigenous antagonists and bio-fungicides tested and this independently of their method of application i.e. incorporation into culture substrate or seed tuber dipping, respectively.

Efficacy of VW biocontrol depends on the method of application of antagonists in the ecosystem, the number of treatments and the capacity of the selected strain to adapt to different environments (Minuto *et al.* 2006). In fact, in the present study, the antagonists tested were incorporated into culture substrate 15 days prior inoculation with *V. dahliae*. Thus, this period seems to be largely sufficient for their establishment and adaptation because normally, as reported by D'Ercole *et al.* (2000), biocontrol agents require a short period for colonizing the environment. Consequently, the well established antagonistic agents may have a double action: preventing pathogen progress, within host tissues, and reducing its spreading via its saprophytic activity and its important capacity of colonizing the rhizosphere outside tissues.

A delay is necessary between protective treatment and occurring of subsequent infection. The duration of this period depended on the nature of *Verticillium*-host plant pathosystem and the type of the protective agent. This conclusion may explain the registered variability of antagonistic potential of biocontrol agents used in the present study and also their successful establishment. In fact, this delay is very short for protection of lucerne with *Gliocladium roseum* and of cotton with *Talaromyces flavus* (Millar *et al.* 1984; Murray *et al*. 1997). In contrast, maple protection with *Bacillus subtilis* requires a delay of three days (Hall *et*

al. 1986) whereas application of *Trichoderma* needs 7 days before aggressive infection of eggplants (Henni 1987).

As practical consequence, application of conidial suspension of *P. oxalicum* by watering tomato plantlets, 7 days prior transplanting, resulted in a significant reduction of Verticillium and Fusarium wilts of tomato (Sabuquillo *et al.* 2005). Similarly, application of antagonists into culture substrate, 15 to 20 days prior inoculation with pathogen, controlled successfully VW of potato caused by *V. dahliae*. Ordentlich *et al.* (1990) also showed that application of *T. harzianum* in sowing rows reduced disease incidence and increased the total yield by 38.5%. Moreover, substrate application of *T. harzianum*, *T. viride* and *T. virens* (10^8 spores/ml) before inoculation with *F. oxysporum* f. sp. *tuberosi* was found to reduce Fusarium wilt severity on potato plants compared to inoculated and untreated plants (Ayed *et al*. 2006).

Effects of antagonists and bio-fungicides on VW development and the subsequent effects on plant growth

According to culture and incubation conditions used in the present study, application of antagonists to potato plants before inoculation did not totally prevent pathogen penetration but had contributed in the delay of its proliferation within tissues. In fact, the relative vascular discoloration extent compared to plant height ranged between 0 and 11% for all antagonists tested whereas for untreated and control plants, it reached 78%. The positive protection achieved by these antagonists was probably due to antagonism of *V. dahliae* microsclerotia leading to inoculum reduction (Jabnounkhiareddine *et al*. 2009a, 2009b) in contact of roots and/or stimulation of defense mechanisms of plant hosts.

Indigenous rhizobacterial isolates used in mixture in the present study had reduced potato wilt severity and enhanced plant growth and production. It seems that these local *Bacillus* sp. isolates acted via antagonism and plant growth promotion as reduced symptom intensity was accompanied with increased plant height and weight compared to the untreated and inoculated control. Similarly, Raupach and Kloepper (1998) found that mixtures of different plant growth promoting rhizobacteria, applied as seed treatment, showed an important enhancement of plant growth and reduction of several cucumber diseases. Haas and Défago (2005) mentioned that mixtures of plant growth promoting rhizobacteria, combining antibiosis and induced systemic resistance may be very efficient in practice. In fact, Hall *et al.* (1986) reported that *Bacillus subtilis* strains, which colonized naturally maple stem tissues, can suppress VW. Furthermore, Tjamos *et al.* (2004) found that three bacterial isolates, among 18 tested under greenhouse conditions and identified as *Bacillus* sp., showed a significant biocontrol activity against *V. dahliae*. The most efficient bacterial isolates, designed as K-165 and 5-127 colonizing naturally the rhizosphere, inhibited the mycelial growth *V. dahliae* in dual-culture experiments and successfully controlled VW of several Solanaceous species. Egamberdiyeva (2006) also found that species of *Pseudomonas*, *Bacillus* and *Arthrobacter* were identified as bacterial antagonists of *V. dahliae*. Berg *et al*. (2001; 2005) showed that various species of the *Pseudomonas*, *Bacillus*, *Trichoderma* genera, isolated from suppressive soils, were effective against *V. dahliae*. In a recent study, Li *et al.* (2008) showed that, in greenhouse experiments, suspension of the cells of the CH2 strain of *Bacillus cereus*, obtained from the rhizosphere of eggplant, reduced the severity of VW on eggplant by 69.69%, and that this strain and its chitinase have good commercial potential in controlling VW.

The reference strain of *P. fluorescens* p 417 had reduced VW severity but its effect on plant height and weight was significantly comparable to that noted on untreated and inoculated control plants. Similar results were reported by Mercado-Blanco *et al.* (2004) who found that application of different strains of the *P. fluorescens* complex reduced VW symptom development in olive trees. Similarly, Berg *et al.* (2000) recorded a significant reduction of VW after dip treatments in bacterial cultures baths, in greenhouse and in fields naturally infested with *V. dahliae*. However, and contrarily to our results, Berg *et al.* (2000) noted an increase of the relative harvest which ranged between 117 (*Streptomyces albidoflavus* S1) and 344% (*P. fluorescens* P6) under greenhouse conditions, and between 113 (*Streptomyces albidoflavus* S1) and 247% (*P. fluorescens* P6) in experimental fields.

The treatment of potato seeds with bio-fungicides based on *T. harzianum* (Biocont-T™) and *Pythium oligandrum* (Polyversum®) had successfully controlled VW and increased tuber weight by 28 and 57%, respectively, compared to the untreated and inoculated control. Similar results were reported by Rekanovic *et al.* (2007) who found that the biofungicide Polyversum® had reduced by 66.6% the severity of VW caused by *V. dahliae*, when applied prior inoculation of pepper plants. However, in a recent study, Giotis *et al.* (2009) mentioned that the biological control product Polyversum®, based on *Pythium oligandrum*, had no positive effect on VW incidence and fruit yield, number and size.

These bio-fungicides tested against *Fusarium oxysporum* f. sp. *tuberosi* had also reduced potato Fusarium wilt incidence compared to the untreated control when incorporated into culture substrate 15 days prior inoculation. Funga stop (based on citric acid and mint oil as Biofolar™) and Biocont-T™ were shown to be the most active during the bioassay whereas Polyversum® had a lesser effect in controlling *Fusarium oxysporum* f. sp. *tuberosi in vivo* (Ayed *et al*. 2007).

The increased plant growth and production observed with some biological treatments (antagonists and bio-fungicides) in the present study may be attributed to an induction of systemic resistance by protective agents involved. According to Van Loon *et al.* (1998), the induced systemic resistance was qualified as an increased plant defence capacity via activation of latent resistance mechanisms induced by various agents including rhizobacteria. In fact, besides its antifungal activities, *T. harzianum* is able to induce, even at a distance of target pathogen, resistance of plants to diseases. In recent studies, application of this antagonist around roots or into soil was shown to induce resistance in plants against their specific pathogens (De Meyer *et al.* 1998; Howell *et al.* 2000; Hibar *et al*. 2007).

Certain *Trichoderma* strains are able to establish durable colonization of roots and to penetrate the epiderm tissue where they produced and released compounds that induced responses of localised or systemic plant resistance. Plants reacted against the fungal invasion via the synthesis and accumulation of phytoalexins, flavonoïds, terpenoids and other antimicrobial compounds. *Trichoderma* strains are generally more resistant to these compounds that the majority of the other fungi.

Similarly, Tjamos *et al.* (2005) reported an induced systemic resistance mechanism in rhizobacteria as is the case of the K165 strain of *Paenibacillus* which induced resistance against *V. dahliae* in eggplant. Moreover, subsequent induction of systemic resistance, K-165 delayed expression of VW symptoms in cucumber and reduced the final development of symptoms by 40 to 50% compared to the untreated control (Tjamos *et al.* 2001). In the same way, Uppal *et al.* (2008) explained the efficacy of the strains DF37 of *P. fluorescens* and M1 of *B. pumilus* in the reduction of VW in potato cultivars 'Kennebec' and 'Russet Burbank', in spite of the weak inhibitory effect against *V. dahliae in vitro*, by the involvement of these bacteria in activating resistance mechanisms in potato.

In the light of the present promising results, further studies will be focused on the assessment of the *in situ* efficacy of these indigenous microorganisms, alone or in combination with other control measures, for reduction of VW severity in areas destinated for seed tubers production.

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