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In vitro and *in vivo* Evaluation of Some Biofungicides for Potato *Fusarium* Wilt Biocontrol

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Abstract: Three biological fungicides, Biocont-T, Funga stop and Polyversum constituted, respectively by *Trichoderma harzianum*, natural extracts (mint oil and citric acid) and *Pythium oligandrum*, were tested against *F. oxysporum* f. sp. *tuberosi* causing potato vascular wilt. Funga stop proved to be the most effective in inhibiting by 72 to 76% the mycelial growth of this pathogen on PDA media after incubation for six days at 25°C. Biocont-T also limited its development by 37 to 63%. However, Polyversum showed a very little activity in controlling this fungus *in vitro*. All bio-fungicides reduced disease incidence compared to the untreated control. Funga stop and Biocont-T were the most active during the bioassay. Whereas, Polyversum had a lesser effect in controlling this disease.

Key words: *Solanum tuberosum* L., *Fusarium oxysporum* f. sp. *tuberosi*, interaction, biological fungicides, inhibition

Introduction

Fusarium wilt of potato (*Solanum tuberosum* L.) caused by *Fusarium oxysporum* f. sp. *tuberosi*, is among the most important diseases in potato production in many parts of the world, especially at relatively high temperatures or when seasons are hot and dry (Hooker, 1981; Venter *et al*., 1992; Manici and Cerato, 1994). This soilborne fungi was detected, in the last years, in Tunisia and was frequently isolated from potato dry rot and wilted plants (Daami-Remadi and El Mahjoub, 2004). It infects plants through the roots via direct penetration or wounds, after which the xylem vascular tissue of the plants is colonized, causing stunting, vascular wilting and death of plants (Daami-Remadi and El Mahjoub, 2004; Ayed, 2005; Ayed *et al*., 2006a). Therefore, economic losses were estimated of 10 to 53% of potato yield (Thanassoulopoulos and Kitsos, 1985).

Currently, preplant soil fumigation and fungicide applications are used, in some parts of the world but not in Tunisia, to control wilts and other diseases caused by soilborne pathogens (Bowers and Locke, 2000). However, the major fumigant used, methyl bromide, has been defined by the Montreal Protocol of 1991 as a chemical that contributes to the depletion of the ozone layer (Ristaino and Thomas, 1997). Other strategies for controlling the disease have been introduced such as solarization, long-term rotations, cultivars and some biological control agents (Katan, 1980; Triki *et al*., 2001; Monnet, 2001; Ayed *et al*., 2006a, 2006c). Chemical application against this soilborne pathogen is absent in Tunisia in spite of the efficiency of some fungicides, tested in further study, in controlling this disease (Anonymous, 2003; Ayed *et al*., 2006b).

As alternatives to these control measures, several reports demonstrated successful use of biological control agents and extracts (Larkin and Fravel, 1998; Elmer and McGovern, 2004; Ayed *et al*., 2006a). Some biological products are commercially available to control plant diseases and can be used as part of an integrated pest management program (Ristaino and Thomas, 1997).

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Since *Fusarium* wilt is a serious threat of a strategic crop in Tunisia, the present research was conducted in order to evaluate the effectiveness, *in vitro* and *in vivo*, of three biological fungicides in controlling *F. oxysporum* f. sp. *tuberosi*.

Materials and Methods

Fungal Cultures and Biofungicides

Isolates of *F. oxysporum* f. sp. *tuberosi* used in this study (Fot_1, Fot_2, Fot_3, Fot_4 and Fot_5) were isolated on 2004 and 2005 from potato tubers showing dry rot symptoms collected from traditional potato-stores (Hammam Ghezaz, Hawaria and Korba in Cap Bon region). They were cultured on Potato Dextrose Agar (PDA) amended with 300 mg L^{-1} of streptomycin-sulfate and incubated at 25°C in the dark. Pathogenicity was achieved by potato plant inoculation. Single spore cultures were maintained on glycerol at -20°C for long-term storage.

Mycelium taken from the edge colony of each isolate was transferred to 150 mL of Potato Dextrose Broth (PDL) and incubated at 25°C for 5 days in a rotary incubator (120 rpm) for plant inoculation. The liquid culture was filtered and the conidial suspension was adjusted to 10^7 spores mL^{-1} by means of a Malassez cystometer.

Effects of three biological fungicides were tested, *in vitro* and *in vivo*, against *F. oxysporum* f. sp. *tuberosi*. The characteristics of these products were listed in Table 1.

Potato Cultivars

Tubers cv. Spunta, the most cultivated in Tunisia, are used in this current study. They are obtained, on 2004, from the Technical Center of Potato of Tunisia, stored in darkness at 6°C. After their germination at 27°C in the dark, tubers were planted in plastic pots.

In vitro Experiments

In vitro bioassay of *F. oxysporum* f. sp. *tuberosi* biocontrol was realized on PDA medium amended with streptomycin sulfate at 200 g L^{-1} and biological fungicides. Three products are tested against five *F. oxysporum* f. sp. *tuberosi* isolates (Fot_1, Fot_2, Fot_3, Fot_4 and Fot_5). A mycelial disc (6 mm diameter) of the pathogen, taken from 6-day-old pathogen culture, was transferred to the center of the solidified PDA media in plates containing biological fungicides (Table 1). The fungal radial growth colonies of all culture plates were measured after four days at 25°C. Eight plates were made per elementary treatment.

Data are arranged as a complete randomized factorial design where treatments (bio-fungicides and untreated control) and isolates are both fixed factors. They were analyzed using SPSS and subjected to analysis of variance and Fisher's least significant difference test LSD (at $p \leq 0.05$).

In vivo Experiments

Each biological fungicide was incorporated to an autoclaved culture substrate. In fact, 2.5 L of water, containing product, were used to humidify 6.74 dm^3 of the autoclaved mixture of perlite and peat (1:3), volume necessary to fill 10 pots. This treatment has been done for each of the three biological fungicides with various concentrations (Table 1).

Table 1: Products used for suppression of *F. oxysporum* f. sp. *tuberosi* development

Active ingredient/microbe	Commercial products	Formulation	Rate of product
Trichoderma harzianum	Biocont-T (Water powder)	More than 14.10^6 spores g^{-1}	200 g m^{-3} of substrate
Citric acid	Funga stop	1.6%	250 cc hL^{-1}
Mint oil		0.8%	
Pythium oligandrum	Polyversum	-	0.5 g L^{-1}

F. oxysporum f. sp. *tuberosi* isolate (Fot_3), being the most aggressive following pathogenicity tests (Ayed *et al*., 2006a), was used for plant inoculation. Potato tubers, cv. Spunta, were planted in plastic pots (6.74 L) containing an autoclaved and treated substrate and kept at 8-32°C (minimum and maximum temperatures, respectively). Two weeks after their emergence, plants were inoculated by irrigation with 150 mL of conidial suspension (10^7 spores mL^{-1}). Ten control plants were non-inoculated. Potato plants were irrigated regularly and fertilized with a nutrient solution (20 N : 20 K_2O : 20 P_2O_5) following Manici and Cerato (1994) method.

Effect of the tested biological fungicides on *Fusarium* wilt development was assessed via a disease severity index. A scale of 0-4 was used to assess disease severity: 0 = asymptomatic leaf, 1 = leaf wilted, 2 = Leaf with hemiplegic yellowing, 3 = leaf with necrosis, 4 = dead leaf. Incidence of *F. oxysporum* f. sp. *tuberosi* was estimated weekly via an Index of Leaf Damage (ILD) calculated per potato plant following formula (Béye and Lafay, 1985) where:

$$ILD = \sum notes/max$$

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

Data are arranged by completely randomized design where treatments (plants treated by each of three biological fungicides, inoculated and non-inoculated) are the only fixed factor. Ten plants per treatment were assessed. Mean comparisons were done following the LSD method ($p \leq 0.05$).

Results

In vitro Experiments

Effects of the three bio-fungicides, amended to the PDA media at the rates showed in Table 1, on mycelial growth of five *F. oxysporum* f. sp. *tuberosi* isolates were noted after four days on incubation at 25°C. Results show that all products reduced significantly mycelial growth of the soilborne pathogen (Table 2 and Fig. 1). A significant interaction is observed between both fixed factors ($p \leq 0.05$).

Funga stop was found to be particularly effective in inhibiting mycelial development by more than 72% compared to pathogen growth on unamended PDA. Biocont-T, including *T. harzianum*, showed lesser efficiency and reduced mycelial growth by 37 to 63% for Fot_2 and Fot_3, respectively. However, a very little inhibition, with a maximum of 12.6% for Fot_1 comparatively to the untreated control, was achieved when Polyversum, constituted by *Pythium oligandrum*, were used.

Fot_2 and Fot_4 were the most resistant isolates to the biological fungicides tested, but Fot_5 was the most sensitive (Table 2).

Table 2: Effect of biological fungicides on mycelial growth of some *F. oxysporum* f. sp. *tuberosi* isolates observed after four days of incubation at 25°C

	Mean colony diameter of *F. oxysporum* f. sp. *tuberosi* colonies (cm)			
Isolates	Control	Biocont-X	Funga stop	Polyversum
Fot_1	6.35	2.8125	1.6375	5.55
Fot_2	6.425	4.0375	1.775	6.46
Fot_3	6.275	2.3625	1.5	5.725
Fot_4	6.925	3.8625	1.8	6.4375
Fot_5	5.9375	2.1875	1.475	5.45

Fot_1, Fot_2, Fot_3, Fot_4 and Fot_5: Isolates of *F. oxysporum* f. sp. *tuberosi*, LSD at $p \leq 0.05$ (Treatments×Isolates) = 0.215 cm

Fig. 1: Effect of bio-fungicides on mycelial growth of *Fusarium oxysporum* f. sp. *tuberosi* isolate (Fot_3) on PDA, observed after four days of incubation at 25°C (Un: Untreated, Po: Polyversum, F: Funga stop and B: Biocont-T)

Table 3: Evolution of Index of Leaf Damage (ILD) of potato plants, cv. Spunta, inoculated by *F. oxysporum* f.sp. *tuberosi* isolate (Fot_3) and previously treated by three biological fungicides (NI: Noninoculated and untreated plants, I: Untreated-inoculated plants)

	Treatments				
Days after planting	NI	I	Biocont-T	Polyversum	Funga stop
45	0^{b}	0.14^{a}	0^{b}	0^{b}	0^{b}
52	0^{c}	0.2169^{a}	0.1127^{b}	0.1405^{b}	0.0592^{bc}
59	0.0275^{c}	0.3279^{a}	0.1665^{b}	0.2797^{a}	0.1939^{b}
66	0.0562^{c}	0.3974^{a}	0.3219^{ab}	0.3622^{ab}	0.2878^{b}
73	0.1016^{c}	2.362^{a}	1.6056^{b}	1.771^{b}	1.8396^{b}

[z]Within lines, means followed by the same letter(s) are not significantly different (p = 0.05) according to S.N.K. test

Fig. 2: Comparison between healthy and inoculated potato plants, cv. Spunta, treated preventively by three bio-fungicides, 73 days after inoculation (1: Untreated plants, 2: Inoculated plants, 3: Biocont-T, 4: Funga stop and 5: Polyversum)

In vivo Experiments

Symptoms typical of *Fusarium* wilt were observed in inoculated plants. Disease symptoms were first noted 30 days after inoculation in untreated and inoculated plants. All treatments were effective in reducing significantly *Fusarium* wilt incidence comparatively to untreated-inoculated control plants during the bioassay (Table 3 and Fig. 2).

A significant reduction in the ILD value was achieved especially by Funga stop and Biocont-T compared to the untreated-inoculated control. This index was, respectively 1.8 and 1.6 but it was 2.36 for the untreated-inoculated plants at the end of the bioassay. However, Polyversum efficiency was lesser than both previous biological products. Moreover, an important development of disease incidence was noted in the last week of the ILD evaluation.

Discussion

Fusarium wilt is a serious threat of a strategic crop causing economic losses of potato yield (Thanassoulopoulos and Kitsos, 1985). As it is a soil-borne pathogen, control of *F. oxysporum* f. sp. *tuberosi* has been restricted to the use of the long-term rotations, solarization and biological antagonists (Monnet, 2001; Triki *et al*., 2001; Ayed *et al*., 2006a). For this reason, biological control of potato *Fusarium* wilt may be aimed against the pathogen by means of bio-fungicides treatments.

In the present experiments, bio-fungicides, especially Funga stop and Biocont-T proved to be effective in controlling this soilborne pathogen *in vitro* and *in vivo*. Polyversum had a lesser efficiency in reducing disease incidence.

Funga stop showed the highest activity, *in vitro* and *in vivo*, against *F. oxysporum* f. sp. *tuberosi*. Jabnoun-Khiareddine (2004) reported the effectiveness of this product in inhibiting the development of *Verticillium dahliae* and *Verticillium alboatrum*. Moreover, Biocont-T provided efficacy in controlling potato *Fusarium* wilt. Daami-Remadi (2001) signalled its activity against *F. roseum* var. *graminearum* and *F. solani* var. *coeruleum* causing potato tuber dry rot. Hibar *et al*. (2006) confirmed its efficiency in reducing disease incidence of *F. oxysporum* f. sp. *radicis-lycopersici* on tomato plants. Inhibitory activity of this bio-fungicide is assigned to *Trichoderma harzianum*. In fact, Ayed *et al*. (2006a) reported an inhibitory activity induced by a local *T. harzianum* isolate against potato *Fusarium* wilt caused by *F. oxysporum* f. sp. *tuberosi*. In the same way, Thangavelu *et al*. (2004) found that soil application of *T. harzianum* effectively controlled *Fusarium* wilt of banana caused by *F. oxysporum* f. sp. *cubense*. Enzymes such as chitinases, β-1,3-glucanases and cellulases produced by the biocontrol agent acted by breaking down the polysaccharides, chitin and β-glucans that are responsible for rigidity fungal cells walls, thereby destroying cell wall integrity and causing a partial lysis (Lorito *et al*., 1994; Howell, 2003). Polyversum showed a very little activity, *in vitro* and *in vivo*, against *F. oxysporum* f. sp. *tuberosi*. These results didn't confirm those found by Hibar *et al*. (2006) that reported efficiency of this biological fungicide in controlling *F. oxysporum* f. sp. *radicis-lycopersici in vitro* and its disease incidence on tomato plants. Furthermore, Benhamou *et al*. (1997, 1999) reported that *Pythium oligandrum* earlier applied to roots can effectively protect plants against soilborne pathogens. Therefore, a weak efficiency of this product can be explained by its particular interaction with pathogens or to the weak application rate.

Increase of disease incidence at the end of the bioassay can be caused by obstruction of the water and nutrient-conducting tissue of inoculated plants (Kucharek *et al*., 2000).

These results revealed efficiency of some biological fungicides in controlling *F. oxysporum* f. sp. *tuberosi* causing *Fusarium* wilt of potato plants. Further investigations on the use, separately or in mixture, of bio-fungicides and tests of other rates may be achieved a better disease control. Use of these products in mixture with some synthetic fungicides should be studied.

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