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Biological Control of Potato Leak with Antagonistic Fungi Isolated from Compost Teas and Solarized and Non-Solarized Soils

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ABSTRACT

Several fungal agents, isolated from compost teas and soil, were tested for their antagonistic activity against *Pythium aphanidermatum* and *P. ultimum* causing potato leak in Tunisia depending on the timing of their application. *In vitro* dual culture experiments showed that *Aspergillus* spp., *Penicillium* sp. and *Trichoderma* sp. isolates had inhibited differently the two *Pythium* species and that their inhibitory effect was improved when plated 24 h prior pathogen. Tested *in vivo* for their ability to control leak development, pooled data of antagonistic treatments revealed that all microbial agents had decreased rot incidence and severity compared with the untreated control. Moreover, their preventive application i.e. 24 h prior to pathogen inoculation has contributed by the reduction of leak severity by about 77.28% as compared to simultaneous application. Both *Pythium* species were differently controlled by the tested fungi. In fact, leak caused by *P. aphanidermatum* was completely suppressed (100% of inhibition) with all microbial agents applied 24 h before inoculation while for *P. ultimum*, the percentage of reduction of leak severity ranged between 15.37% and 95.94%.

Keywords: biosuppression, microbial agents, *Pythium* spp., *Solanum tuberosum* L., timing of treatment, watery wound rot

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most economically important vegetable crop in the world (Wang *et al*. 2008; Schieber and Aranda Saldaña 2009). It is an important nutrient source in human nutrition (Öztürk *et al*. 2010) and even considered as a cereal substitute (Fuglie 1994). In Tunisia, it is a strategic crop; it occupies yearly 25 000 ha which represent approximately 16% of the total cultivated vegetables area (Djébali and Tarhouni 2010). Per capita potato consumption has more than doubled in Tunisia during the past three decades (Fuglie 1994).

For the season, early and extra-early crops, seeds are mainly provided via seed import from European countries. For the late season crop which is the second main crop season in Tunisia, locally produced tubers issued from the season crop production are used. Storing the crop for several months is crucial. In fact, for seed and local market supplies potatoes are stored in refrigerated and unrefrigerated traditional storage, under open-air (under trees) or controlled conditions, from June to November (Khamassy *et al*. 2002; Rejeb Gharbi and El Fahem 2004, 2007). However, tuber storing is a potentially risky phase as growers can encounter numerous problems if management of the stored crop is not done right. Even though monitoring of storage environment could be efficient in controlling post-harvest diseases (Tivoli and Jouan 1981; Bartz and Kelman 1984; Barr *et al*. 1996; Triki *et al*. 2001; Daami-Remadi *et al*. 2006a). In fact, many bacterial and fungal diseases causing tuber rots and blemishing can increase significantly during the storage period such as soft rot (Triki *et al*. 1996a, 1996b), pink rot (Triki *et al*. 1996a, 1996b), Pythium leak (Priou *et al*. 1997; Daami-Remadi 2001a; Triki *et al*. 2001), dry rot (Daami-remadi and El Mahjoub 1996; Chérif *et al*. 2001; Daami-Remadi *et al*. 2006a), black scurf (Daami-Remadi *et al*. 2008), black dot (Daami-Remadi *et al*. 2010a), and stem rot (Daami-Remadi *et al*. 2010b). These increasingly important post-harvest diseases are caused by soilborne fungi due to the absence of crop rotation, since 78% of potato farmers have a total surface lower than one hectare and 14% of < 2 ha (Rezig *et al*. 2010).

Pythium leak or watery wound rot caused by *Pythium aphanidermatum* and *P. ultimum* is a serious disease in Tunisia that may cause significant losses early after harvest and/or during storage (Triki *et al*. 1996b, 2001). In fact, the average loss of stored tubers reached 2.0% among a total of 90 potato heaps from various production areas over a two-year survey, and 3.3% average losses at harvest were recorded from 35 field observations (Priou and El Mahjoub 1994, 1999).

Strategies used to manage Pythium leak and watery rots are generally focused on avoiding infection and disease development via crop rotation, planting in well-drained soils, avoiding excessive irrigation at the end of the growing season, allowing sufficient time between vine killing and harvest for proper periderm development, harvesting when tuber pulp temperatures are below 21°C, reducing tuber injury, and favouring tuber healing (Hooker 1981; Secor and Gudmestad 1999; Salas *et al*. 2003). Nevertheless, such practices alone are not always sufficient to effectively control this disease particularly in the absence of fungicides registered for the use against tuber leak and alternative strategies are needed.

Suppressive soils were largely used as potential sources of effective biocontrol agents for the management of soilborne plant pathogens such as *Pythium* spp. (Chet and Baker 1981; Jones and Samac 1996), *Phytophthora* spp. (Jones and Samac 1996; Peters *et al*. 2003), *Rhizoctonia solani* (Chet and Baker 1981; Mazzola 2002; Peters *et al*. 2003), *Helminthosporium solani* (Martinez *et al*. 2002; Peters *et al*. 2003), *Verticillium dahliae* (Tjamos *et al*. 2004), *Fusarium* spp. (El-Masry *et al*. 2002; Peters *et al*. 2003), *Gaeuman-*

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nomyces graminis var. *tritici* (Mazzola 2002), *Rosellinia necatrix* (Pliego *et al*. 2011), *Sclerotium rolfsii* (Chet and Baker 1981), and others (Hornby 1983; Deacon 1991). As an alternative plant disease control measure, compost amendments were also employed in both organic and conventional agriculture to maintain soil fertility and plant health (Hoitink and Fahy 1986; Hoitink *et al*. 1991; Hoitink and Zuniga de Ramos 2004). For example, the incidence of anthracnose fruit rot, caused by *Colletotrichum coccodes*, was reduced in organic tomato (*Lycopersicon esculentum*) plots amended with a high rate of composted cannery wastes as compared with nonamended control plots. This reduction in disease incidence was also associated with an increase in the marketable yield and fruit quality in compost-amended organic plots (Abbasi *et al*. 2002). Composts may be applied in their solid form or as extracts or compost teas for the control of several soilborne (Zhang *et al*. 1998; El-Masry *et al*. 2002; Scheuerell and Mahaffeee 2002; 2004; Hibar *et al*. 2006; Termorshuizen *et al*. 2006; Kerkeni *et al*. 2007a; Sang and Kim 2011) and foliar plant diseases (Elad and Shteinberg 1994; Zhang *et al*. 1998).

Inhibition induced by composts resulted from a combination of chemical and biological mechanisms. Biological factors included populations of compost (Muhammad and Amusa 2003; Suárez-Estrella *et al*. 2007), microbial competition for nutriments with pathogen (Chen *et al*. 1988a, 1988b), production of antibiotics (Hoitink *et al*. 1991), production of extra cellular lytic enzymes (El-Masry *et al.* 2002), parasitism (Hoitink *et al*. 1991), and induced systemic acquired resistance (Zhang *et al*. 1998; Pharand *et al*. 2002). In this paper, we evaluate the efficacy of some fungi isolated from several types of composts and soils on the development of two *Pythium* species causing potato leak in Tunisia. The effect of the timing of their application on their effectiveness is also assessed.

MATERIALS AND METHODS

Plant material

Apparently healthy and undamaged potato tubers cv. 'Spunta' were used. This cultivar is the most cultivated in Tunisia and known to be susceptible to *Pythium* species causing leak (Priou *et al*. 1997). Tubers, kindly provided by the Technical Center of Potato and Artichoke, Essaïda, Tunisia, were stored at 6°C for one month before use. Just before inoculation, tubers were washed to remove excess soil, superficially sterilized in 10% sodium hypochlorite solution (Aiglol Production, Zaouiet Sousse, Tunisia) during 5 min, rinsed in distilled water and air dried.

Pythium species

Pythium ultimum and *P. aphanidermatum* were isolated from potato tubers showing typical leak symptoms. All isolates were identified based on described morphological characteristics (Van der Plaats-Niterink 1981). Their virulence was maintained by inoculation of freshly wounded tubers and re-isolation on Potato Dextrose Agar (PDA) plates.

Pythium species were cultured on PDA supplemented with 100 mg/l of streptomycin sulphate (Pharmadrug Production Gmbh, Hamburg, Germany) and incubated at 25°C for one week before use in the bioassays and stored at 4°C for preservation.

Antagonistic fungi: Source, isolation techniques and culture

Antagonistic fungi tested in the present study were isolated from several compost teas obtained from mature composts differing in their basic ingredients (**Table 1**) and kindly provided by the Technical Center of Organic Agriculture, Tunisia. Some fungi were collected from solarized and non-solarized soil samples (**Tables 1, 2**). Compost teas were prepared from 4-month-old composts according to the method of Brinton and Droffner (1995) and Siddiqui *et al*. (2009) where compost and tap water were mixed in the ratio of 1:5 (w/v), in polyethylene non-degradable containers with covers. The mixtures were under natural aeration and left at ambient temperature for 10 days prior filtering through double layered cheese cloth.

Table 1 Basic ingredients of composts used for isolation of fungal antagonists.

Compost type	Bovine manure	Chicken manure	Ovine manure	Equine manure	Chopped straw
CT1	50%	25%	25%	-	-
CT2	60%	-	30%	-	10%
CT3	30%	30%	30%	-	10%
CT4	25%	25%	25%	15%	10%

Table 2 Antagonists tested against *Pythium* spp. and their origins.

Isolate	Fungal identification	Main source
CH1	*Aspergillus niger*	Solarized soil
CH2	*Aspergillus terreus*	Solarized soil
CH3	*Aspergilus* sp.	Compost type CT3
CH4	*Aspergillus* sp.	Compost type CT2
CH5	*Penicillium* sp.	Compost type CT2
CH6	*Penicillium* sp.	Compost type CT4
CH7	*Penicillium* sp.	Compost type CT1
CH8	*Aspergillus* sp.	Non-solarized soil
CH9	*Penicillium* sp.	Non-solarized soil
CH10	*Trichoderma harzianum*	Non-solarized soil
CH11	*Penicillium* sp.	Non-solarized soil
CH12	*Aspergillus niger*	Non-solarized soil

For fungi isolation from compost teas, 2 ml were incorporated into 200 ml of PDA cooled to 45°C and amended with 60 mg of streptomycin sulphate before pouring into each of the 90 mm Petri dishes and allowing it to solidify before use. Inoculated plates are incubated at 25°C for 10 to 15 days before fungal isolation as described in Daami-Remadi *et al*. (2006b).

For fungi isolation from soil, 5 g of composite soil sample were transferred into a 100-ml Pyrex glass bottle containing sterile distilled water (final soil/water ratio of 1:10 [w/v]), and bottles were agitated for 60 min on a rotary shaker (300-500 rpm). The supernatant was removed, filtered through double layered cheese cloth and serial diluted up to 10^{-4} dilution by using sterile distilled water. About 20 µl of the diluted soil extract were spread on each Petri dish containing PDA medium supplemented with streptomycin sulphate (300 mg/l) and cooled to 45°C, rotated gently by hand and allowed to solidify. Cultures were incubated at 25°C for one week.

The purified fungal cultures were identified using cultural and morphological features with reference to Banrnet and Hunter (1972) and Moreau (1968). The fungi collected were provided in **Table 3**. They were cultured at 25°C for one week before use and stored at 4°C for preservation.

For tuber inoculation, the culture plates were flooded with sterile distilled water and the conidia dislodged with a scalpel by applying only slight pressure for avoiding removal of conidiophores or pieces of the medium from the plate. The conidial suspension was recuperated, filtered through double layered cheese cloth and then the final concentration was adjusted to10^7 conidia/ml by using a Malassez cell counting chamber (Fa. Laboroptik. Friedrichsdorf, Tiefe 0.2 mm, 0.0025 mm^2, HBG, Germany).

These fungi were previously tested for their pathogenicity on wounded potato tubers cv. 'Spunta' and were found to be non-pathogenic.

Assessment of the *in vitro* antagonism of fungi tested against *Pythium* spp.

The isolated fungi were screened *in vitro* for antagonism against *Pythium* species by the dual culture technique on PDA added with streptomycin sulphate (100 mg/l). In fact, agar plugs (diameter 6 mm), removed from 5-day-old PDA cultures of the pathogen, were plated at 2 cm from the edges of the Petri dishes (diameter 9 cm) whereas those of the tested fungi were placed at the opposite sides. Untreated control plates were plated with pathogen plugs only.

For testing the variation of efficacy depending on the timing of antagonistic treatments, two assays were conducted where fungi

to be tested were plated at the same time as the pathogen or 24 h before the pathogen.

The mean diameter of the pathogen developing colonies was noted after 2 days of incubation in the dark at 25°C. Hyphal *in vitro* interactions were studied based on several macroscopic and microscopic observations of pathogen colonies confronted with antagonists for qualifying the damages occasioned on pathogen mycelium in comparison to untreated controls. There were three replicate plates for each dual culture.

Assessment of the *in vivo* antagonism of fungi tested against *Pythium* spp.

Apparently healthy tubers (cv. 'Spunta') were used in this study. They were washed in running tap water to remove excess soil, dipped in 0.5% sodium hypochlorite for 30 min, rinsed twice with sterile distilled water (5 min each) and air-dried.

Tubers were wounded at two sites along the tuber longitudinal axis by a 6 mm diameter disinfected cork borer occasioning wounds of 6 mm in diameter and in depth, which serve as sites of infection. Tuber inoculation was made by deposing a mycelial agar disc (6 mm diameter) colonized by the pathogen removed from a 7-day-old culture at 25°C.

Conidial suspensions (10^7 conidia/ml) of fungi tested individually were applied by inoculating each wound with 100 µl. Sterile distilled water (the same volume) was used as negative control and fungal suspensions were used as positive controls. Tubers that had been similarly wounded and inoculated but untreated were used as control.

Similarly, as for the *in vitro* experiment, the effect of the timing of application on the antagonistic agent's efficacy was studied based on two assays where pathogen was inoculated, as described above, either simultaneously with antagonistic treatments or 24 h later.

Ten tubers were used per elementary treatment (i.e. per timing of application, per antagonistic treatment tested and per *Pythium* species).

All inoculated and treated tubers were incubated at 25°C for 48 h at high relative humidity. Tubers were rated for disease incidence by estimating the percentage of tubers showing visible leak symptoms on their surface (around the inoculation sites). In addition, tubers were cut in two (along the longitudinal axis), across the inoculation sites, and the maximum width (W, mm) and depth (D, mm) of the rot was assessed to compute the penetration (P, mm) of tissues following the formula presented by Lapwood *et al*. (1984) where P = [W/2 + (D-5)]/2.

Statistical analyses

For the *in vitro* (on Petri plates) and the *in vivo* (on potato tubers) assays, the data were collected as mean colony diameter (average of two perpendicular diameters) values in each replication and the mean pathogen penetration, respectively. For each tuber, penetration value represents a mean of two inoculation sites. Data were subjected to one-way analysis of variance (ANOVA) by using SPSS 10.0 for Windows according to a completely randomised factorial design where the timing of application, the antagonistic treatments (fungi species and untreated control) and *Pythium* species were the three fixed factors. Means were separated using Student-Newman-Keul's (SNK) or Least Significant Difference (LSD) test (at $P \leq 0.05$).

The whole experiment was conducted twice and only data of one experiment was analyzed due to the detection of latent bacterial infections in some tubers used.

RESULTS

The ability of the fungal agents isolated from compost teas and soil to suppress *P. aphanidermatum* and *P. ultimum*, and to control potato leak, was investigated as a function of the timing of agent application and pathogen inoculation.

Effect of fungi tested on the mycelial growth of *Pythium* spp.

Results analyzed by ANOVA revealed significant effects of the three fixed factors (timing of application, antagonistic treatments and *Pythium* species), and highly significant (at $P \leq 0.01$) interactions i.e. timing of application × antagonistic treatment, and antagonistic treatment × *Pythium* species (**Table 3**).

Data of the main effects of the timing of application and antagonistic treatments and their interaction for both *Pythium* species combined are given in **Table 4**. In fact, for pooled data of the timing of application, the percentage of radial inhibition of *Pythium* spp. varied from 17.89% (CH3 isolate) to 37.80% (CH6 isolate). Moreover, 9 out of the 12 isolates tested had reduced the *in vitro* pathogen growth by more than 20%. However, for combined data of the antagonistic treatments, the application of antagonists 24 h prior to pathogen had decreased the radial growth of *Pythium* spp. by 26.61% compared to 21.45% recorded with simultaneous application. Furthermore, when both agents (antagonist and pathogen) were plated at the same time, the radial growth decrease of *Pythium* species, recorded after 48 h of

Table 4 Effects of antagonistic treatments and the timing of their application on the radial growth of *Pythium* spp. recorded after 48 h of incubation at 25°C.

Timing/ Antagonistic treatment	Timing of antagonistic application		Average per antagonistic treatment
	Simultaneous with pathogen	24 h prior to pathogen	
Control	7.63	7.65	7.64 a
CH1	5.93 (22.29)	5.87 (23.31)	5.90 bc (22.80)
CH2	6.09 (20.10)	5.96 (22.11)	6.03 bc (21.11)
CH3	6.32 (17.15)	6.23 (18.62)	6.27 b (17.89)
CH4	6.20 (18.68)	6.09 (20.37)	6.15 ab (19.53)
CH5	5.80 (23.93)	5.28 (31.04)	5.54 c (27.49)
CH6	4.23 (44.59)	5.28 (31.04)	4.75 d (37.80)
CH7	6.25 (18.03)	5.15 (32.67)	5.70 bc (25.36)
CH8	6.55 (14.09)	5.95 (22.22)	6.25 b (18.16)
CH9	6.38 (16.28)	5.56 (27.37)	5.97 bc (21.82)
CH10	6.15 (19.34)	5.08 (33.66)	5.61 c (26.51)
CH11	6.18 (18.90)	5.08 (33.55)	5.63 c (26.24)
CH12	5.79 (24.04)	5.86 (23.42)	5.83 bc (23.73)
Average per application timing	6.11 a (21.45)	5.77 b (26.61)	

CH_1 and CH_{12}: *Aspergillus niger*; CH_5, CH_6, CH_7, CH_9, and CH_{11}: *Penicillium* sp.; CH_2: *Aspergillus terreus*; CH_8, CH_4, CH_3: *Aspergillus* sp.; CH_{10}: *Trichoderma* sp. Numbers in parenthesis indicate the percentage (in %) of the radial growth inhibition of *Pythium* spp. as compared to the untreated control. For the averages per application timing and per antagonistic treatment tested, means followed by the same letter are not significantly different according the SNK test at $P \leq 0.05$.

Table 3 Analysis of variance of mean colony diameter of the pathogen depending on fixed factors tested and their interactions.

Source	df	Mean square	*F value* [a]
Timing of application	1	4.621	20.246 **
Antagonistic treatment	12	4.990	21.860**
Pythium species	1	18.245	79.933**
Timing of application × Antagonistic treatment	12	1.125	4.929**
Timing of application × *Pythium* species	1	0.548	2.403 NS
Antagonistic treatment × *Pythium* species	12	1.130	4.950**
Timing of application × Antagonistic treatment × *Pythium* species	12	0.353	1.546 NS
Error	104	0.228	

[a] Values followed by ** are statistically significant at $P \leq 0.01$. df: degree of freedom; NS: Not significant.

Table 5 Effects of antagonistic treatments on the radial growth of *Pythium* spp. recorded after 48 h of incubation at 25°C.

Pythium species /Antagonistic treatment	*P. ultimum*	*P. aphanidermatum*	Average per antagonistic treatment
Control	7.78	7.50	7.64 a
CH1	6.13 (21.11)	5.66 (24.55)	5.90 bc (22.80)
CH2	6.23 (19.82)	5.82 (22.44)	6.03 bc (21.11)
CH3	6.32 (18.75)	6.23 (17)	6.27 b (17.89)
CH4	6.18 (20.57)	6.12 (18.44)	6.15 ab (19.53)
CH5	6.41 (17.57)	4.67 (37.77)	5.54 c (27.49)
CH6	5.63 (27.65)	3.88 (48.33)	4.75 d (37.80)
CH7	6.36 (18.22)	5.04 (32.77)	5.70 bc (25.36)
CH8	6.49 (16.50)	6.01 (19.88)	6.25 b (18.16)
CH9	6.15 (20.90)	5.79 (22.77)	5.97 bc (21.82)
CH10	5.70 (26.68)	5.53 (26.33)	5.61 c (26.51)
CH11	6.28 (19.18)	4.98 (33.55)	5.63 c (26.24)
CH12	6.05 (22.18)	5.60 (25.33)	5.83 bc (23.73)
Average per *Pythium* species	6.28 a (20.76)	5.60 b (27.43)	

CH_1 and CH_{12}: *Aspergillus niger*; CH_5, CH_6, CH_7, CH_9, and CH_{11}: *Penicillium* sp.; CH_2: *Aspergillus terreus*; CH_8, CH_4, CH_3: *Aspergillus* sp.; CH_{10}: *Trichoderma* sp. Numbers in parenthesis indicate the percentage (in %) of the radial growth inhibition of *Pythium* spp. as compared to the untreated control. For the averages per application timing and per antagonistic treatment tested, means followed by the same letter are not significantly different according the SNK test at $P \leq 0.05$.

incubation at 25°C, ranged between 14.09 and 44.59% with only 5 out of 12 antagonists tested reducing by more than 20% the pathogen growth as compared to the untreated control. However, when plated 24 h prior to pathogen, 11 out of 12 antagonists tested had inhibited by more than 20% the growth of *Pythium* spp.; the percentage of inhibition varied between 18.62% (isolate CH3) and 33.66% (isolate CH11). Thus, the effectiveness of the antagonists tested was found to vary depending on the timing of their application and was improved when they were applied 24 h prior to inoculation as is illustrated by the case of *Trichoderma* sp. (isolate CH10) in **Fig. 1**.

Table 5 indicates that, independently of the timing of application, the effectiveness of the antagonists varied depending on *Pythium* species tested. Indeed, the radial growth inhibition of *P. ultimum* ranged between 16.50% (CH8) and 27.65% (CH6) where 6 out of 12 antagonists tested had reduced this parameter by more than 20%. However, the *in vitro* development of *P. aphanidermatum* decrease varied from 17% (CH3) to 48.33% (CH6) with 9 out of 12 fungal antagonists reducing this parameter by more than 20% as compared to the untreated control. Pooled data of all antagonists and timings of application tested showed an average percentage of *P. ultimum* growth inhibition of 20.76% compared to 27.43% for *P. aphanidermatum*. However, for combined data of both *Pythium* species, the highest inhibitory effect (37.80%) was recorded with CH6 treatment; for 9 out of 12 antagonistic treatments tested, the pathogen growth was inhibited by more than 20%. As pictorial illustration of the inhibitory effect of CH2 (*A. terreus*) and CH6 (*Penicillium* sp.) isolates recorded *in vitro* when dual cultured with *Pythium* species is given in **Fig. 2**. This figure clearly demonstrates the strong antagonistic potential of *A. terreus* when plated at the same time as pathogen and the important decrease in *Pythium* spp. mycelial growth when applied preventively (i.e. 24 h before pathogen).

Macroscopic studies of the hyphal interactions between microbial agents tested and *Pythium* species indicated a strong reduction of the hyphal density of the pathogen induced by CH5, CH6 (**Fig. 2**), and CH11 (*Penicillium* sp.), CH12 (*A. niger*), and CH10 (*Trichoderma* sp.) isolates. Some of the microbial agents tested are also able to grow over pathogen colonies as is the case of *Trichoderma* sp. (CH10) whereas for some agents (CH2 for example in **Fig. 2**), an inhibition zone separated pathogen and antagonist colonies suggesting the involvement of antibiosis mechanism. At the level of the light microscope, some agents (*Trichoderma* sp.) exerted a typical mycoparasitic coiling around a pathogen's mycelium. A strong hyphal lysis was also induced by the majority of fungal isolates tested which may adversely affect pathogen viability and consequently, virulence.

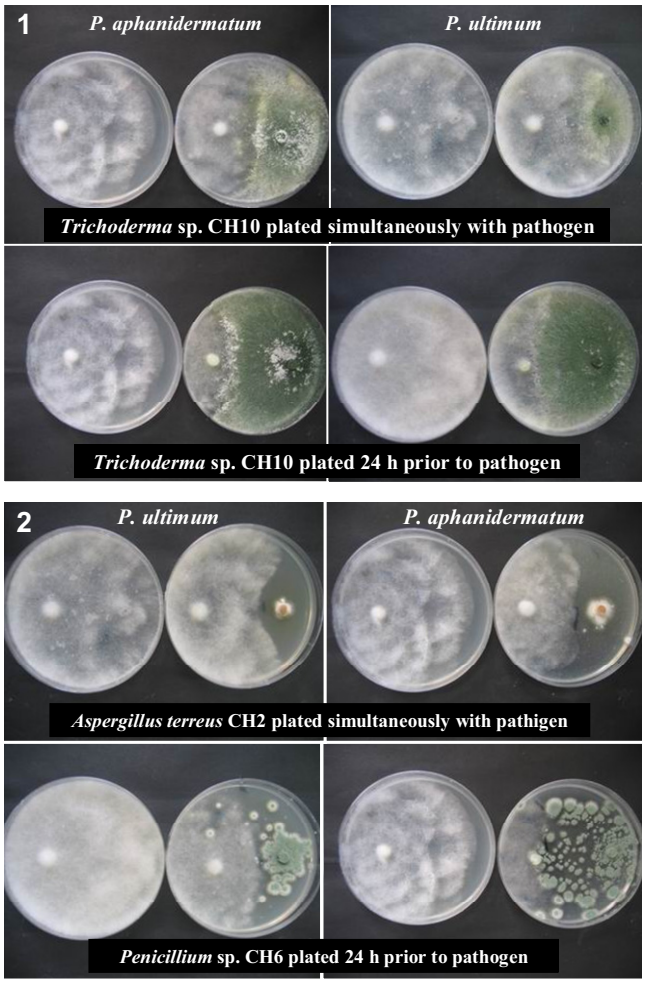

Fig. 1 Variation in the inhibitory effect of *Trichoderma* sp. against *Pythium* spp. depending on its timing of application observed after 48 h of incubation at 25°C.

Fig. 2 Antagonistic activity of *Aspergillus terreus* and *Penicillium* sp. isolates against *Pythium* spp. depending on their timing of application observed after 48 h of incubation at 25°C.

Effect of fungi tested on leak incidence and severity

Data shown in **Table 6** indicate the variations of mean leak incidence (on the ten tubers used) depending on fixed factors tested. In fact, for all antagonistic treatments combined, leak incidence seems to be reduced by about 42.78% when treatments were applied 24 h before pathogen inoculation. This reduction varied depending on *Pythium* species used for tuber inoculation. In fact, it decreased by 23% for *P. ultimum* compared with 91.52% for *P. aphanidermatum* with preventive treatments. It should be signalled that leak incidence due to *P. aphanidermatum* was completely annulled, as compared to the untreated control (disease incidence 100%), with all antagonistic fungi when applied 24 h prior to pathogen. These effects on disease incidence may influence disease severity too. In fact, as indicated in **Table 7**, the ANOVA analysis of pathogen penetration data revealed a highly (at $P \leq 0.01$) significant timing of application by antagonistic treatment by *Pythium* species interaction suggesting that antagonist's effectiveness is dependent on the timing of its application and on the target leak causal agent. Moreover, the effects of the three fixed factors tested (mains effects) as well as the different two factor interactions were found to be also highly significant.

Table 6 Pythium leak incidence (%) noted on potato tubers cv. 'Spunta' depending on *Pythium* species used for tuber inoculation, antagonistic treatment tested and the timing of its application recorded after 48 h of incubation at 25°C.

Application timing	**Simultaneous with pathogen**		**24 h before pathogen**		**Average incidence per**
Antagonistic treatments/*Pythium* species	***P. ultimum***	***P. aphanidermatum***	***P. ultimum***	***P. aphanidermatum***	**antagonistic treatment ***
Control	100	100	100	100	100
CH1	100	100	90	0	72.5
CH2	100	100	100	0	75
CH3	100	90	100	0	67.5
CH4	90	0	100	0	47.5
CH5	100	20	90	0	52.5
CH6	90	60	90	0	60
CH7	100	100	100	0	75
CH8	100	70	80	0	62.5
CH9	90	70	60	0	52.5
CH10	100	90	80	0	67.5
CH11	100	90	90	0	70
CH12	100	100	100	0	75
Average incidence per *Pythium* species and per application timing	96.92	74.61	90.76	7.69	
Average incidence per application timing**	85.76		49.23		

CH_1 and CH_{12}: *Aspergillus niger*; CH_5, CH_6, CH_7, CH_9, and CH_{11}: *Penicillium* sp.; CH_2: *Aspergillus terreus*; CH_8, CH_4, CH_3: *Aspergillus* sp.; CH_{10}: *Trichoderma* sp.

Table 7 Analysis of variance of mean penetration of the pathogen depending on fixed factors tested and their interactions.

Source	**df**	**Mean square**	***F value*** [a]
Timing of application	1	29115.156	1165.879**
Antagonistic treatment	12	549.620	22.009**
Pythium species	1	6993.556	280.048**
Timing of application × Antagonistic treatment	12	170.025	6.808**
Timing of application × *Pythium* species	1	3220.069	128.944**
Antagonistic treatment × *Pythium* species	12	204.988	8.208**
Timing of application × Antagonistic treatment × *Pythium* species	12	116.937	4.683**
Error	468	24.973	

[a] Values followed by ** are statistically significant at $P \leq 0.01$. df: degree of freedom; NS: Not significant.

Table 8 Pythium leak severity (Penetration, in mm) noted on potato tubers cv. 'Spunta' depending on *Pythium* species used for tuber inoculation, antagonistic treatment tested and the timing of its application recorded after 48 h of incubation at 25°C.

Application timing	**Simultaneous with pathogen**		**24 h before pathogen**		**Average penetration per**
Antagonistic treatments/*Pythium* species	***P. ultimum***	***P. aphanidermatum***	***P. ultimum***	***P. aphanidermatum***	**antagonistic treatment ***
Control	26.65	24.25	16.30	23.88	22.77 a
CH1	31.65 (0)	11.73 (55.05)	3.88 (83.95)	1.50 (100)	12.19 bcd (59.75)
CH2	27.80 (0)	9.43 (65.16)	9.73 (44.42)	1.50 (100)	12.11 bcd (52.39)
CH3	23.13 (14.01)	13.93 (45.38)	5.25 (74.66)	1.50 (100)	10.95 bcde (58.51)
CH4	26.30 (1.39)	1.50 (100)	3.58 (85.97)	1.50 (100)	8.22 e (71.84)
CH5	24.50 (8.54)	4.80 (85.49)	2.75 (91.55)	1.50 (100)	8.39 e (71.39)
CH6	22.30 (17.29)	9.75 (63.73)	3.10 (89.18)	1.50 (100)	9.16 de (67.55)
CH7	22.75 (15.50)	19.00 (23.07)	2.73 (91.72)	1.50 (100)	11.49 bcde (57.57)
CH8	26.25 (1.59)	14.23 (44.06)	2.60 (92.56)	1.50 (100)	11.14 bcde (59.55)
CH9	22.38 (16.99)	13.53 (47.14)	2.10 (95.94)	1.50 (100)	9.88 cde (65.02)
CH10	25.40 (4.97)	16.23 (35.27)	14.03 (15.37)	1.50 (100)	14.29 b (38.90)
CH11	23.88 (11.03)	15.45 (38.68)	3.15 (88.85)	1.50 (100)	10.99 bcde (59.64)
CH12	28.80 (0)	17.93 (27.80)	3.35 (87.5)	1.50 (100)	12.89 bc (53.82)
Average penetration per *Pythium* species and per application timing	25.52	13.21	5.58	3.22	
Average penetration per application timing**	19.37 a		4.40 b		

CH_1 and CH_{12}: *Aspergillus niger*; CH_5, CH_6, CH_7, CH_9, and CH_{11}: *Penicillium* sp.; CH_2: *Aspergillus terreus*; CH_8, CH_4, CH_3: *Aspergillus* sp.; CH_{10}: *Trichoderma* sp.
Numbers in parenthesis indicate the percentage (in %) of reduction of leak severity (penetration) as compared to the untreated control. For the averages per application timing and per antagonistic treatment tested, means followed by the same letter are not significantly different according the SNK test at $P \leq 0.05$.

Pooled data for application timings, for all *Pythium* species and antagonistic treatments combined, presented in **Table 8** reveal that the lowest penetration (4.40 mm) was recorded on tubers treated 24 h prior to inoculation. These preventive treatments have contributed by the reduction of leak severity by about 77.28% as compared to simultaneous application during inoculation. Moreover, as also noted with disease incidence, *Pythium* species seemed to respond differently depending on the timing of antagonistic treatments; leak was less severe when tubers were treated preventively.

Pooled data of the timing of their application, all fungi tested had significantly reduced leak severity as compared to the untreated and inoculated control; the highest percentages of inhibition of disease development (71.39 and 71.84%) were recorded with CH4 (*Aspergillus* sp.) and CH5 (*Penicillium* sp.) isolates, respectively (**Fig. 3**). Moreover, 11 out the 12 fungal isolates tested had suppressed leak development by more than 50% independently of *Pythium* species and application timings (pooled data).

The more interesting inhibitory effects were obtained with all antagonists when applied preventively. In fact, leak caused by *P. aphanidermatum* was completely suppressed (100% of inhibition) with all fungi applied 24 h before inoculation while for *P. ultimum*, the percentage of reduction of leak severity ranged between 15.37 and 95.94% and 10 out of the 12 antagonists had decreased rot severity by more than 80%.

It is important to note that some fungal isolates have

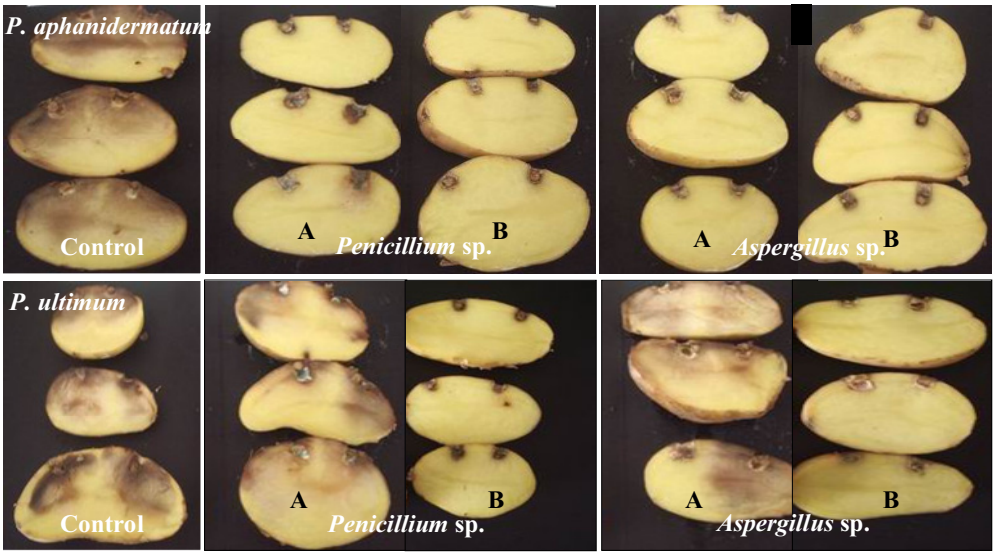

Fig. 3 Variation in the suppression of leak development by *Aspergillus* sp. and *Penicillium* sp. depending on the timing of their application noted after 48 h of incubation at 25°C. (**A**) Antagonistic treatment applied simultaneously with pathogen inoculation; (**B**) Antagonistic treatment applied 24 h prior pathogen inoculation.

showed a relatively important effectiveness even when applied simultaneously with *P. aphanidermatum*; this is the case of CH4 (*Aspergillus* sp.) and CH5 (*Penicillium* sp.) isolates (100% and 85.49%, respectively) and at a lesser degree CH2 (*A. terreus*), CH6 (*Penicillium* sp.) and CH1 (*A. niger*) isolates for which percentage of inhibition varied between 55.05 and 65.16%. However, for *P. ultimum*, the highest inhibitory effects subsequent to simultaneous application (17.29, 16.99, and 15.50%) were obtained with treatments based on CH6, CH9 and CH7 (*Penicillium* sp.) isolates, respectively.

DISCUSSION

Involvement of a *Pythium* species in development of potato leak, absence of resistant potato cultivars together with the absence of registered fungicides for post-harvest use and the increasingly serious losses are the most important factors that incited us to begin the present biocontrol experiments. In previous attempts to reduce leak incidence, metalaxyl and soilborne strains of *T. harzianum* have shown successful control of leak in potatoes (Triki and Priou 1997; Daami-Remadi 2001a, 2001b) but their application under commercial conditions is still uncommon.

Given that composts and compost teas are able to control soil-borne pathogens (Hoitink *et al*. 1991, 1997), the numerous published reports on their suppressive effects against *Pythium* sp. (Chen *et al*. 1988a, 1988b; Mandelbaum and Handar 1990) and their capability to protect plants against diseases due to their variable physiochemical and biological properties (Boulter *et al*. 2000; Fuchs and Larbi 2004), several compost types were exploited in the current study as source of potentially important biocontrol agents. Moreover, their ability to suppress *P. aphanidermatum* and *P. ultimum*, and to control potato leak, was investigated as a function of time of agent application and pathogen inoculation.

The *in vitro* interaction revealed the suppressive effects of the microbial agents used against *Pythium* species. The mycelial growth of the pathogen was significantly inhibited by *Aspergillus* spp., *Penicillium* sp. and *Trichoderma* sp. isolates. Several mechanisms of action were deployed during antagonism i.e. mycoparasitism, hyphal lysis and antibiosis. These last phenomena were the most known for the widely used biocontrol agents such as *Trcihoderma* spp. whereas for *Aspergillus* spp., mycoparasitism has not been widely reported in literature. In fact, findings from *in vitro* studies concerning the inhibitory effects of *Trichoderma* sp. are in agreements with several previous studies (Harman *et al*. 1980; Triki and Priou 1997; Daami-Remadi 2001a; Atef and Haikal 2008) whereas those dealing with the suppressive effects of *Aspergillus* species isolated from composts were in accordance with Venkatasubbaiah and Safeeulla (1984) results who found that *A. niger*, isolated from the rhizosphere of coffee (*Coffea arabica*) seedlings, was antagonistic to *Rhizoctonia solani in vitro* and exhibited hyperparasitism against this collar rot pathogen in dual culture. Similarly, a mycoparasitic coiling was also observed during *Aspergillus* sp. isolates and *Fusarium* spp. hyphal interactions (Daami-Remadi *et al*. 2006b; Suárez-Estrella *et al*. 2007). However, *A. niger* did not induce inhibitory zones on any of the tested fungus including *P. aphanidermatum* (Muhammad and Amusa 2003). *Aspergillus* species are ubiquitous in most agricultural soils and they generally produce a variety of secondary metabolites exhibiting inhibitory effects on several soil-borne microorganisms (Siddiqui *et al*. 2004).

Several microorganisms have been reported to be biocontrol agents for suppression of the pathogenic *Pythium* species (Harman *et al*. 1980; Harman and Hader 1983; Hoitink and Boehm 1999). The present study showed the evidence of the inhibitory effect of some compost microorganisms. In fact, *Aspergillus*, *Penicillium*, *Trichoderma*, and other fungi are among the most abundant in composts (El-Masry *et al*. 2002; Molla *et al*. 2002; Naidu *et al*. 2010) and they play a significant role in the composting process as well as in suppressing the growth of pathogenic fungi (Phae *et al*. 1990; Raimbault 1998; Muhammad and Amusa 2003; Suárez-Estrella *et al*. 2007).

For all microbial agents tested in the present study, the need of their preventive application for the improvement of their effectiveness was evidenced based on dual culture experiments. Overall, the percentages of inhibition have interestingly increased when antagonists were plated 24 h prior *Pythium* species as compared to simultaneous inoculation. These conclusions are in accordance with other *Pythium* biocontrol studies (Triki and Priou 1997; Daami-Remadi 2001a).

Data from our study also showed the presence of important microorganisms that may play an interesting role in Pythium leak biocontrol such as *Aspergillus* sp., *A. niger*, *A. terreus* and *Penicillium* sp. as well as the globally known antagonist *Trichoderma* sp. In fact, watery wound rot incidence due to *P. aphanidermatum* was totally reduced by the majority of the microbial agents tested when applied 24 h prior to pathogen inoculation. However, more interesting results were recorded for penetration (leak severity) where some isolates such as CH4 (*Aspergillus* sp.) had totally in-

hibited rot development when applied simultaneously with *P. aphanidermatum* and all microbial agents had completely suppressed leak when applied preventively. Similar conclusions were previously reached with *T. harzianum* use for *Pythium* spp. biocontrol (Triki and Priou 1997; Daami-Remadi 2001a; Kerkeni *et al*. 2007b). Results concerning *in vivo* effectiveness of *A. niger* as biocontrol agent joined those of Venkatasubbaiah and Safeeulla (1984) who recorded an increase in germination and a reduction in the incidence of coffee collar rot disease by seed treatment with this rhizospheric agent. Similarly, the antagonistic potential of *Aspergillus* sp. against both leak agents confirmed previous local reports on biocontrol of soil-borne pathogens with *Aspergillus* sp. isolated from compost teas (Daami-Remadi *et al*. 2006b; Kerkeni *et al*. 2007c).

The present study also highlighted the importance of the timing of antagonist application on its effectiveness in leak control. It was clearly evidenced that some compost fungi are able to completely suppress rot development when applied preventively following tuber wounding. Numerous reports indicated similar attempts in improving biocontrol agent's effectiveness in different pathosystems. In fact, the influence of antagonist/pathogen contact time on the effectiveness of biocontrol was reported by Pratella *et al*. (1993) in stone fruit/*Monilinia laxa* pathosystem. Moreover, in apple (*Malus domestica*)/*Botrytis cinerea* pathosystem, introduced populations of *Candida oleophila* was found to increase about 32 times during the initial 24 h following their introduction to fresh wounds, and remained stable afterwards. The biocontrol of gray mold rot was more effective when this antagonist agent was applied to fresh wounds rather than one-day-old wounds. Thus, its application should follow the occurrence of wounding as soon as possible in order to obtain optimal disease control during storage (Mercier and Wilson 1995) as also shown in our study. In the pepper (*Capsicum annuum* L.)-*Pythium* spp. pathosystem, *Pseudomonas chlororaphis* suppressed disease most effectively when applied 3 days before roots were inoculated with *P. aphanidermatum* and *P. dissotocum* (Chatterton *et al*. 2004) although significant suppression was observed for all treatment times.

The use of these fungal biocontrol agents isolated from compost and/or soils to control potato leak may constitute a promising alternative to manage this increasingly important disease because postharvest conditions provide an ideal niche for microbial development and consequently, disease control. Moreover, their testing under conditions conducive for leak expression such as use of susceptible potato cultivar ('Spunta'), tuber wounding before inoculation, incubation under high moisture (Stanghellini and Hancock 1971; Lifshitz and Hancock 1984; Barr *et al*. 1996; Priou *et al*. 1997; Taylor *et al*. 2008) and temperature altogether have normally favoured pathogen expression and consequently, rot development. Thus, results presented here concerning the *in vivo* inhibitory effects of the microbial agents tested proved their effectiveness under extremely favourable conditions for *Pythium* spp. development but due to differences in optimal thermal requirements for both species (Ben-Yephet and Nelson 1999; Triki *et al.* 2001; Salas and Secor 2001; Lui and Kushalappa 2003), the assay may give more additional precisions if it will be conducted under different temperatures as this abiotic factor may play an important role in disease dynamic and evidently in leak control.

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