International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 6, No. 4, p. 44-53, 2015

RESEARCH PAPER OPEN ACCESS

Antagonistic effect of *Trichoderma harzianum* against *Phytophthora infestans* in the North-west of Algeria

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Article published on April 01, 2015

Key words: *Phytophthora infestans, Trichoderma harzianum,* Biological Control, Tomato, Potao.

Abstract

The oomycete, *Phytophthora infestans* is considered one of the most important pathogens of potatoes and tomatoes worldwide. A total of 38 *Phytophthora infestans* isolates were obtained from leaves, tubers and stems of infected crops of potato and tomato in different regions of the North West of Algeria in 2010, 2011 and 2012. Based on morphological and physiological characteristics, they were tested for the virulence test on potatoes tubers and tomatoes leave then, for a biological control by using *Trichoderma spp*. as antagonistic agent. *Trichoderma* species are among important antagonists of plant pathogenic fungi. The main purpose of this study was to evaluate the biocontrol potential of native *Trichoderma harzianum*. Their antagonistic activities including competition and colonization against *Phytophthora infestans* with an inhibition rate of 86%.

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Introduction

The late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary 1876 is the most important and most destructive disease of potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*) worldwide since 1990. The asexual life cycle of *P. infestans* is short; and sporulating foliar lesions develop three to seven days after successful infection under conducive conditions resulting in a polycyclic epidemic (Rekanović *et al.*, 2011). The annual economic losses caused by the disease worldwide have been about 170 billion US dollars (Haverkort *et al.*, 2009; Wu *et al.,* 2012). Seed tubers infected with *P. infestans* will either rot in storage, after planting in the field or survive and initiate new epidemics of potato late blight (Kirk *et al.*, 2009; Stevenson *et al.*, 2007). The disease is widespread in North and South America, Europe; Asia (Fry *et al.*, 1993; Guenthner *et al.*, 2001; Chowdappa *et al.*, 2013). In recent years late blight has become a significant epidemic problem in North Africa: Moroco (Sedgui *et al.*, 1999; Andrivon *et al.*, 2007), Tunisia (Jmour and Hamada, 2006; Harbaoui *et al.*, 2013) and Algeria (Corbière *et al.*, 2010). *P.infestans* is a heterothallic oomycete, which presence of hyphae of opposite mating types A1 and A2 are necessary for sexual reproduction and oospores forming (Drenth, 1995; Mazáková *et al.*, 2006). The disease can be managed in many ways. For effective black shank control, growers use a combination of crop rotation, cultivar resistance and fungicide applications (Melton , 1998). Fungicides such as metalaxyl, are however expensive and growers tend to use lower than optimum dosages (Csoinos and Bertrand, 1994). Furthermore, the use of fungicides, besides being expensive and involving risks to the environment associated with the application of chemicals, is not totally effective and may lead to the appearance of new, resistant strains of pathogens (Bruin and Edgington, 1980). It is therefore necessary to develop alternative ways of control. One such alternative is biological control, in which microorganisms are selected for their ability to antagonize pathogens. *Trichoderma* spp. Species of the genus *Trichoderma* are considered as potential biological control agents (BCAs), and the modes of action include mycoparasitism, antibiosis, competition, enzyme activity and induced plant defence (Howell, 2003). *Trichoderma* has considerable activity against many pathogenic fungi, e.g. *Rhizoctonia solani* (Beagle-Ristaino and Papavizas, 1984), *Pythium ultimum* (Besnard and Davet, 1993), *Armillaria* (Osando and Waudo, 1994), *Fusarium oxysporum* ((Perveen and Bokhari, 2012), *Fusarium solani* (Rojo *et al.*, 2007), *Botrytis cinerea* (Bendahmane *et al.*, 2012), *Verticillium dahliae* (Mouria *et al.*, 2013) and *Phytophthora palmivora* (Mpika *et al.*, 2009). The present experiment was undertaken to evaluate the efficacy of the mycelial growth inhibition of *P. infestans* by *Trichoderma harzianum* isolates that could be used in the future as the biological control for late blight in the fields.

Materials and methods

Sampling of P. infestans isolates

The diseased samples were collected during potato and tomato growing seasons between 2010 and 2012 from the blighted potato and tomato leaves, with freshly sporulation lesions of *P. infestans*. The samples were kept in plastic bags brought to the laboratory.

Isolation of P. infestans

To stimulate sporulation, the infected potato and Tomato leaflets were placed in a plastic box containing moist filter paper and incubated in darkness at 18°C for 24 hours. Infected tissues were surface disinfected in 0,5% NaOCl for 2 to 3 min, then placed on selective media amended with antibiotics (Hollomon, 1965) or PARP (1L of deionized water containing 17g of cornmeal agar amended with the following antibiotics: (ampicillin 50 µg/ml, rifampicin 50 µg/ml and benomyl 10 µg/ml). The plates were then incubated at 18°C for 5-7 days for mycelial growth. The hyphal tips were cut from the colony margins and transferred to amended rye A agar to get pure cultures (Ribeiro, 1978). Pure cultures were maintained in unamended rye A agar for further study (Deahl *et al.*, 1993; 1995).

Pathogenicity test

Inoculum preparation

The isolates were grown in rye B media for 14 days in the dark at 18°C for sporangia production, and transferred to the light for 2 days to encourage sporulation. Sporangia and mycelium were harvested by flooding with cold sterile water (4°C) and gentle scraping of the surface of the culture using a rubber policeman. The sporangia suspension was stirred with a magnetic stirrer for 1 h. Sporangial suspensions of each isolate were prepared from 15-days-old cultures on pea agar, and adjusted to 5.10^4 sporangia per ml using a haemacytometer. Before inoculation, the sporangial suspensions were stored for 6 h at 4°C to encourage zoospore release from the sporangia.

Whole tuber inoculation with P. infestans

Tuber late blight development caused by the different *P. infestans* genotypes on the tuber cultivars were evaluated at different commonly used post-harvest potato storage temperatures (3, 7 and 10°C) using whole tuber sub–peridermal inoculation. All tubers were washed in distilled H_2O to remove soil. The tubers were then surface sterilized by soaking in 2% sodium hypochlorite solution for 4 h. Tubers were dried in a controlled environment with forced air ventilation at 5950 l min^{-1} at 15°C in dry air (30% relative humidity) for four hours prior to inoculation. The washed, surface-sterilized tubers were inoculated by a sub-peridermal injection of a sporangia suspension of 2 x 10^{-5} ml (delivering zoospores released from about 20 sporangia inoculation-1) with a hypodermic syringe and needle at the apical end of the tuber about 1 cm from the dominant sprout to a maximum depth of 1 cm.

Four tubers of each cultivar were inoculated with each *P. infestans*. Four control tubers per cultivar were inoculated with cold (4°C) sterile distilled H_2O. After inoculation, tubers were placed in the dark in sterilized covered plastic crates and returned to controlled environment. The chambers were set at 3, 7 or 10°C and 95% humidity and the sample tubers were incubated for 40 days until evaluation (Kirk *et al.*, 2009).

Biological control

In this study, the reduction in growth and inhibition zone in the dual culture test was used as the criteria to evaluate the in vitro antagonistic property of *T. harzianum*. The in vitro evaluation consisted of placing 4mm diameter discs of the pathogen and antagonists taken from the peripheries of expanding colonies grown on media. Other, we prepare the control test by without placing the disc of the antagonist, only pathogen was kept for comparison.Types of interactions were studied in dual culture on seventh day. After both the fungi came in contact with each other, the contact/inhibition zone cut using sharp blade. The reduction in mycelia growth was recorded and the percentage of inhibition over control for each treatment was calculated in this dual plate culture test as given in Table 2. The following formula was used for calculation the percentage reduction in growth, which is:

% Reduction in growth = 100 * (X-Y/X)

Where:

X= Growth of pathogen alone without antagonist (control).

Y= Growth of pathogen along with the antagonist

Statistical Analysis

The laboratory experiment consisted of 1 treatments including control with three replications. The design was completely randomizes (CRD) which is used for statistical analysis. Data from the *P. infestans* mycelial growth inhibition were analyzed by ANOVA.

Result

A total of thirty eight isolates were obtained from infected potato and tomato leaves that collected from some tomato and potato cultivating areas. This isolates (Table 1) are maintained in a collection of Plant pathology laboratory, department of Sciences, University of Es-Senia and that is the initial and the starting point of our research.

Table 1. Origins of the isolates studied.

Isolates	Year	Host	Kind	Localization
P1	2010	Potato	Tuber	Sidi Kada (Mascara)
P2	2010	Potato	Stem	Sidi Kada (Mascara)
P3	2010	Potato	Tuber	Ghriss (Mascara)
P4	2010	Potato	Soil	Ghriss (Mascara)
P5	2010	Potato	Tuber	Matmour (Mascara)
P6	2010	Potato	Soil	Matmour (Mascara)
P7	2010	Potato	Tuber	Maoussa (Mascara)
P8	2010	Potato	Leaves	Maoussa (Mascara)
P9	2010	Tomato	Soil	Mesreguine (Oran)
P9'	2010	Tomato	fruits	Mesreguine (Oran)
P10	2010	Potato	Tuber	Teghanif (Mascara)
P11	2011	Potato	Soil	Teghanif (Mascara)
P12	2011	Potato	Tuber	Mohammadia (Mascara)
P13	2011	Tomato	leaves	Chammouma (Mosta)
P14	2011	Potato	Tuber	Chammouma (Mosta)
P15	2011	Tomato	Soil	Chammouma (Mosta)
P16	2011	Tomato	stem	Chammouma (Mosta)
P17	2011	Potato	Tuber	Ain Rayes (Mosta)
P18	2011	Tomato	Leaves	Ain Rayes (Mosta)
P19	2011	Potato	Tuber	Stidia (Mosta)
P20	2011	Tomato	Leaves	Stidia (Mosta)
P21	2012	Potao	Leaves	Oued Fodda (Chlef)
P'21	2012	Potao	Tuber	Oued Fodda (Chlef)
P22	2012	Potao	Leaves	Boukadir (Chlef)
P'22	2012	Potao	Tuber	Boukadir (Chlef)
P23	2012	Potato	Soil	Chlef
P30	2012	Potao	Leaves	Ramchi (Tlemcen)
P'30	2012	Potao	Tuber	Ramchi (Tlemcen)
P''30	2012	Potao	Soil	
P31	2012	Potao	Leaves	Heennaya (Tlemcen)
P'31	2012	Potao	Tuber	Heennaya (Tlemcen)
P32	2012	Potao	Leaves	Meghnia (Tlemcen)
P'32	2012	Potao	Tuber	Meghnia (Tlemcen)
P''32	2012	Potao	Soil	Tlemcen
P33	2012	Potao	Leaves	Oued Djamea (Relizane)
P'33	2012	Potao	Tuber	Oued Djamea (Relizane
P34	2012	Potao	Leaves	Hamadna(Relizane)
P'34	2012	Potao	Tuber	Hamadna(Relizane)

Pathogenicity test

The three varieties of tubers inoculated and incubated at 10° C showed a significant difference in their resistance against *Phytophthora infestans* and approve its aggressive power and its pathogenicity on its host. All isolates tested in this study on *Spounta* and *Bartina* cultivars are pathogenic with a higher degree of aggressiveness and this result showed discoloration and deterioration of tubers after inoculation. Although, all isolates tested on *Condor* cultivars are not really significant with less degree of aggressiveness than other cultivars. So, for conclusion, we can say that *Condor* cultivars are more resistant to disease by contribution than other cultivars (Fig.1).

Biological control

The result of *Trichoderma* tested for their *in vitro*

antagonism shows that there is a significant difference in radial growth of the pathogen alone in the box as a test and in confrontation with antagonist (Fig.2).

In this study, *T. harzianum* was consistently found to be the effect inhibitor reduction in radial growth of all pathogens of *Phytophthora infestans* with an highest inhibition rate (85 %) with P10 and less inhibition rate (57%) with P19 (Table 02).

Table 2. *In-vitro* antagonism of *Trichoderma harzianum* against *Phytophthora infestans*.

Isolats	Control	test	% inhibition
P1	1,33	7,83	82,98
P2	2,17	7,40	70,71
P3	1,73	8,30	79,12
P4	1,53	7,53	79,65
P5	1,23	7,80	84,19
P6	2,27	7,60	70,17
P7	2,43	6,97	65,02
P8	1,83	8,30	77,91
P9	3,20	8,30	61,45
P10	1,20	8,30	85,54
P11	2,43	8,30	70,68
P12	1,83	8,30	77,91
P13	1,10	8,30	86,75
P15	1,26	5,73	77,98
P16	1,73	7,40	76,58
P17	1,90	7,13	73,35
P18	2,73	7,33	62,73
P19	3,20	7,43	56,94
P20	3,00	7,77	61,38
P21	3,00	7,73	61,19
P22	1,80	7,37	75,55
P30	3,20	8,30	61,45
P31	2,53	8,27	69,36
P32	2,07	6,53	68,37
P33	2,70	6,47	58,24
p34	3,37	7,87	57,19

The result of dual culture show that *Trichoderma harzianum* is a highly significant antagonistic agent (P≤0.05) inhibited the mycelial growth of pathogen *P. infestans*.

Discussion

Infection of potato tubers by *P. infestans* may be initiated by zoospores, sporangia or oospores washed in precipitation or irrigation water from plant foliage and deposited in soil (Fry, 2008).

In the first step, our results indicate that all our strains are pathogenic, aggressive and violent on tubers cultivars (Spounta/Bartina) but we mark that it have a relationships between inoculums concentration and resistance in potatoes cultivars (Condor) to the late blight and the same studies founded by (Kroll and Eide, 1981; Kuhn *et al.*, 2013) have proven that the mildew can attack all varieties regardless of their level of tolerance, however, there are less sensitive than other varieties. The same result were found by (Liu, 2009) that a most accessions of *S. verrucosum* displayed high levels of foliar resistance when compared to the susceptible *S.*

tuberosum cv. 'Katahdin' control and were significantly ($P<0.05$) more resistant than control.

In the second step, our research was based on biological control shows that *P. infestans* mycelia growth inhibition caused by the antagonistic strains of *Trichoderma harzianum* indicate that *T. harzianum* were able to reduce disease incidence of pathogen, among these isolates there are physiological differences and these variations could be due to the mechanism involved in the antagonistic activity by differential secretion of antifungal substances various toxic and antibiotic metabolites (Dennis and Webster, 1971a, b; Claydon *et al.* 1987; Lorito *et al.*, 1994) and enzymes (Lorito *et al.*, 1993) which are involved in the inhibition and lysis of pathogenic fungi. Similar reactions were reported previously by (Barnett and Binder, 1973; Elad *et al.*, 1983) who noticed inhibition of growth, lysis and parasitism by *Trichoderma spp*. of some species of *Phytophthora*. (Smith *et al.*, 1990; Fang and Tsao, 1995) have shown the biocontrol capacity of these fungi *(T. harzianum, Gliocladium spp*. or *Pythium nunn* and *Penicillium funiculosum) In vitro* and *in vivo* studies on *Phytophthora spp*. (Bowers *et al.*, 2001b) have shown that the application of *Trichoderma sp* on leaf discs allowed the reduction of susceptibility leaf to *Phytophthora megakarya*. (Singh and Islam, 2010) proving that the *in vitro* culture of *P. nicotianae and T. harzianum* together led to a variety of interactions. *P. nicotiana* growth was generally inhibited, the hyphae lysed on dual culture media and hyphae were intensely parasitized by *T. harzianum*. (Mpika *et al.*, 2009) were tested the inhibitor effect of *Phytophthora palmivora* agent of brown rot cocoa pods in Côte d'Ivoire by *Trichoderma sp*.

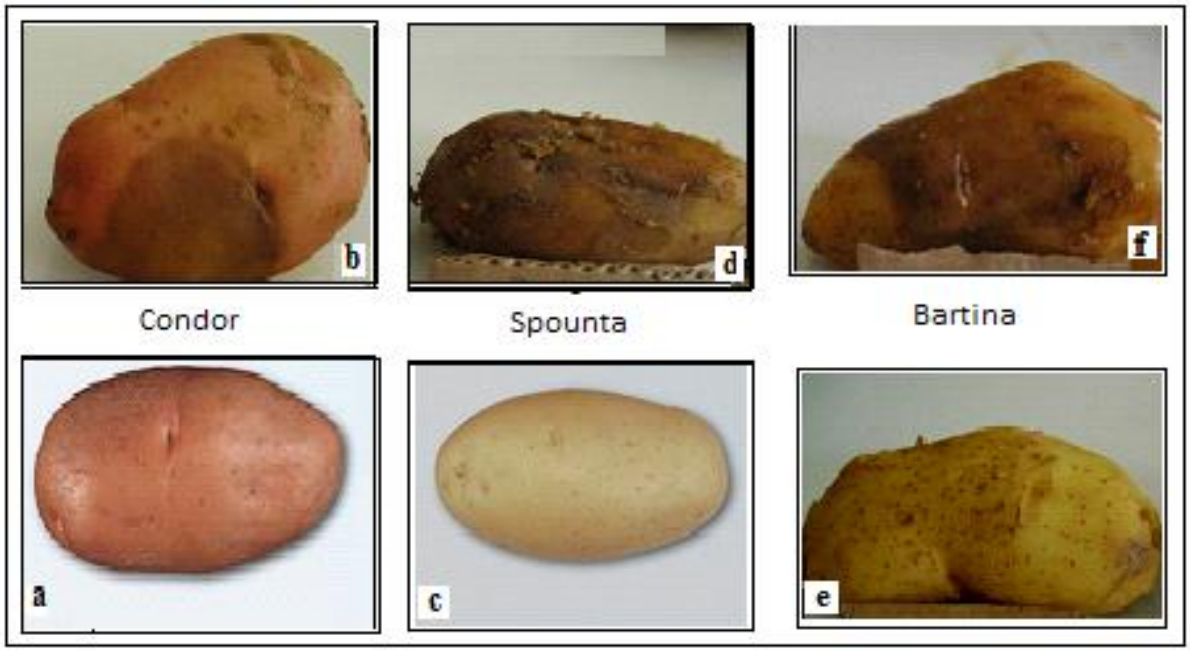

Fig. 1. (b, d & f) showing the aggressiveness degrees of the pathogen in the inoculated tubers; (a, c & e) screening the control tubers.

The same studies of (Loliam *et al.*, 2010) showed the antagonistic activity of *S. rubrolavendulae* against *P. infestans* with 83.33% of growth inhibition. Furthermore, (Michel *et al.*, 2005) with *Trichoderma harzianum* against *Sclerotium rolfsii*. As well, (Osorio-Hernández *et al.*, 2011) and their studies on the *In vitro* behavior of *Trichoderma spp*. against *Phytophthora capsici*. Also, (Moayedi and Ghalamfarsa, 2010) have reported the isolation of a variety of mycoparasitic *Trichoderma* isolates that have the potential to suppress *Phytophthora root rot* of Sugar Beet on antagonistic Activities of

Trichoderma sp. (Ezziyyani *et al.*, 2009) their results showed that *T.harzianum* demonstrated a dearly antagonistic affect against *Phytophthora capsici root rot* of papper, especially on PDA medium enriched with laminarine-glucose (3:1,v/v), which is reported to increase the antifungal activity through secretion of the hydrolytic enzyme, β-1,3-glucanase.

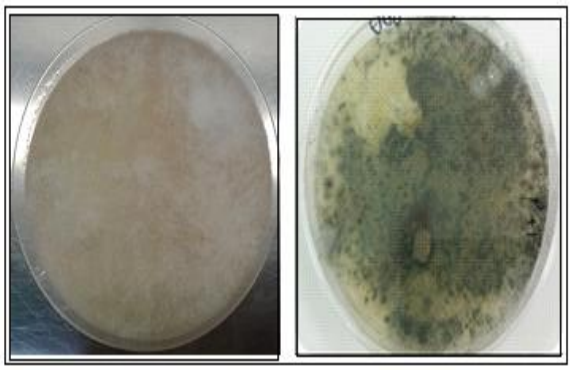

Fig. 2. Dual culture of Antagonist and pathogen. A. *P. infestans* (control: without antagonist), B. with antagonist *T. harzianum*.

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