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Biocontrol of Wheat *Fusarium* Crown and Root Rot by *Trichoderma* spp. and Evaluation of Their Cell Wall Degrading Enzymes Activities

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Fourteen strains of *Trichoderma* spp. were isolated from Algerian desert soils and assessed for their antagonistic activity against *Fusarium* crown and root rot of wheat. Biocontrol efficiency of *Trichoderma* spp. was studied by *in vitro* and *in vivo* based bioassay against three pathogenic species: *F. culmorum, F. graminearum* and *F. verticillioides*. *In vitro* based bioassay (dual culture) results obtained with all *Trichoderma* spp. isolates showed significant decrease in colony diameter of *Fusarium* species compared to the control. The highest percentages of reduction in colony diameter were obtained with *T. harzianum* Thr.4 causing a growth reduction of 70.68%, 67.05 and 70.57% against *F. culmorum*, *F. graminearum* and *F. verticillioides*, respectively. All *Trichoderma* spp. isolates were able to overgrow and sporulate above *F. culmorum* colonies but no overgrowth was observed with *F. graminearum* and *F. verticilliodes*. The seed treatment by *Trichoderma* spp. isolates before sowing in a soil already infested by the pathogens led to a significant decrease of disease severity compared to the untreated control. The highest disease index decrease (> 70%) was obtained with two isolates of *T. harzianum* (Thr.4 and Thr.10) and *T. viride* Tv.6 against the three fungal pathogens. Lytic enzymes production by *Trichoderma* spp. isolates was tested in liquid cultures containing fungal cell walls of each pathogen as sole carbon source. Higher levels of protease and chitinase activities were induced by hyphal cell walls of *F. graminearum* than cell walls of *F. verticillioides* and *F. culmorum*. *T. harzianum* Thr.4 exhibited the highest enzyme activities with hyphal cell walls of *F. graminearum* and *F. culmorum*. However, in the medium amended with cell wall of *F. verticillioides,* maximal lytic activities were recorded for *T. viride* Tv.6.

Keywords: *Trichoderma* spp., biological control, *Fusarium* spp., wheat, cell wall degrading enzymes.

Root and crown rot of wheat caused by *Fusarium* species is a very common and economically important disease in many wheat-producing area of the world, especially in low and medium rainfall areas (Cook, 1980; Wiese, 1987; Moya-Elizondo, 2013). Invasion of crown and root tissues by root and crown rotting fungi reduces the plant's capacity for efficient nutrient and water uptake, causing significant losses in wheat production (Tunali et al., 2008). In addition to the quantity losses, *Fusarium* species produce a wide range of mycotoxins, which are hazardous to humans and animals (Nelson

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et al., 1993; Parry et al., 1995). As *Fusarium* species are soil-borne in nature, application of fungicides to control these pathogens is not practical. In addition, resistance to *Fusarium* crown rot in commercial cultivars is only partial (Cook, 2010). These reasons have increased the need to find alternative methods for pathogens control as biological control methods.

Trichoderma is one of the most exploited fungal biocontrol agents in agriculture to manage plant diseases caused by a wide number of fungal pathogens (Harman et al., 2008; Vinale et al., 2008). Several mechanisms have been associated with *Trichoderma* spp. ability to control plant pathogens including nutrient competition, antibiotics and antifungal metabolites production, and mycoparasitism by cell wall degrading enzymes (Howell, 2003; Vinale et al., 2008). Extracellular chitinase and protease production increases significantly when *Trichoderma* spp. is grown in medium supplemented with either autoclaved mycelium or fungal cell walls (Elad et al., 1982; Geremia et al., 1993) and a correlation between biocontrol ability of *Trichoderma* spp. and lytic enzymes activities has been reported (Flores et al., 1997; Herrera-Estrella and Chet, 1999).

Trichoderma isolated from agricultural crops rhizosphere have been the subject of several researches. However, those isolated from extreme environment remain poorly studied. Although fungal isolates from hot desert were revealed to play an important role in seeds germination by breaking dormancy and increasing water uptake (Delgado-Sánchez et al., 2011). The objective of this investigation was to evaluate the potential of fourteen strains of *Trichoderma* isolated from Algerian desert soils for their biocontrol ability against three causal agent of root and crown rot of wheat in Algeria, *Fusarium graminearum* Schwabe, *Fusarium culmorum* (W.G. Smith) Saccardo and *Fusarium verticillioides* (Saccardo) Nirenberg (Boureghda et al., 2010; Lounaci and Athmani-Guemouri, 2014), focusing on the relationship between their ability to reduce disease index of pathogens and their cell wall degrading enzymes activities.

Materials and Methods

Isolation and identification of pathogens and antagonists

The pathogen strains used in this study are originated from culture collection of the Laboratory of Phytopathology and Molecular Biology of the National High School of Agronomy (ENSA), El-Harrach, Algiers, Algeria. *Fusarium* strains were isolated from wheat diseased plant (root rot) and identified based on morphological characters using the literature Tousson and Nelson (1976), Booth (1977), Leslie and Summerell (2006). *F. culmorum* identification was confirmed by DNA analysis with Fco1 primers (Fco1 Forward: ATGGTGAACTCGTCGTGGC, Fco1 Revers: CCCTTCTTACGCCAATCTCG). Strains of the three *Trichoderma* species *T. harzianum*, *T. viride* and *T. hamatum* were isolated from desert soil of six sites in the southern east of Algeria (Table 1) by the dilution plate method described by Davet and Rouxel (1997). *Trichoderma* isolates were identified at species level based on phenotypic and molecular approaches. Phenotypic identification was performed by observation of colony characteristics at different temperatures and by

Table 1

List of *Trichoderma* spp. strains used in the present investigation

Isolate code	Species	Origin	Isolation year
Thr.1	*Trichoderma harzianum*	Ain Ben Naoui	2012
Thr.2	*Trichoderma harzianum*	Doucen	2012
Thm.3	*Trichoderma hamatum*	Foughala	2012
Thr.4	*Trichoderma harzianum*	Foughala	2012
Thr.5	*Trichoderma harzianum*	Foughala	2012
Thv.6	*Trichoderma viride*	Foughala	2012
Thm.7	*Trichoderma hamatum*	El-Hadjeb	2012
Thr.8	*Trichoderma harzianum*	Doucen	2013
Thr.9	*Trichoderma harzianum*	Loutaya	2013
Thr.10	*Trichoderma harzianum*	Tighdidine	2013
Thr.11	*Trichoderma harzianum*	El-Hadjeb	2013
Thr.12	*Trichoderma harzianum*	Loutaya	2013
Tv.13	*Trichoderma viride*	Doucen	2013
Thr.14	*Trichoderma harzianum*	Loutaya	2013

Thr: *T. harzianum*, Thm: *T. hamatum*, Tv: *T. viride*

microscopic studies of conidiophores, phialides and conidia. Phenotypic results were compared to a taxonomic key of the genus *Trichoderma* as described by Rifai (1969), Bissett (1984; 1991a, b, c), Gams and Bissett (1998), Chaverri et al. (2003). This investigation was completed by molecular identification performed by sequencing the ITS1-5.8S-ITS2 rRNA region using the primers ITS1 and ITS4 as described by White et al. (1990). The single cultures spores of isolates were maintained on Potato Dextrose Agar (PDA) slants at 4 °C and subcultured at 2 month intervals.

Plant material

The wheat cultivar Vitron known for its sensitivity to the *Fusarium* root rot and head scab of wheat was used in the present study.

Antagonistic activity of Trichoderma *strains* in vitro

Interactions between antagonistic fungi and pathogenic fungi were carried out by direct confrontation method described by Benhamou and Chet (1996). One mycelial disc (5 mm) of each fungus was placed at opposite poles on PDA plates, keeping a distance of 4 cm between the two fungi and incubated at 25 °C for 7 days. Three replicates were made for each combination of *Trichoderma* isolate/pathogen. Plates containing only the pathogens were used for control. Growth of pathogenic strains was measured at two days intervals and the hyphal interactions were studied under optical microscope. Inhibition percentage of the pathogen mycelial growth was estimated using the following formula: $I\,(\%) = (1 - Cn/C0) \times 100$. Cn: diameter of pathogen colony in the presence of the antagonist (mm). C0: Colony diameter of the control (mm).

Enzyme production

For hydrolytic enzyme production, discs (5 mm diameter) from 5-day-old cultures of *Trichoderma* isolates previously grown on potato dextrose agar (PDA) were inoculated into synthetic medium (SM) containing the following components (g/l): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; $FeSO_4.7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The medium was supplemented with cellular wall (2 g/l) of each fungal pathogen as carbon source prepared according to Sivan and Chet (1989). The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml SM medium with constant shaking (120 rpm) at 30 °C. After 72 h, the cultures were filtered through Whatman 1 filter paper at 4 °C. All experiments were performed in triplicate.

Estimation of protein and enzyme activity in the culture filtrates

The protein content in the culture filtrates was estimated by Bradford (1976) method using bovine serum albumin (BSA) as standard. The chitinase assay mixture consisting of 1 ml culture filtrate and 1 ml suspension of colloidal chitin (0.5% in 50 mM sodium acetate buffer, pH 5.0) was incubated at 37 °C under constant shaking. After 30 min, the released N-acetyl glucosamine in reaction mixture was estimated by the dinitrosalicylic (DNS) method described by Miller (1959). One unit (U) enzyme activity was defined as the amount of enzyme, which produced 1 μmole of N-acetylglucosamine per minute. Protease activity was measured by the method of Kembhavi et al. (1993) using casein as a substrate. A 0.5 ml of the culture filtrate was mixed with 0.5 ml solution of casein (1% in 50 mM phosphate buffer, pH 7.0) and incubated for 15 min at 50 °C. The reaction was stopped by addition of 0.5 ml of trichloroacetic acid (20%; w/v). One unit enzyme activity was defined as the amount of enzyme, which produced 1 μg of tyrosine per minute during the hydrolysis reaction under standard assay conditions. Specific activity is given in units per mg protein.

Antagonistic activity of Trichoderma *strains* in vivo

Soil infestation with pathogens

Soil infestation with fungal pathogens was carried out according to the method described by Khalifa (2006). A mixture of 54 g wheat seed and 22 ml of distilled water was distributed in flasks of 250 ml and then autoclaved at 120 °C for 20 minutes. The mixture was inoculated with five discs (5 mm) from 7-day-old culture of the pathogen. The mixture was incubated at 25 °C under continuous light for 15 days. Wheat seeds sown at a rate of 5 grains per pot were covered with 2.5 g of the mixture then covered by autoclaved soil.

Wheat seed treatment by Trichoderma *strains*

This test was conducted following the technique described by Harman et al. (1989). Wheat seeds were treated by *Trichoderma* spp. spore suspensions at a concentration of 10^6–10^7 spores/ml and at rate of 1 ml/4 g of seed. The treated seeds were incubated for

24 h at 25 °C in the dark. Four pots were used for each treatment as well as controls. Two controls were used, a positive control with untreated wheat seeds sown in pathogen-infested soil and a negative control with untreated seeds sown in non-infested soil. Severity disease estimated by a disease scale ranging from 0 to 3 based on the presence of black or brown lesions extending on the crown surface: 0: no symptom; 1: 1 to 25%; 2: 25 to 50%; 3: More than 50%.

Results

In dual culture, all *Trichoderma* isolates exhibited significant inhibition on growth of pathogens compared to the control (Table 2). Among *Trichoderma* isolates tested, *T. harzianum* Thr.4 showed the strongest growth inhibition activity against *F. culmorum, F. graminearum* and *F. verticillioides* by 70.68%, 67.05% and 70.57%, respectively. In fact, isolates Thr.4, Thr.10 and Thr.8 of *T. harzianum* showed high potential antagonistic activity against the three pathogens, and inhibited their growth with a percentage higher than 60%. Low growth rate of the three pathogens tested compared to controls was observed in the presence of *T. viride* Tv.6. In addition, these isolates were able to overgrow and sporulate above *F. culmorum* colonies after 5 to 6 days of incubation but no overgrowth was observed with *F. graminearum* and *F. verticilliodes*. Microscopic examination of the interaction zone between antagonistic and pathogenic fungi revealed the coiling of

Table 2

Effect of *Trichoderma* spp. on colony diameter of *Fusarium* strains in dual culture after 7 days of incubation

Isolates	*F. culmorum*		*F. graminearum*		*F. verticillioides*	
	Colony diameter (mm)	% of reduction	Colony diameter (mm)	% of reduction	Colony diameter (mm)	% of reduction
Thr.1	37.00 de	55.42	40.00 de	54.02	41.33 h	46.32
Thr.2	38.33 e	53.81	36.00 c	58.62	32.66 d	57.58
Thm.3	42.00 fg	49.39	44.33 f	49.04	39.33 fg	48.92
Thr.4	24.33 a	70.68	28.66 a	67.05	22.66 a	70.57
Thr.5	33.33 c	59.84	37.66 cd	56.71	31.66 cd	58.88
Tv.6	30.00 b	63.85	35.66 c	59.01	30.66 c	60.18
Thm.7	41.33 f	50.20	51.66 h	40.62	40.33 gh	47.62
Thr.8	32.33 c	61.04	32.33 b	62.83	27.33 b	64.50
Thr.9	35.66 d	57.03	44.00 f	49.42	32.33 cd	58.01
Thr.10	28.66 b	65.46	31.33 ab	63.98	24.33 a	68.40
Thr.11	43.66 h	47.39	48.00 g	44.82	46.66 i	39.40
Thr.12	43.00 gh	48.19	43.66 f	49.81	31.33 cd	59.31
Tv.13	33.66 c	59.44	48.66 gh	44.06	37.66 f	51.09
Thr.14	41.00 f	50.60	41.33 ef	52.49	35.33 e	54.11
Control	83.00 i	–	87.00 i	–	77.00 j	–

Thr: *T. harzianum*, Thm: *T. hamatum*, Tv: *T. viride*

Values in the column followed by the same letter are not significantly different according to Tukey test at $P < 0.05$

Table 3

Effect of *Trichoderma* spp. on disease severity

Isolates	*F. culmorum*		*F. graminearum*		*F. verticillioides*	
	Disease index	Severity reduction	Disease index	Severity reduction	Disease index	Severity reduction
Thr.1	1.25 de	52.83	1.93 ef	35.66	1.69 de	41.52
Thr.2	1.60 g	39.62	2.40 g	20.00	1.45 d	49.82
Thm.3	0.99 c	62.64	1.17 c	61.00	1.10 c	61.93
Thr.4	0.27 a	89.81	0.48 a	84.00	0.30 a	89.61
Thr.5	1.05 cd	60.37	1.24 c	58.66	1.12 c	61.24
Tv.6	0.33 a	87.54	0.60 a	80.00	0.64 b	77.85
Thm.7	1.03 cd	61.13	1.15 c	61.66	1.14 c	60.55
Thr.8	0.98 c	63.01	1.24 c	58.66	1.15 c	60.20
Thr.9	1.56 fg	41.13	1.68 d	44.00	1.48 d	48.78
Thr.10	0.64 b	75.84	0.85 b	71.66	0.75 b	74.04
Thr.11	1.96 h	26.03	2.11 f	29.66	1.86 e	35.64
Thr.12	1.78 gh	32.83	2.01 ef	33.00	1.51 d	47.75
Tv.13	1.05 cd	60.37	1.24 c	58.66	1.13 c	60.89
Thr.14	1.35 ef	49.05	1.90 e	36.66	1.86 e	35.64
Control	2.65 i	–	3.00 h	–	2.89 f	–

Thr: *T. harzianum*, Thm: *T. hamatum*, Tv: *T. viride*
Values in column followed by same letter are not significantly different according to Tukey test at $P < 0.05$

the *Trichoderma* spp. around hyphae of the pathogenic fungus and finally segmentation of their mycelia, showing the process of mycoparasitism.

The efficiency of all *Trichoderma* spp. isolates under greenhouse conditions (pot trial) was evaluated during the first week of germination and 30 days after sowing. Disease incidence and severity of the seedlings treated with *Trichoderma* spp. were significantly less than the control inoculated only with pathogen (Table 3). Seed treatment with *T. harzianum* Thr.4 resulted in the highest percentage of disease decrease for the three pathogens, followed by *T. viride* Tv.6 and *T. harzianum* Thr.10. *F. graminearum* isolate was the most aggressive pathogen with disease index of 3.00, followed by *F. verticillioides* and *F. culmorum* with 2.89 and 2.65, respectively. The isolates Thr.5, Thr.8 (*T. harzianum*), Thm.3, Thm.7 (*T. hamatum*) and *T. viride* Tv.13 also strongly decreased disease index (by ~60%) for the three fungal pathogens. In addition, this study has shown variability in the antagonistic ability of the isolates belonging to *T. harzianum* and *T. viride* species compared to *T. hamatum* towards the same pathogenic agent.

The hydrolytic enzymes production by *Trichoderma* isolates was tested using cell walls of the three *Fusarium* pathogens as sole carbon source. Cellular walls of fungal pathogens were degraded by all *Trichoderma* isolates and the specific proteolytic and chitinolytic activities were calculated (Fig. 1). Highest enzyme activities were exhibited by Thr.4 and Thr.8 of *T. harzianum* followed by *T. hamatum* Thm.3 with *F. graminearum* cell wall. When Thr.4, Thr.8 and *T. hamatum* Thm.3 were grown in media amended with hyphal cell walls of *F. graminearum*, the levels of chitinase were 4.10, 3.75 and 3.22 U/mg,

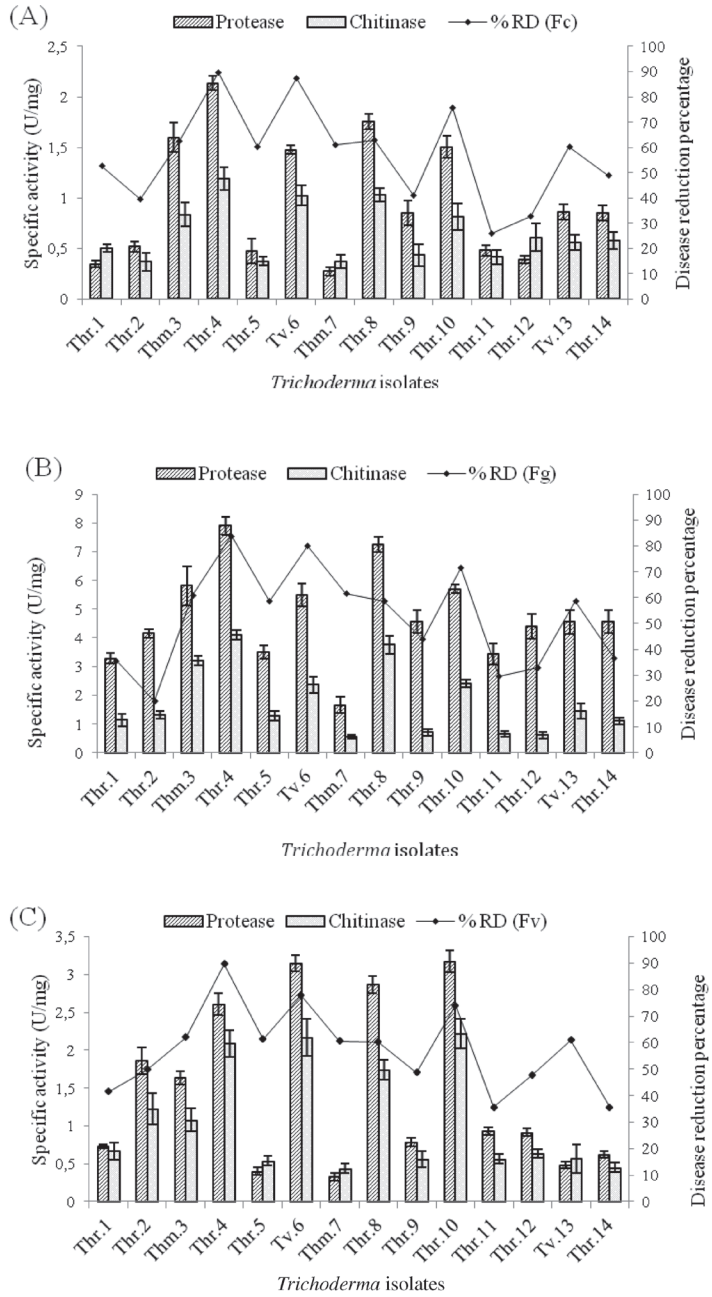

Fig. 1. Specific proteolytic and chitinolytic activities of *Trichoderma* isolates grown on SM supplemented with cellular wall of each fungal pathogen, (A): *F. culmorum*. (B): *F. graminearum.* (C): *F. verticillioides*. Thr. *T. harzianum,* Thm. *T. hamatum,* Tv. *T. viride*. *%* RD: Disease reduction percentage. Each result is the mean of three replicates ± SD

respectively. On the other hand, high level of proteolytic activity was shown by these isolates in the same medium, specific proteolytic activity of *T. harzianum* Thr.4 (7.90 U/mg) was also higher than *T. harzianum* Thr.8 (7.25 U/mg) and *T. hamatum* Thm.3 (5.81 U/mg). High lytic activities were also recorded for *T. viride* Tv.6 and *T. harzianum* Thr.10 in the medium amended with cell wall of *F. graminearum.* Both isolates produced maximal lytic activities when hyphal walls of *F. verticillioides* were used as sole carbon source. However, minimum enzyme activities were observed by the most of *Trichoderma* isolates with *F. culmorum* cell wall. The specific activities of protease and chitinase of *T. hamatum* Thm.7 were the lowest with the three fungal cell walls.

Discussion

In the present investigation *in vitro* and *in vivo* antagonistic activity of fourteen isolates of *Trichoderma* spp. against three causal agents of wheat *Fusarium* crown and root rot were studied. Dual culture experiments have shown highly significant growth reduction of pathogens compared to the controls. Nevertheless, isolates Thr.4, Thr.8 and Thr.10 of *T. harzianum* and *T. viride* Tv.6 were more effective than other isolates used for reducing growth of the three fungal pathogens. Furthermore, these isolates were able to overgrow and sporulate on *F. culmorum* colonies after 5 to 6 days of incubation, showing their highest mycoparasitic capacity towards this fungal pathogen. Similar phenomenon was described by Benhamou and Chet (1996) during confrontation between *T. harzianum* and soil born fungus, *Pythium ultimum*. However, *Trichoderma* spp. used in this study were not able to invade *F. graminearum* and *F. verticillioides* colonies with formation of lytic zones between the pathogen and *Trichoderma* spp. colonies. Formation of clear zones in dual cultures could be explained by the production of extracellular hydrolytic enzymes rather than production of growth inhibiting substances by *Trichoderma* spp. (Benhamou and Chet 1993; Haran et al., 1996). These isolates have a rapid growth compared to the other antagonists, especially the two isolates of *T. hamatum*. Competitiveness of *Trichoderma* spp. based on rapid growth and germination is a decisive feature for antagonism (Camporota, 1985; Chet et al., 1998).

Dual culture tests have also shown that all *Trichoderma* isolates were able to inhibit the mycelial growth of the three fungal pathogens prior to mycelial contact, indicating a possible production of diffusible components by the antagonists, such as lytic enzymes or water-soluble metabolites (Grondona et al., 1997; Anees et al., 2010). Previous studies have demonstrated that before mycelia of fungi interact, *Trichoderma* spp. produces low level of extracellular exochitinases (Kullnig et al., 2000; Brunner et al., 2003). The diffusion of these lytic enzymes dissolves cell fragments of pathogen cells. The degraded cell wall components from pathogens induced the production of further enzymes and the expression of gene related to mycoparasitism increasing the antagonism of *Trichoderma* spp. allowing them to be more antagonistic (Vinale et al., 2008).

The hydrolytic enzymes production by fourteen isolates of *Trichoderma* spp. in the presence of fungal pathogen cell walls as sole carbon source, indicates that these substrates can be used as inducers of lytic enzymes production. This result was also supported by Elad

et al. (1982), Sivan and Chet (1989) and Geraldine et al. (2013). Most of *Trichoderma* spp. isolates tested in this study were able to produce a high level of chitinase and protease when grown in the presence of *F. graminearum* cell wall, whereas cell walls of *F. verticillioides* and particularly *F. culmorum* were less effective as inducers of these two enzymes. Sivan and Chet (1989) have explained difference in cell wall degrading enzymes activities of *T. harzianum* against *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium* species by protein content in their cell walls, they have argued that cell walls of *Fusarium* species contain more protein (7–28%) than walls of other fungi. Chemical composition analysis indicates that the hyphal wall of *F. culmorum* contains more protein than the cell walls of *F. graminearum* with only 4.5% (Laborda et al., 1974; Barbosa and Kemmelmeier, 1993). Several studies have reported that chitinase and protease involved in mycoparasitism of *Trichoderma* strains play an important role in the destruction of the plant pathogens. These enzymes are responsible of breakdown of the host cell wall, since fungal cell walls contain chitin embedded in a protein matrix (Elad et al., 1982; Sivan and Chet, 1989; Peberdy, 1990).

The results obtained *in planta* tests by seed treatment with *Trichoderma* spp. have shown the effectiveness of all *Trichoderma* ssp. isolates used in this study on biocontrol of wheat crown and root rot. The highest disease index reduction was obtained with Thr.4 and Thr.10 of *T. harzianum* and Tv.6 of *T. viride* against the three fungal pathogens. Sivan and Chet (1986) reported that application of *T. harzianum* under greenhouse conditions resulted in a significant control of *F. culmorum* on wheat. Other investigations have also shown the effectiveness of these two species as biocontrol agents in reduction of *Fusarium* diseases on wheat (Dal Bello et al., 2002; Salehpour et al., 2005; Foroutan, 2013). In general, data obtained *in planta* bioassay were consistent with the clear inhibitory effects exhibited by the antagonists on pathogen growth in dual culture tests, and with their cell walls degrading enzymes activities. However, we cannot correlate high biocontrol capacity of disease with chitinase and protease specific activities because isolates, such as *T. harzianum* Thr.5, *T. hamatum* Thm.7, and *T. viride* Tv.13, showed important (≈ 60%) disease reduction percentage but low levels of protease and chitinase activities. Despite this finding, Elad and Kapat (1999) have shown a correlation between protease activity and biocontrol capacity of *T. harzianum* against *Botrytis cinerea* under greenhouse conditions. In addition, authors studies suggest that increased secretion of extracellular enzymes such as chitinase, β-1,3-glucanases and proteases should be key components of *Trichoderma* spp. action in biocontrol of phytopathogens in the field and may be used as markers to hasten the selection of new promising isolates (Benítez et al., 2004; Woo et al., 2006; Geraldine et al., 2013).

Seed treatment by the antagonist agents enables them to invade the roots and the rhizosphere colonization which is an important characteristic of antagonists of soil borne pathogens. Studies showed a variability in root colonization ability of *Trichoderma* spp., some strains can colonize only some sites located on the roots level (Metcalf and Wilson, 2001), but other can colonize the whole surface of the roots for several weeks (Thrane et al., 1997) or month (Harman, 2000). *Trichoderma* species showed a widespread capacity to act against pathogens through various mechanisms including antibiosis, mycoparasitism and rhizosphere competence. These species showed also their capacity to induce systemic resistance against various classes of plant pathogens including fungi, bacteria and viruses in a variety of plants (Harman, 2000; Howell, 2003; Harman et al., 2004).

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