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# Antagonistic effect of *Trichoderma harzianum* VSL291 on phytopathogenic fungi isolated from cocoa (*Theobroma cacao* L.) fruits

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In this study we evaluated the antagonism *in vitro* of *Trichoderma harzianum* strain VSL291 against 18 pathogens of cocoa fruits in dual culture. *T. harzianum* VSL291 inhibited the growth of the phytopathogenic fungi tested between 10.54 and 85.43%. The mycoparasitism of *Moniliophthora roreri* by *T. harzianum* VSL291 was studied by light and scanning electron microscopy. *T. harzianum* VSL291 hyphae grew in parallel with the hyphae of *M. roreri* and in some places these were united with the hyphae of the cocoa pathogen through small structures like apresorios that tangled in the pathogenic fungus preventing its growth. *T. harzianum* VSL291 produced lytic enzymes:  $\beta$ -1,3-glucanases, chitinases, proteases, xylanases and lipases, when grown in minimal medium, with fungal cell walls as the sole carbon source. The highest proteolytic activities detected in *T. harzianum* VSL291 broth with *M. roreri*, *Penicillium expansum* and *Byssoschlamys spectabilis* cell walls appear to be associated with increased activities of  $\beta$ -1,3 glucanases, chitinases, lipases, proteases and xylanases and biocontrol index derived from the experiments of confrontation. These results suggest that proteolytic enzymes according to their degree of induction could participate in the antagonistic effect of *T. harzianum* VSL291 against the fungi tested.

**Key words:** Antagonism, *Trichoderma harzianum*, mycoparasitism, phytopathogenic fungi

## INTRODUCTION

In the year 2010, the world wide cocoa was estimated in 3.7 millions of ton, of which Africa was the main producer with 2.51 millions of ton, Asia and Oceania 0.66 millions of ton and Latin America and the Caribbean with 0.51 millions of ton (FAO, 2004). However, in cocoa plantations a lot of wastes are made that generally remains scattered into the plantation and causing the

phytopathogen fungi propagation that affect the cacao plant and play an important role in the destruction of the important natural resources of the cocoa industry. In the last decades, the worldwide cocoa production was seriously affected by diseases caused by phytopathogenic fungi. For example, in tropical America the more important were the frosty pod rot caused by *Moniliophthora roreri* (Phillips-Mora et al., 2006) and the witches broom caused by *Crinipellis perniciososa* (de Marco et al., 2003). On the other hand, diseases caused by *Phytophthora* strains produce cocoa losses worldwide between 45 to 100% of the production (Djocogue et al., 2010). The cocoa plants cultivated in the Chontalpa sub region located in the state of Tabasco, Mexico, are

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**Abbreviations:** BCI, Biocontrol index; PDA, potato dextrose agar; SD, standard deviation

affected by frosty pod rot disease (Phillips-Mora et al., 2006), which cause losses higher than 80% (Phillips-Mora and Wilkinson, 2007). Many chemical products applied to control this disease are very effective in most of the case, but caused serious damage to the environment, soil, and humans (Lee et al., 2004). Other techniques of control are the plant management with cultural practices and the genetic resistance (Howell, 2003), but these techniques only reduce the incidence of fungal pathogens into the plantations.

*Trichoderma* strains are antagonistic to some phytopathogenic fungi because they have the ability to suppress the diseases they cause (Harman, 2006). *Trichoderma* uses several biocontrol mechanisms such as mycoparasitism, antibiosis, and competition for space and nutrients, and is also able to promote plant growth and development, and induce the defense response of plants (Shoresh et al., 2010). It has also been demonstrated that it has the ability to produce lytic enzymes that can act in a synergistic way increasing its antagonist action (Benitez et al., 2004). Several *Trichoderma* species have been extensively studied for their biocontrol potential of diseases in different crops (Harman et al., 2004). However, despite the importance of *M. royeri* in cocoa farming, little is known about the interaction of *Trichoderma*-cocoa phytopathogenic fungi (Samuels et al., 2006). The aim of this study was therefore to study the antagonistic effect of *T. harzianum* VSL291 on fungal plant pathogens isolated from cacao fruits with symptoms of disease.

## MATERIALS AND METHODS

### Fungal strains

The microbial strains used in this study included the following phytopathogenic fungi: *M. royeri*, *Phytophthora megasperma*, *P. capsici*, *Colletotrichum gloeosporioides*, *Fusarium solani*, *F. coeruleum*, *F. verticillioides*, *Corynespora cassiicola*, *Cochliobolus lunatus*, *C. hawaiiensis*, *Cladosporium cladosporioides*, *Byssosclamyces spectabilis*, *B. nivea*, *Penicillium chrysogenum*, *P. expansum*, *Rhizopus oryzae*, *Neurospora crassa* and *Aspergillus niger*. The strains were isolated previously from diseased tissue of cocoa fruits with symptoms of frosty pod rot and pod rot disease, from Huimanguillo, Tabasco State in Mexico. The antagonistic fungi *T. harzianum* strain VSL291 was isolated from the soil cultivated with *Agave tequilana* cv. 'Azul' in the State of Jalisco, Mexico (Sánchez and Rebolledo, 2010). All were obtained from the culture collection of the Genetic Laboratory of the Instituto Tecnológico de Veracruz.

### Confrontation experiments

The confrontation experiments of the interactions between eighteen phytopathogenic fungi and the antagonistic *T. harzianum* strain VSL291 was evaluated by using the technique described by Szekeres et al. (2006). Briefly, in Petri dishes with PDA medium, three-day-old *T. harzianum* mycelia discs of 5 mm in diameter were

put at equidistant points and each fungal pathogen was left to confront. The Petri dishes were incubated at 25°C in darkness and then the antagonist activities were recorded on day 7 of incubation and digital images were taken at a distance of 18 cm with a Cyber-shot DSC-P72. The percentage of inhibition of the growth of the pathogen fungi was calculated using the biocontrol index (BCI) according to the formula:  $BCI = [A / B] \times 100$  (Szekeres et al., 2006); where A is the area of the colony of *T. harzianum* and B is the total area occupied by colonies of *T. harzianum* and each pathogen fungus. The Image software (<http://www.ansci.wisc.edu/equine/parrish/index.html>) was used to calculate the area for the BCI. Data of inhibition were compared among the phytopathogenic fungi by ANOVA, followed by Tukey's tests as warranted, and inhibition areas values were compared among the phytopathogenic strains using 95% confidence intervals. All statistical analyses were conducted with the Statistix 9.0 software (Analytical Software, 2008).

### Mycoparasitic assays

In order to complement the macroscopic observations already observed, microscopic descriptions were made on the mycoparasitism exerted by *T. harzianum* on the fungal pathogen *M. royeri* known to be responsible for the greatest number of losses in the cocoa plantations in Mexico. To observe the region of interaction between *Trichoderma* and *M. royeri*, observations were made by scanning electron microscopy (SEM). Electron micrographs were taken at the Institute of Ecology (INECOL, Xalapa, Veracruz, Mexico). The samples analyzed came from the interaction zone of confrontation between pathogen and antagonist in the potato dextrose agar (PDA) medium. The processing of samples was done by the following method: agar cuts of 2 × 2 mm were prepared from the zone of interaction between *T. harzianum* and *M. royeri*. The samples were immersed in an aqueous solution of 1% agar and set at 4°C for 2 h by immersion in a dissolution consisting of glutaraldehyde (25%) to 3% in sodium cacodylate buffer, 0.1M at pH 7.2, followed by three washes with the same buffer for 30 min in the dark, then fixed by immersion in osmium tetroxide 1% for 2 h at 4°C in the dark. Dehydration was carried out in stages of 15 min with increasing ethanol dissolutions of 30, 50, 70, 90 and 100%, and dried by the critical point method (ethanol/CO<sub>2</sub> liquid). The cuts were mounted on a pedestal with graphite conductive paint and coated with gold by evaporation method and sputtering. The test was done with a SEM JEOL, JSM-5600LV scanning electron microscopy (Bozzola, 2007).

### Enzyme assays

To test the ability of *T. harzianum* VSL291 to grow and degrade cell walls of fungal pathogens, the following fungi were selected: *C. hawaiiensis*, *C. lunatus*, *C. gloeosporioides*, *C. cassiicola*, *C. cladosporioides*, *B. spectabilis*, *P. expansum*, *A. niger*, *F. verticillioides* and *M. royeri*. Fungal cell walls were prepared according to Vazquez-Garcidueñas et al. (1998). On 250 ml Erlenmeyer flask was placed 50 ml of minimum medium (MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.24 g; KCl, 0.24 g; NH<sub>4</sub>NO<sub>3</sub>, 1.2 g; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.0024 g; MgCl<sub>2</sub>·7H<sub>2</sub>O, 0.0024; K<sub>2</sub>HPO<sub>4</sub>, 1.08 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.0024 g; pH 5.5; for 1200 ml) and 1% of cellular wall of fungal pathogen. Medium was inoculated with a 5 mm mycelia disc of *T. harzianum* VSL291 and was incubated for 3 days (250 rpm, 30°C).

Also, enzymatic activities were measured every 24 h. The β-1,3-glucanase activity was measured using the method described by Miller et al. (1959). Using laminarin as substrate, one unit of enzyme activity was defined as the amount of enzyme that

produced 1 mmol of reduced sugar (using glucose as a standard) per ml per minute. Xylanase activity was determined according to Rawashdeh et al. (2005). Using xylan as substrate, one unit of enzyme activity was defined as the amount of enzyme required to release 1  $\mu$ mol of reducing sugar (using xylose as a standard) per ml per min. Chitinase activity was determined according to the method described by Monreal and Reese (1969). Using colloidal chitin as substrate, one unit of enzyme activity was defined as the amount of enzyme required to release 1  $\mu$ mol of reducing sugar (using N-acetylglucosamine as a standard) per ml per min. Protease activity was measured using the method described by Kunitz (1946). The enzyme activity was defined as the amount of enzyme required to release 1  $\mu$ g of tyrosine per ml per min. Lipase activity was measured using the method described by Nawani et al. (1998). The enzyme activity was defined as the amount of enzyme that produced 1 mg of p-nitrophenol per ml per ml. The protein content was estimated according to Bradford (1976). Statistical analyses of the data were done as aforementioned.

## RESULTS AND DISCUSSION

### Confrontation experiments

On the digital images, the colonies of *T. harzianum* VSL291 strain as well as the total areas occupied by the colonies of both *Trichoderma* and the cocoa tree pathogen were drawn around and measured by the ImageJ software with the use of freehand selection tool. During the analysis, the scale was set to 28.346 pixels per cm; accordingly the unit of the calculated areas was  $\text{cm}^2$ . The areas of *Trichoderma* VSL291 colonies were measured daily for one month and the changes of the occupied areas were followed (data not shown). *T. harzianum* VSL291 have control over most of the phytopathogenic fungi strains tested. An initial rapid increase of the *Trichoderma* VSL291 colonies was observed in most cases at the 5 to 7 days, while the areas did not change considerably during the following period and remained approximately the same from the seventh day to the end of the investigation. A zone of progressive inhibition produced by *T. harzianum* VSL291 against, *C. lunatus*, *C. gloeosporioides*, *C. cassicola*, *C. cladosporioides*, *F. verticillioides* and *M. roleri* was observed, while *R. oryzae*, *P. megasperma*, and *P. capsici* produced less inhibition.

The digital images taken on the seventh day were used to calculate the BCIs values. The average and the standard deviation (SD) values were calculated from the three replicate measurements for both areas; *T. harzianum* area and *T. harzianum* + phytopathogenic area (Table 1). The higher effect of *T. harzianum* on the growth of the phytopathogenic fungi were obtained with *P. chrysogenum*, *C. gloeosporioides*, *P. expansum*, *B. nivea*, and *C. lunatus*. The percentage of inhibition ranged from 76.37 to 85.43%. On the other hand, the lower inhibition effect was observed with *N. crassa*, *P. capsici*, *P. megasperma*, and *R. oryzae*, which ranged from 10.54 to 35.06%. The percentage of inhibition that

we found for a microorganism of the genus *Penicillium* (*P. chrysogenum*) was higher than that previously reported for the biocontrol of *P. digitatum* by *T. harzianum*. However, for *P. expansum* and *B. nivea*, our results were lower than those reported for the same microorganisms (Guédez et al., 2009). Quiroz et al. (2008) found that the highest inhibition of the growth of *Penicillium* sp. and *Fusarium* spp. were obtained when the confrontation experiments were carried out with the *Trichoderma* sp., strains RP-12b and ST-2 whose percentage of inhibition ranged from 70 to 100%. Those percentages of inhibition agreed with our results. For the microorganisms of the genus *Phytophthora* however, our results were lower to that obtained by other authors for biocontrol with *T. harzianum* strains (Aryantha and Guest, 2006; Villegas and Castaño, 1999). For the genus *Fusarium*, our results of BCI values agreed with that obtained by Suárez et al. (2008).

### Observation of mycoparasitism by scanning electron microscope

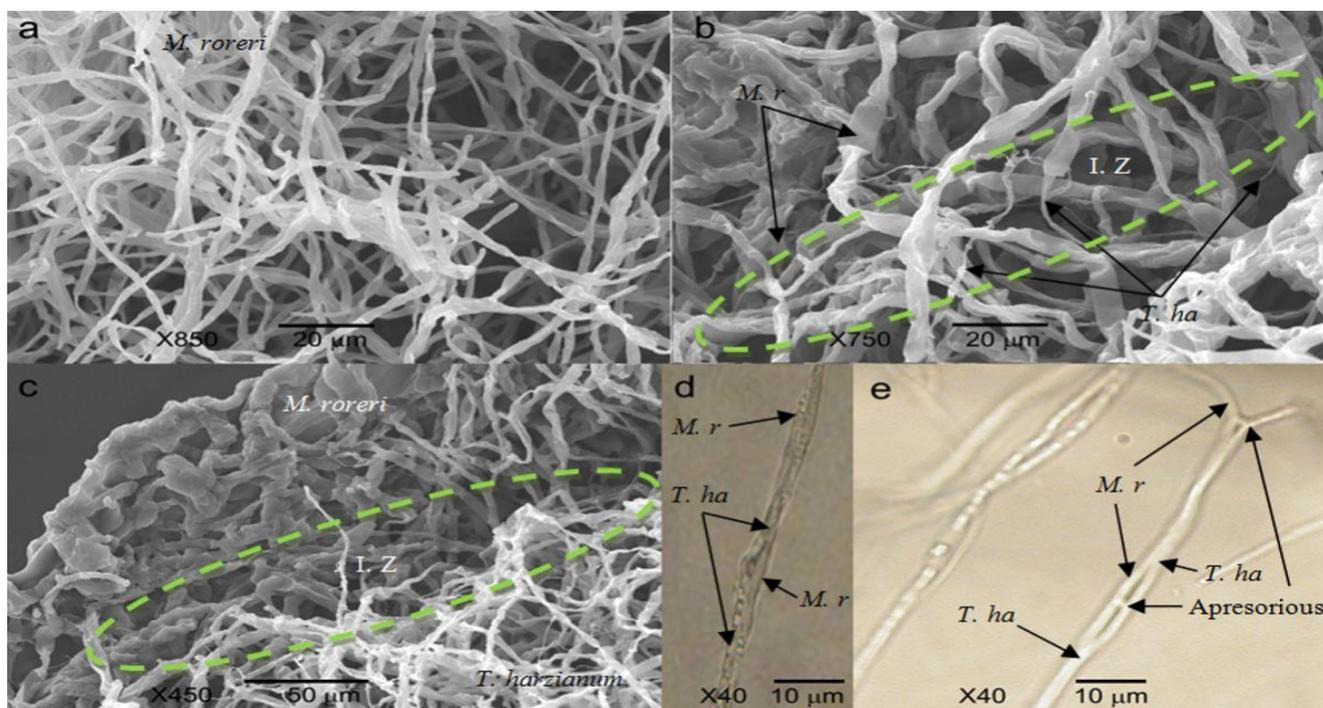
The mycoparasitism was analyzed by observing the preparations by scanning electron microscope. It was possible to observe the morphological structure and distribution of the hyphae of *M. roleri* in pure culture (Figure 1a) and the changes that these structures experienced when the pathogen fungi was confronted in dual culture with *T. harzianum* (Figure 1b and c). During the confrontation period in dual culture, the hyphae of *T. harzianum* grew on the hyphae of *M. roleri*, causing morphological deformations and disorganization in the structure of their cell wall so that its appearance becomes rough, probably due to the secretion of antifungal substances (enzymes and antibiotics) by *T. harzianum* (Figure 1b and 1c). The disintegration of mycelial walls resulted in the total destruction of the colony of *M. roleri*. Moreover, optical microscopy examination of the hyphae of *T. harzianum* in the interaction zone, showed that hyphal interactions exist, which would demonstrate a parasitic behavior of strain *T. harzianum* VSL291 on *M. roleri*. It envisioned the growth surrounding the hyphae of *T. harzianum* on *M. roleri* (Figure 1d), wrapping them either loosely or tightly. Also, it was observed that hyphae of *T. harzianum* normally grew in parallel to those of *M. roleri* (data not shown) and that at certain intervals were connected to them with small branches like apresorous (Figure 1e). These results demonstrate the particular parasitic ability of the antagonist that finally is able to inhibit the growth of the pathogen.

These morphological changes in the structure of the phytopathogenic fungi caused by *T. harzianum* were similar to those observed with other phytopathogenic fungi. Benhamou et al. (1999) reported some events of the mycoparasitism between *Pythium oligandrum* and

**Table 1.** Biocontrol index of *T. harzianum* VSL 291 (inhibition zone) against phytopathogenic fungi.

Phytopathogenic fungi	Area <sup>a</sup> <sub>T</sub> (cm <sup>2</sup> ) ± SD	Area <sup>b</sup> <sub>T+P</sub> (cm <sup>2</sup> ) ± SD	BCI <sup>c</sup>
<i>P. chrysogenum</i>	48.05 ± 2.31	56.24 ± 0.33	85.43 <sup>a</sup>
<i>C. gloeosporioides</i>	46.001 ± 0.55	56.44 ± 0.04	81.49 <sup>a</sup>
<i>C. hawaiiensis</i>	45.69 ± 0.25	56.35 ± 0.17	81.08 <sup>a</sup>
<i>P. expansum</i>	44.83 ± 3.74	56.45 ± 0.25	79.40 <sup>ab</sup>
<i>B. nivea</i>	44.31 ± 0.98	56.25 ± 0.32	78.7 <sup>ab</sup>
<i>C. cladosporioides</i>	43.19 ± 0.21	56.42 ± 0.08	76.56 <sup>ab</sup>
<i>C. lunatus</i>	43.01 ± 0.050	56.32 ± 0.03	76.3 <sup>ab</sup>
<i>C. cassicola</i>	42.26 ± 3.94	56.50 ± 0.13	74.81 <sup>ab</sup>
<i>F. verticillioides</i>	41.36 ± 0.94	56.35 ± 0.37	73.39 <sup>ab</sup>
<i>M. roleri</i>	40.83 ± 0.19	56.15 ± 0.17	72.72 <sup>ab</sup>
<i>F. solani</i>	39.17 ± 0.07	56.19 ± 0.08	69.71 <sup>ab</sup>
<i>F. coeruleum</i>	38.12 ± 0.36	56.48 ± 0.01	67.50 <sup>abc</sup>
<i>B. spectabilis</i>	32.87 ± 1.51	56.16 ± 0.16	58.53 <sup>bc</sup>
<i>A. niger</i>	26.93 ± 5.21	56.61 ± 0.02	47.57 <sup>cd</sup>
<i>N. crassa</i>	19.76 ± 10.12	56.45 ± 0.33	35.06 <sup>de</sup>
<i>P. capsici</i>	16.04 ± 0.71	56.08 ± 0.09	28.60 <sup>def</sup>
<i>P. megasperma</i>	11.65 ± 0.28	56.12 ± 0.13	20.77 <sup>ef</sup>
<i>R. oryzae</i>	5.91 ± 0.29	56.15 ± 0.18	10.54 <sup>f</sup>

<sup>a</sup> Area<sub>T</sub>: Area of the *Trichoderma* colony (blue point line in Figure 1); <sup>b</sup> Area<sub>T+P</sub>: total area occupied by the colonies of *Trichoderma* and pathogen fungi (yellow line in Figure 2); <sup>c</sup> Biocontrol index at day 7. Different letter within the column indicate significant differences ( $P < 0.05$ , ANOVA and Tukey's tests).



**Figure 1.** Scanning electron microscopy micrographs (a, b and c) showing mycoparasitic effect by *T. harzianum* VSL291 and optical microscopy images (d and e) on PDA medium: (a) Mycelia of *M. roleri* in pure culture; (b) and (c) *T. harzianum* parasitizes *M. roleri* inducing lysis and deformation of *M. roleri* hyphae (I. Z, interaction zone between *T. harzianum* and *M. roleri*); (d) Mycoparasitism by envelopment of *M. roleri* hyphae by *T. harzianum*, (e) Parallel growth of hyphae of *T. harzianum* with structures similar to aporesoriums on hyphae of *M. roleri*.

**Table 2.** Production of enzymes by *T. harzianum* VSL291 in minimum medium and fungal cell wall as carbon source.

Fungal cell wall	Enzyme specific activity <sup>a</sup>				
	$\beta$ -1,3-glucanases <sup>b</sup>	Chitinases <sup>b</sup>	Proteases <sup>b</sup>	Xylanases <sup>b</sup>	Lipases <sup>b</sup>
<i>C. hawaiiensis</i>	1.60 <sup>b</sup>	0.65 <sup>ab</sup>	0.28 <sup>bc</sup>	0.68 <sup>b</sup>	0.20 <sup>bc</sup>
<i>C. gloeosporioides</i>	0.65 <sup>cde</sup>	0.11 <sup>c</sup>	0.08 <sup>e</sup>	0.22 <sup>cd</sup>	0.11 <sup>bc</sup>
<i>C. cassiicola</i>	0.46 <sup>de</sup>	0.02 <sup>c</sup>	0.10 <sup>de</sup>	0.08 <sup>d</sup>	0.10 <sup>bc</sup>
<i>B. spectabilis</i>	1.44 <sup>b</sup>	0.32 <sup>bc</sup>	0.33 <sup>ab</sup>	0.67 <sup>b</sup>	0.12 <sup>bc</sup>
<i>C. cladosporioides</i>	0.52 <sup>de</sup>	0.07 <sup>c</sup>	0.27 <sup>bc</sup>	0.31 <sup>c</sup>	0.14 <sup>bc</sup>
<i>C. lunatus</i>	1.25 <sup>bc</sup>	0.01 <sup>c</sup>	0.20 <sup>cd</sup>	0.56 <sup>b</sup>	0.14 <sup>bc</sup>
<i>M. roreri</i>	3.22 <sup>a</sup>	0.89 <sup>a</sup>	0.42 <sup>a</sup>	1.82 <sup>a</sup>	0.60 <sup>a</sup>
<i>P. expansum</i>	1.10 <sup>bcd</sup>	0.26 <sup>bc</sup>	0.37 <sup>ab</sup>	0.74 <sup>b</sup>	0.27 <sup>b</sup>
<i>A. niger</i>	0.21 <sup>e</sup>	0.06 <sup>c</sup>	0.01 <sup>e</sup>	0.06 <sup>d</sup>	0.01 <sup>c</sup>
<i>F. verticillioides</i>	0.06 <sup>e</sup>	0.05 <sup>c</sup>	0.04 <sup>e</sup>	0.19 <sup>cd</sup>	0.29 <sup>b</sup>

<sup>a</sup>Specific activity measured at 24 h of incubation; <sup>b</sup>mU/mg of protein experiments by triplicate. Different letter within the column indicate significant differences ( $P < 0.05$ , ANOVA and Tukey's tests).

other phytopathogenic oomycetes (*Rhizoctonia solani*, *F. oxysporum*, *P. megasperma*, and *P. ultimum*). The damages made on the different fungal structures of the pathogenic fungus were related with the presence of increased sizes of cells derives from the disorganization of the cytoplasm, retraction and rupture of the plasmatic membrane and the alteration and distortion of the cell wall in the place of penetration of the antagonist. All these action therefore trigger the massive colonization of the pathogen and causes cellular lyses.

### Mycoparasitism simulated experiments

*T. harzianum* VSL291 was grown in minimum medium and fungal cell walls as sole carbon source.  $\beta$ -1,3-glucanase, chitinase, protease, xylanase and lipase activity were detected in the culture medium. In general, the maximum enzyme activity was detected at 24 h and the level of enzymatic activity produced by *T. harzianum* depended on the phytopathogenic cell wall (Table 2). *T. harzianum* VSL291 broths with cell walls of *M. roreri*, *C. hawaiiensis* and *B. spectabilis* had the highest  $\beta$ -1,3-glucanase specific activity (3.22, 1.60 and 1.44, respectively); *C. gloeosporioides*, *A. niger*, and *F. verticillioides* had the lowest specific activity (0.65, 0.21 and 0.06, respectively); *C. lunatus*, *P. expansum*, *C. cladosporioides*, and *C. cassiicola* had intermediate specific activity (1.25, 1.10, 0.52, and 0.46, respectively). In the same way the highest chitinolytic activity was detected in *T. harzianum* VSL291 broth with *M. roreri*, *C. hawaiiensis* and *B. spectabilis* cell walls (0.89, 0.65 and 0.32, respectively); *C. gloeosporioides*, *C. cladosporioides*, *A. niger*, *F. verticillioides*, *C. cassiicola* and *C. lunatus* had the lowest specific enzyme activity (0.11, 0.07, 0.06, 0.05, 0.02 and 0.01, respectively); *P.*

*expansum* had intermediate specific activity (0.26). The highest proteolytic activity was detected in *T. harzianum* VSL291 broth with *M. roreri*, *P. expansum*, *B. spectabilis*, *C. hawaiiensis* and *C. cladosporioides* cell walls (0.42, 0.37, 0.33 and 0.28, respectively); *C. cassiicola*, *C. gloeosporioides*, *F. verticillioides* and *A. niger* had the lowest specific enzyme activity (0.10, 0.08, 0.04 and 0.01, respectively); and *C. lunatus* had intermediate specific activity (0.20). The highest xylanolytic activity was detected in *T. harzianum* VSL291 broth with *M. roreri*, *P. expansum*, *C. hawaiiensis* and *B. spectabilis* cell walls (1.82, 0.74, 0.68 and 0.67, respectively); *F. verticillioides*, *C. cassiicola* and *A. niger* had the lowest specific enzyme activity (0.19, 0.08 and 0.06, respectively); *C. lunatus* and *C. cladosporioides* had intermediate specific activity (0.56 and 0.31, respectively). Finally, the highest specific lipolytic activity was detected with *M. roreri*, *F. verticillioides*, *P. expansum* and *C. hawaiiensis* cell walls (0.60, 0.29, 0.27 and 0.20, respectively) and *C. lunatus*, *C. cladosporioides*, *B. spectabilis*, *C. gloeosporioides*, *C. cassiicola*, and *A. niger* (0.14, 0.14, 0.12, 0.11, 0.10 and 0.01, respectively).

Previous studies have shown that *T. harzianum* chitinases and glucanases activities were induced when the cultures were supplemented with cellular walls of *Sclerotium rolfsii* (Elad et al., 1982), *F. oxysporum*, *R. solani* (Sivan and Chet, 1986), *Botrytis cinerea* (Schirmbock et al., 1994) and *C. perniciososa* (de Marco et al., 2003). On the other hand, Suárez et al. (2005) using the strain CECT 2413 of *T. harzianum* grown with cell walls of *B. cinerea*, *R. solani* and *P. ultimum* as a sole carbon source, found that *T. harzianum* proteomic response varies both qualitatively and quantitatively on the different fungal cell walls, suggesting that *T. harzianum* is able to modify the production of these proteins according to the fungal host. Our results are

consistent with the results of these groups, *T. harzianum* VSL291 induced the synthesis of enzymes according to the cell wall of fungi tested. Our results of  $\beta$ -1, 3-glucanase activities of cell wall tested are similar to those obtained by Sivan and Chet (1989) with mycelia of *R. solani* and by de la Cruz et al. (1995). This group reported activities of 0.700 to 1.0 mU/ $\mu$ g protein for cell walls of *B. cinerea*, *Gibberella fujikuroi*, *R. solani*, *Phytophthora citrophthora*, and *Saccharomyces cerevisiae* using a strain of *T. harzianum*. However, Vazquez-Garcidueñas et al. (1998) reported  $\beta$ -1, 3-glucanase specific activities from 1 to 27 mU/ $\mu$ g protein in 48-h *T. harzianum* broth with fungal cell walls of *Macrophthalmus rouxii*, *Neurospora crassa*, *R. solani*, and *S. cerevisiae*.

On the other hand, Küçük, and Kivanç (2008) obtained chitinolytic activities values similar to those obtained by us, using *T. harzianum* strains grown in liquid cultures containing *Gibberella zeae* and *Aspergillus ustus* cell walls as sole carbon source. The values obtained in our study for *A. niger* and *F. verticillioides* were lower to that reported by this group. In another study, Rey et al. (2000) using wild and modified strains of *T. harzianum* grown in cell walls of *S. cerevisiae* and *B. cinerea* as sole carbon source obtained higher chitinolytic than that obtained in our study. Xylanase and lipase activities detected in this study are low probably because the concentration of xylan and lipids in the cell walls are zero or very low and hence the induction is low. To the best of our knowledge there are no xylanase and lipase activities reported by *Trichoderma* using fungal cell walls as sole carbon source, probably because the concentrations of xylan or lipids in the cell walls are very low. Levels of xylanase activity of our work ranged from 0.08 to 1.82 mU/mg protein, significantly lower than those reported from xylan as carbon source ranging from 206 to 24 400 mU/mg protein (Stricker et al., 2006; Seiboth et al., 2003). With regard to lipase, values obtained in this study ranged from 0.01 to 0.60 mU/mg protein, significantly lower than that reported by Kashmiri et al. (2006) from olive oil as carbon source. The results of the low levels of induction of lipolytic and xylanase enzymes by *T. harzianum* VSL291 are congruent because these enzymes are inducible by xylan and lipid concentration and those in cell walls of fungi are low (Sentandreu et al., 2004).

More also, we chose the cell walls of fungi as a carbon source and measured products released to determine if the amount of products released by the hydrolysis of cell walls could be related to its BIC. We found no correlation between BCI and the degree of enzyme induction (data no shown), probably because in confrontation experiments there are other control mechanisms such as production of antibiotics (Tijerino et al., 2011) and competition for nutrients (Elad et al, 2000). However, our results show that the cell walls of strains *M. roreri*, *P. expansum*, *B. spectabilis* and *C. hawaiiensis* have the highest proteolytic activity in the *T. harzianum* broth.

Interestingly, *M. roreri*, *P. expansum*, and *B. spectabilis* also have high BCI (Table 1). These results therefore show that proteolytic activity of *T. harzianum* VSL291 could play an important role in mycoparasitism. We suggest that *T. harzianum* VSL291 proteases could expose other components of the fungi cell wall, inducing the expression of other enzymes and consequently increasing the antifungal activity. In relation to fungi cell walls that had low levels of proteolytic activity we suggest that the affinity for the substrate and the degree of exposure to cell wall proteins of *T. harzianum* VSL291 proteases could have influenced these low activity.

These results are consistent with other reports. Suarez et al. (2004) have shown that protease PRA1 from *T. harzianum* CECT2413 has different degrees of expression according to the substrate used and has additive or synergistic effects with other proteins produced during the antagonistic activity. Howell et al. (2003) have attributed in part the action of proteases produced by *Trichoderma* strains to inactivate hydrolytic enzymes produced by pathogens. Benitez et al. (2004) also reported that alkaline protease *Prb1* from *T. harzianum* IMI 206040 plays an important role in biological control and *Prb1* transformants showed an increase of up to five-fold in the biocontrol efficiency of *Trichoderma* strains against *R. solani*. On the other hand Sivan and Chet (1989) reported that the cell walls of the *Fusarium* species contain more proteins than the cells of other fungi and this makes it difficult for the degradation of their cell wall. In general, our results therefore show that the cell walls that induced in *T. harzianum* VSL291 higher levels of proteolytic activity also induced a higher activity of other enzyme activities tested.

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