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Changes in *Trichoderma asperellum* enzyme expression during parasitism of the cotton root rot pathogen *Phymatotrichopsis omnivora*

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ABSTRACT

The present study determined the activity of hydrolytic enzymes and the gene expression during direct interaction of the novel*Trichoderma asperellum*strains TC74 (high antagonistic capacity) and Th1 (low antagonistic capacity) with the plant pathogenic fungus*Phymatotrichopsis omnivora*. The*T. asperellum*strains produced the lytic enzymes endochitinase, N-acetylglucosaminidase and β-1,3-glucanase when grown on two different carbon sources. The response of strain TC74 was more rapid than that of strain Th1. When directly exposed to*P. omnivora*, the*T. asperellum*strains expressed one endochitinase and one N-acetylglucosaminidase, as shown by RT-PCR experiments. The strains also expressed two β-1,3-exoglucanases (designated as exg290 and exg343). TC74 and Th1 were able to express their chitinases and β-1,3-exoglucanase activities when grown on the different carbon sources tested. When the*T. asperellum*strains were grown in the presence of*P. omnivora*, qRT-PCR experiments revealed that mycoparasitism-related genes were first expressed prior to contact between the antagonist and the pathogen's mycelium. As a general response, the transcription level of these genes was increased at the post-contact stage. Although TC74 and Th1 both express the assayed genes when grown in direct contact with*P. omnivora*, significant differences in the time, intensity and kinetics of the response were observed.

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Introduction

The plant pathogenic fungus, *Phymatotrichopsis omnivora* (Duggar) Hennebert is a major pathogen of several crops, including pecans (*Carya illinoensis*), cotton (*Gossypium* spp.), alfalfa (*Medicago sativa*), peanuts (*Arachis hypogaea*), apples (*Mallus domestica*), peaches (*Prunus persicae*) and ornamental trees and shrubs (Uppalapati et al., 2010). Each year, *P. omnivora* causes significant economic losses in northern Mexico and the southern United States. In the United States, this pathogen causes an average of $100 million in annual losses for the cotton crop alone (Marek et al., 2009). In Mexico, one million dollars in annual losses were estimated due to the reduction of the nut yield from affected pecan trees (Samaniego-Gaxiola, 2009). The cost of replanting and treating infested trees also contributes to the total losses. Current estimates indicate that approximately 350 000 pecan trees could be infected with *P. omnivora* in northern Mexico.

Various approaches, including deep tillage, flooding, and root barriers, have been implemented to control *P. omnivora* and reduce its dissemination (Torto-Alalibo et al., 2005). A wide range of chemical control measures has also been tested with some success (Isakeit et al., 2007). However, this approach necessitates the injection of large quantities of chemicals into the soil, making it expensive and therefore not commercially feasible. Such is the case for the application of systemic fungicides such as benzimidazoles and sterol biosynthesis inhibitors; although both of these have been shown to reduce the incidence of root rot (Matheison & Lyda, 1984; Whitson & Hine, 1986), their use is relatively expensive, and they exhibit poor basipetal translocation to the roots and poor persistence and penetration in the soil.

Several antagonists of *P. omnivora* have been suggested as candidate biological control agents for the pathogen (Samaniego-Gaxiola, 2009; Uppalapati et al., 2010). As an example, fungi in the genera *Trichoderma* have been an interesting studied microorganism (Samaniego-Gaxiola, 2008). Several native *Trichoderma* strains have displayed significant biocontrol efficiency against soil-borne pathogens such as *P. omnivora* (Harman, 2006). In previous studies, *Trichoderma asperellum* strain TC74 was shown to have a significant antagonistic capacity against *P. omnivora* while strain Th1 was deficient in antagonism (Guigón-López et al., 2010b). Why some strains are efficient in this regard and others are not remains unknown. The antagonistic activity of strain TC74 against *P. omnivora* was shown to be primarily related to mycoparasitism of both mycelium and sclerotia (Guigón-López et al., 2010a). Mycoparasitism, one of the most important currently used biocontrol mechanisms, involves direct parasitism of one fungus by another by means of a complex sequence of events that includes sensing the presence of the prey, attachment to the prey's hyphae, defense responses of mycoparasitic fungus and killing of the prey (Druzhinina et al., 2011). Many genes that encode hydrolases and oligopeptides are specifically expressed before and during contact with the prey. The hydrolases, which are expressed sequentially, include enzymes that degrade the fungal cell wall (Seidl et al., 2009). For *T. asperellum*, the activity of various enzymes including chitinase (Viterbo et al., 2002a; Sanz et al., 2004) and β-1,3-glucanase (Elad et al., 1983; Bara et al., 2003) has been described. Gene regulation of chitinases and glucanases in *T. asperellum* has also been studied. *T. asperellum* possesses two N-acetyl-D-glucosaminidase (NAGase) genes and two endochitinase genes. The NAGase genes *exc1y* and *exc2y* are upregulated by glucosamine, whereas the endochitinase gene *chit36Y* is regulated by nitrogen and glucose levels (Viterbo et al., 2002b). Expression of the endochitinase gene *ech42* is induced by carbon depletion and/or by stress (Mach et al., 1999).

Although mycoparasitism is a key mechanism used in the biocontrol of *P. omnivora*, the process has not been studied during the interaction of this species with *T. asperellum*. Because studies of the changes in *T. asperellum* gene expression during direct confrontation with *P. omnivora* are lacking, the present study was conducted to determine the activity of hydrolytic enzymes and to measure enzyme gene expression during direct interaction of *T. asperellum* and *P. omnivora*.

Materials and methods

Microorganisms and culture conditions

The fungus *Phymatotrichopsis omnivora* was isolated from root-rotted pecan (*Carya illinoensis*) Wangenh. (Koch) trees. Two *Trichoderma asperellum* strains, TC74 and Th1 (Fig 2A), which differ in efficiency as antagonists (Guigón-López et al., 2010b), were used in this study. These strains are the property of the Natural Resource Research Center (Lopez, Chihuahua, Mexico) and Temperate Zone Microorganisms collection (CIAD, Cuauhtemoc, Chihuahua, Mexico), respectively. For enzyme assays, *Trichoderma harzianum* T22 (ATCC 20847) was used as a reference strain.

Two different media based on minimal medium (MM) (0.680 gL^{-1} of KH_2PO_4, 0.870 gL^{-1} of K_2HPO_4, 1.0 gL^{-1} of NH_4NO_3, 0.2 gL^{-1} of $CaCl_2$, 0.2 gL^{-1} of $MgSO_4.7H_2O$, 0.2 gL^{-1} of $MnSO_4$, 0.2 gL^{-1} of $FeSO_4$, 0.2 gL^{-1} of $ZnSO_4$) were used to assay enzyme production. These media were (1) MM-AF: MM + 0.5 % lyophilized basidiocarps of *Agaricus bisporus* and 0.3 % cereal fiber (waste products of wheat flour) and (2) MM-PO: SM + 0.5 % dried powdered *P. omnivora* sclerotia.

Enzyme assays

Trichoderma asperellum strains were pre-cultured in potato-dextrose broth (PDB) by inoculating 100 mL of medium with 10^6 conidia and incubating the mixture for 3 d at 25 °C with shaking at 150 RPM. The mycelium was then collected by centrifugation for 13 min at 4°C, 2880 ×*g* and transferred to 250-mL Erlenmeyer flasks containing 100 mL of MM-AF or MM-PO medium. The cultures were incubated at 25 °C with shaking at 150 RPM, and 14-mL samples were removed at various times: 4, 6, and 10 after inoculated days (aid) for cultures in MM-AF and 3, 5, and 10 aid for those in MM-PO. The samples were centrifuged for 13 min at 2880 ×*g*, and the supernatant was transferred to 15-mL tubes. The crude extracts were stored at −4 °C until use.

N-acetylglucosaminidase (NAGase) activity was determined according to (Lorito et al., 1994) using 10 μg μL^{-1} *p*-

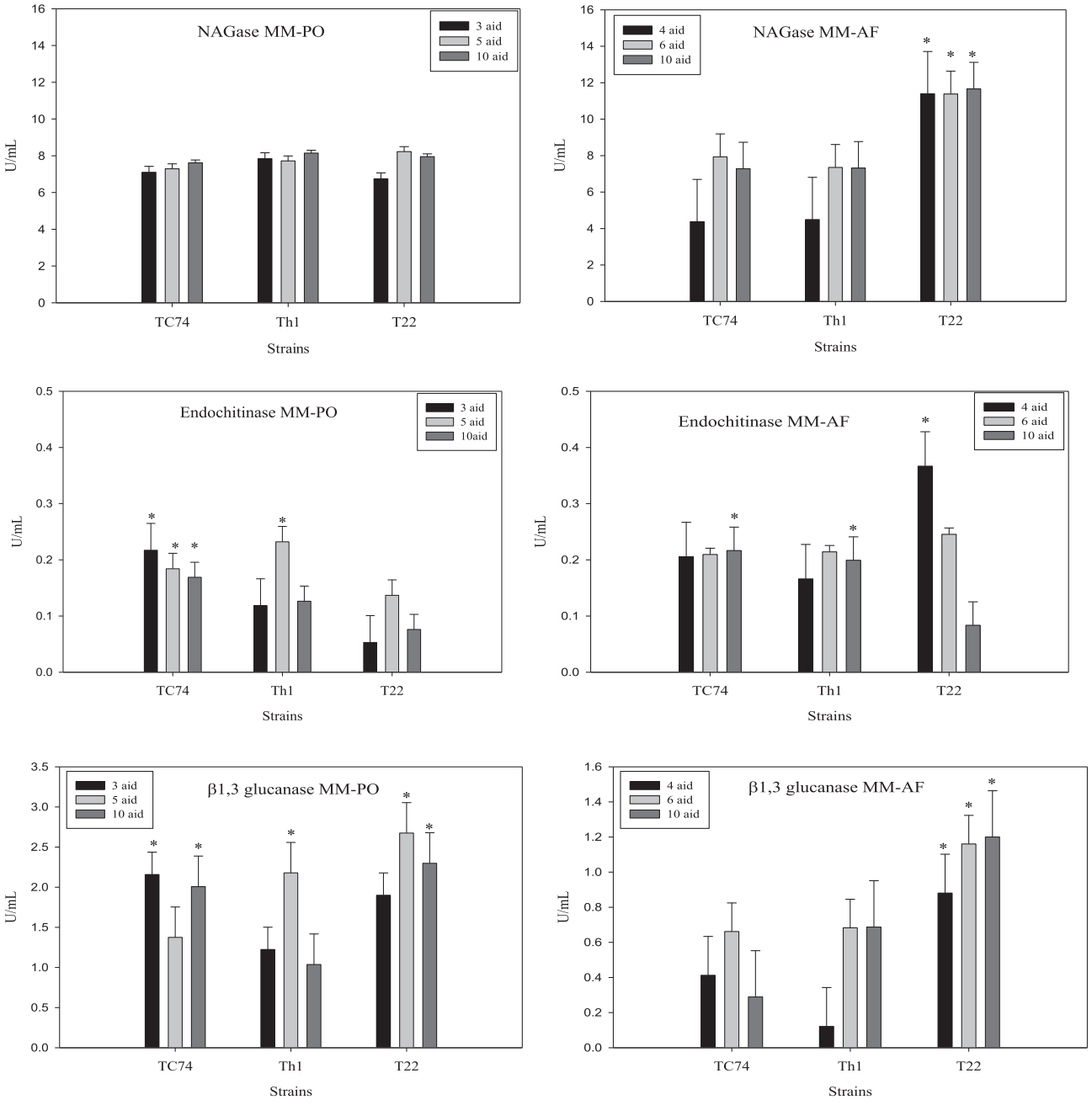

Fig 1 – Enzymatic activity of *T. asperellum* growth in different substrates: in *P. omnivora* powdered sclerotium (MM-PO) and in *A. bisporus* plus fiber (MM-AF). Vertical bars indicate mean standard error. The statistical significance (Tukey $P < 0.05$) among the same after inoculated days (aid) is shown by stars.

nitrophenyl-β-D-N-acetyl-glucosaminide (Sigma, St. Louis, MO) as a substrate. Briefly, the substrate was dissolved in 50 mM phosphate buffer (pH 6.7); 30 μL of this solution was then placed in each well of a 96-well microplate, and 60 μL of culture filtrate was added to each well. The plate was incubated at 50 °C for 30 min, and the reaction was stopped by adding 30 μL of 0.4 M Na_2CO_3 before determining the absorbance of the solution at 405 nm using a Microplate Reader 550 (Bio-Rad). Glucanase activity was determined using the same method (Lorito et al., 1994) with *p*-nitrophenyl-β-D-glucopyranoside (Sigma, St Louis, MO) as a substrate and incubating the reaction at 37 °C for 30 min. One nanokatal of NAGase or glucanase activity is equivalent to the liberation of 1 nmol of *p*-nitrophenol per second per milliliter under the conditions described. Endochitinase activity was determined by measuring the decrement in turbidity of a colloidal chitin solution at 10 mg mL^{-1} in 50 mM phosphate buffer (pH 6.7) (Lorito et al., 1994). Five hundred microliters of colloidal chitin solution were mixed with 500 μL of enzyme filtrate, and the mixture was incubated at 30 °C for 24 h with agitation (Thermomixer 5436 Eppendorf). The mixture was then diluted with 4 mL of distilled water, and its absorbance at

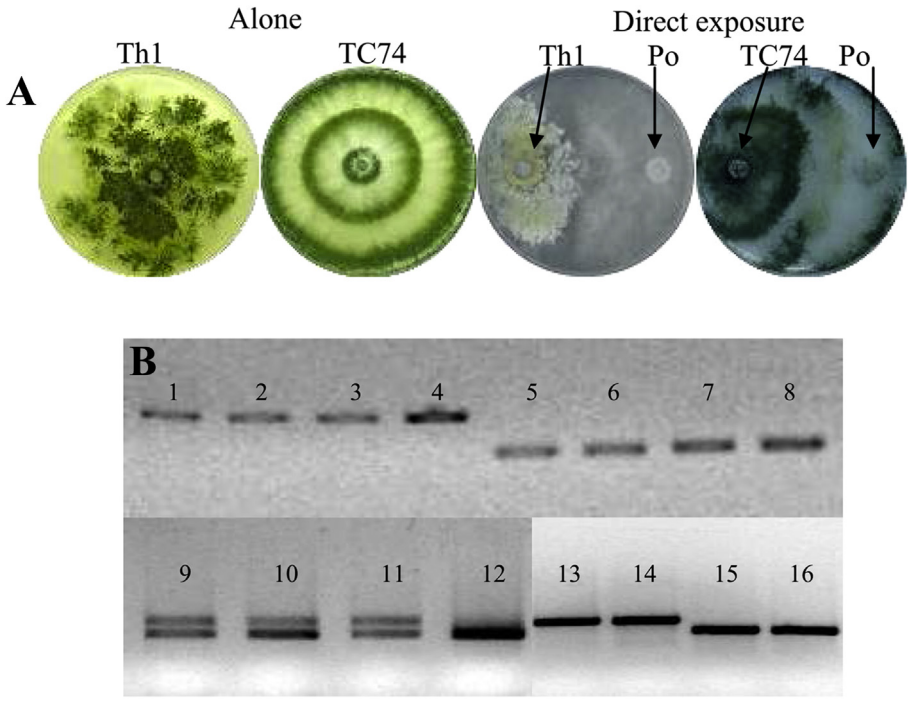

Fig 2 – (A) *T. asperellum* Th1 and TC74 growth alone and during direct exposure against *P. omnivora* (Po). (B) Amplified products during *T. asperellum* and *P. omnivora* interaction. Exochitinase: in sclerotium TC74 (1), Th1 (3); in confrontation TC74 (2), Th1 (4). Endochitinase: in sclerotium TC74 (5), Th1 (7); in confrontation TC74 (6), Th1 (8). β-1,3-exoglucanase: in sclerotium Th1 (9), TC74 (11); in confrontation Th1 (10), TC74 (12); purified: exg343 Th1 (13), TC74 (14); exg290 Th1 (15), TC74 (16).

510 nm was determined. One unit of endochitinase activity is defined as the amount of enzyme required to reduce the turbidity of a chitin suspension by 5 %. β-1,3-glucanase activity was determined as the amount of reducing sugar released after incubation of the enzyme filtrate with laminarin (Napolitano et al., 2006). Five hundred microliters of laminarin (1 mg mL^{-1} in 50 mM phosphate buffer pH 6.7) was mixed with 500 μL of culture filtrate, and the mixture was incubated for 2 h at 30 °C with agitation at 200 RPM followed by the addition of 500 μL of reactive copper solution and further incubation for 20 at 120 °C. Subsequently, 500 μL of ammonium arseniomolybdate was added, the volume of the sample was brought to 8.5 mL, and its absorbance at 510 nm was measured. The amount of reducing sugars released was determined as glucose equivalents based on the calibration curve (20, 40, 80, 120, 160, and 200 μg mL^{-1}). One unit of β-1,3-glucanase activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars per minute under the assay conditions.

Each determination was performed twice, with three replicates for NAGase and glucanase and four replicates for endochitinase and β-1,3-glucanase. The data obtained were subjected to analysis of variance (ANOVA) and Tukey's test ($P < 0.05$) using SAS software (SAS Institute, NC, USA).

Gene expression

Direct exposure

Exposure of TC74 and Th1 to *Phymatotrichopsis omnivora* was performed on solid MM supplemented with 0.3 % glucose; the cultures were covered with a cellophane membrane (Cortes et al., 1998). Disks of potato-dextrose-agar medium (PDA), 8 mm in diameter and containing mycelium, were obtained with a punch and placed directly on the solid MM containing the *P. omnivora* cultures. The disks were placed 5 cm apart with a cellophane membrane covering the pathogen mycelium and the antagonist fungus. For the RT-PCR experiments, the antagonist mycelium was collected at the stage of first contact between the mycelia. As a control, plates covered with a cellophane membrane and inoculated only with *Trichoderma asperellum* were used. For real-time RT-PCR experiments, the antagonist's mycelium was collected before contact (pre-contact), at first contact stage (contact) and 48 h after first contact (post-contact).

Substrate induction experiments

The *Trichoderma asperellum* strains (10^6 conidia mL^{-1}) were cultured in 25 mL of potato-dextrose broth (PDB) for 48 h at 28–30 °C with shaking at 150 RPM. The mycelium was then collected by centrifugation for 13 min at 2880 ×*g* and washed twice, first with a 2 % $MgCl_2$ solution and then with sterile distilled water. The mycelium was transferred to 50-mL tubes containing 25 mL of MM complemented with 0.5 % of the following substrates: laminarin, oat (containing 1, 3 and 1, 4-β-glucans, Megazyme™), barley (containing 1, 3- and 1, 4-β-glucans, Megazyme™) and dried powdered *Phymatotrichopsis omnivora* sclerotia. As before, gene expression was evaluated on MM containing 0.5 % and 2 % glucose. After 48 h of incubation, the mycelium was collected by centrifugation, washed and transferred to 50-mL tubes for RNA extraction.

RNA extraction and RT-PCR

Mycelium from *Trichoderma asperellum* strains growing in the treatment test plates was collected from the interaction zone after the cellophane membrane was removed. Hyphae collected by centrifugation were used for liquid culture. Each mycelium was ground in liquid nitrogen to a fine powder. Total RNA was extracted using Trizol™ (Invitrogen), following the manufacturer's instructions. The RNA concentration and quality were determined by spectrophotometry at 260 and 280 nm; only good-quality samples (as determined by ratio $A_{260nm}/A_{280nm} = 1.8-2.0$) were used. The cDNA synthesis was performed using Super Script III Reverse Transcriptase™ (Invitrogen) and 2 μg of total RNA according to the manufacturer's instructions. The cDNA was used as a template for the PCR runs. The primers (Table 1) utilized in these reactions were designed based on the analysis of sequences reported in the GenBank (NCBI) database. The actin primers used are described in (Ruocco et al., 2009). The PCR runs were performed under the following conditions: one cycle of 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing for 45 s and extension at 72 °C for 30 s, followed by final extension for 8 min at 72 °C. The annealing temperature was 59 °C for actin primers, 62 °C for enqui and exqui primers and 70 °C for exglu primers. Each reaction contained the following amounts of each component: 1 μL of cDNA (800–1900 ng/μL^{-1}, $A_{260nm}/A_{280nm} = 1.8-2.0$), 1 μL of primer mix, 12.5 μL of master mix, and nuclease-free water necessary to reach a volume of 25 μL. The chitinase and actin amplified products were purified using the PureLink PCR Purification Kit (Invitrogen) and sent for sequencing to the Genomic Analysis and Technology Core ARL-Biotechnology, University of Arizona, Tucson, Arizona, USA. Two isoforms of β-1,3-exoglucanase were cloned by TOPO TA Cloning™ (Invitrogen) and purified using a QIAprep Spin Miniprep Kit (Genelute Plasmid Mini-Prep Kit, Sigma) before being subjected to sequencing. Sequence analysis was performed using the BioEdit Clustal W and CAP programs.

Quantitative reverse transcription real-time pcr for gene amplification

The relative expression of the chitinase and glucanase genes during direct exposure of *Trichoderma asperellum* strains TC74 and Th1 to *Phymatotrichopsis omnivora* was measured by quantitative reverse transcription real-time PCR (qRT-PCR). The primers used in these relative expression experiments are shown in Table 1. For the *exglu*343 amplified product, the primers used were Fw: 5′- AGCTTGGCGACCGCAACTGG-3′ and Rv: 5′-AATATCGAGCTGGAGTTAAACTTAC-3′; these two primers amplified a ~230-bp product. The actin gene was used as a housekeeping gene. The conditions for the PCR runs were as follows: 94 °C for 10 min, 40 cycles of 94 °C for 30 s, and 70 °C for 1 min. Chitinase (endo- and exo-) annealing temperature was 62 °C and for β-1,3-exoglucanase was set at 70 °C. For *exglu*343, the PCR settings were 94 °C for 10 min, 40 cycles of 94 °C for 30 s and 57 °C for 45 s, and 72 °C for 30 s. The volumes of the PCR runs were 25 μL and 12.5 μL and included the SYBR Green qPCR Master Mix, 1 μL of primer mix, 1 μL of cDNA and the amount of nuclease-free water required for each volume. Each sample was run in triplicate, and analysis was performed by relative quantification with the actin gene as an internal control (Livak & Schmittgen, 2001). The data obtained were analyzed by ANOVA and Tukey's test ($P < 0.05$) using SAS software.

Results

Enzymatic activity

The enzymatic profile, and, therefore, the antagonistic potential of *Trichoderma asperellum* strains, was compared by their response to different substrates. MM-AF medium is a more complex substrate than MM-PO medium, a *Phymatotrichopsis omnivora* sclerotia-based substrate. The *T. asperellum* strains and the reference strain *Trichoderma harzianum* T22 produced the lytic enzymes N-acetylglucosaminidase (NAGase), endochitinase and β-1,3-glucanase when grown on the two carbon sources for various incubation times (Fig 1). The general response of TC74 was more rapid than that of Th1. The highest observed enzymatic activity differed depending upon whether the strains were cultured in MM-AF or in MM-PO.

The NAGase activity was similar for all strains with respect to the enzyme kinetic response. Just in MM-AF, T22 showed the highest response. Endochitinase production by TC74 and Th1 exceeded that of T22 in MM-PO, whereas in MM-AF medium this was only at 10 aid. β-1,3-glucanase activity was twice as high in cultures grown in MM-PO as in those grown in MM-AF. In TC74, the changes in enzyme activity with time differed from those in Th1 and T22.

Gene expression

In *Trichoderma asperellum*, the expression at least four different gene products was increased when the fungus was co-

Table 1 – Designed primers used for expression analysis of chitinases, glucanases and actin during direct confrontation of *T. asperellum* against *P. omnivora*.

Name	Primer	Sequence (5′→3′)	Long	Tm	GC %
Enqui	Forward	ATCAACGGAGGCGTTCCCGC	20	59.77	65.00
Long: 168 bp	Reverse	GACTGTAGCGCCGGCCTTGG	20	60.11	70.00
Exqui	Forward	TTCGGAGCGCATGCAGCCAA	20	59.97	60.00
Long: 294 bp	Reverse	ACGCGGGGCCAGCCAAATAC	20	60.04	65.00
Exglu	Forward	AGCTTGGCGACCGCAACTGG	20	60.25	65.00
Long: 290 bp	Reverse	GCGTTGCCCGCGCAATTCAT	20	59.84	60.00
Actin	Forward	GGTATGGGTCAGAAGGACT	19	57	52.63
Long: 750 bp	Reverse	CCTTTCGGACGTCGACATC	19	49	57.89

cultured with *Phymatotrichopsis omnivora*. One of the amplified products was ~160 bp in length and was related to an endochitinase (enq160), while another of the amplified products (290 bp) was related to an exochitinase (exq290) (Fig 2). On alignment with GenBank reported sequences, the sequences of the amplified products showed 94 %–98 % homology with *T. asperellum* endochitinase and *Trichoderma harzianum* exochitinase (Table 2). The expression responses for enq160 and exq290 were the same whether the two organisms were co-cultured on plates or grown in liquid MM medium with powdered sclerotia added.

The *T. asperellum* strains also expressed two β-1,3-exoglucanase-related products during direct co-culture with *P. omnivora* (Fig 2). One of these products corresponded to a 290- bp (exg290) species with 96 % homology to the *T. asperellum* β-1,3-exoglucanase gene (Table 2); the other, a 343-bp product (exg343), exhibited 97 % homology to the *H. lixii* (anamorph: *T. harzianum*) β-1,3-exoglucanase gene. The region between exg290 and exg343 was used to design new primers for subsequent exg343 expression.

The expression of exg290 and exg343 varied depending on the growth conditions. This difference was more pronounced for TC74 than for Th1. Under conditions of no antagonism (control), the two strains amplified the exg290 and exg343 products to similar levels; when the strains were grown in the presence of dried powdered sclerotium, the exg290 and exg343 transcripts were also expressed at similar levels. However, during direct co-culture with *P. omnivora*, the exg290 product in the TC74 strain was amplified more extensively, while exg343 expression was reduced to a minimum. To confirm these changes in the expression of β-1,3-exoglucanase, direct co-cultures of TC74 and Th1 with *P. omnivora* were again prepared, and co-cultures with the plant pathogenic fungi *Rhizoctonia solani* and *Botrytis cinerea* were also tested (Fig 3A). The results of these experiments confirmed that exg290 was amplified more extensively in the TC74 strain whereas both isoforms were amplified to similar levels in Th1; when Th1 was directly co-cultured with *R. solani*, the expression of exg290 was more intense than the expression of exg343. When *T. asperellum* was grown on either oat or barley 1–3, 1-4-β-glucans or on laminarin, both exg290 and exg343 were expressed at levels similar to those observed when it was grown on dried powdered sclerotium (Fig 3B). When growth occurred on 0.5 % and 2 % glucose, only exg343 was expressed; under these conditions, exg290 expression was inhibited.

RT-qPCR analysis

When grown under conditions involving direct exposure to *Phymatotrichopsis omnivora*, the TC74 strain responded more strongly than the Th1 strain; the response of the latter was slow and weak (Fig 4A). The expression of mycoparasitism-related genes was first detected at the pre-contact stage. As a general response, transcription levels were increased at the post-contact stage. The kinetic response of gene expression varied for the exg343 β-1,3-exoglucanase, the transcript level of which was higher at the mycelium-contact stage; this was followed by a low level of transcription in Th1 and a stage during which non-significant changes in expression occurred after this point in TC74. For strain TC74, endochitinase transcripts reached their highest level at the time of mycelium contact, followed by a significant decrease in endochitinase expression at the post-contact stage.

When transcript expression in the two strains was compared, it was found to be statistically higher in TC74 than in Th1 (Tukey $P < 0.05$) (Fig 4B). The expression of endochitinase was almost five times higher, NAGase almost four times, β-1,3-exoglucanase five times and exg343 three and a half times.

Discussion

Biocontrol of the plant pathogen fungus *Phymatotrichopsis omnivora* by application of *Trichoderma* spp. is a highly viable alternative to other methods for the control of this pathogenic fungus. Previous studies of the biocontrol of *P. omnivora* (Samaniego-Gaxiola, 2008) have focused on the identification of active strains and on the evaluation of antagonism *in vitro* and have reported contrasting results with the TC74 and Th1 strains, as found in this study. The species *Trichoderma asperellum* is widely distributed in Mexico and across America. However, despite the fact that it represents a highly viable alternative for the biocontrol of phytopathogenic fungi, it has not often been used in research. Because *T. asperellum* is one of the species that has been isolated and characterized by this research group as a biocontrol agent, the present study

Table 2 – Amplified products of *T. asperellum* when challenged against *P. omnivora* and their mycoparasitism related gene.

Amplified product	TC74			Th1		
	Gene	Species and GenBank no.	Homology (%)	Gene	Species and GenBank no.	Homology (%)
Endochitinase	*chit42*	AY265452.1 *T. asperellum* T203	98	*ech42*	AY665688.1 *T. asperellum* CPK810	96
Exochitinase	*exc1y*	AJ314642.2 *T. harzianum*	98	*exc1y*	AJ314642.2 *T. harzianum*	94
β 1, 3 Exoglucanase		EU314718.1 *T. asperellum*	96		EU314718.1 *T. asperellum*	96
β 1, 3 Exoglucanase		AJ002397.1 *Hypocrea lixii*	97		AJ002397.1 *Hypocrea lixii*	97
Actin	*Actin*	AM231150.1 *T. harzianum*	96	*Actin*	AM231150.1 *T. harzianum*	95

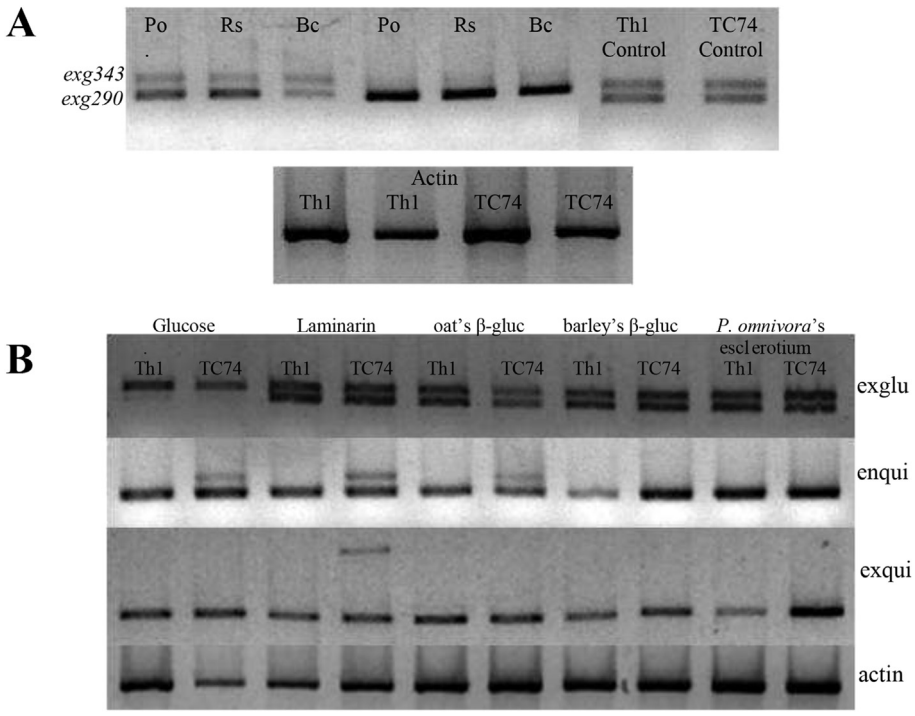

Fig 3 – (A) RT-PCR analysis of expression of β-1,3-exoglucanase exg290 and exg343 during direct exposure of *T. asperellum* against different plant pathogens fungi *P. omnivora* (Po), *R. solani* (Rs) and *B. cinerea* (Bc). (B) RT-PCR analysis of expression of *T. asperellum* enzyme genes in different substrates.

was undertaken in an attempt to understand the mechanisms by which *T. asperellum* exerts its mycoparasitic effects on *P. omnivora*. To achieve these objectives, two *T. asperellum* strains with different biocontrol capabilities were evaluated as antagonists; one of these, TC74, is an efficient biocontrol strain, while the other, Th1, is a defective biocontrol strain. These strains were selected based on the results of previous studies by our working group (Guigón-López et al., 2010a,b). With the goal of achieving a more thorough understanding of the biocontrol capabilities of the selected strains, various experiments were conducted.

The induction of chitinolytic and glucanolytic enzymes by *P. omnivora* was tested using powdered sclerotium as a substrate. Growth on MM-PO and MM-AF triggered the expression of chitinase and β-1,3-glucanase activities in both strains. However, the type of substrate affected the enzymatic activity. *P. omnivora* sclerotium triggered similar responses in TC74, Th1 and the reference strain *Trichoderma harzianum* T22, whereas when the cells were grown in MM-AF, the enzymatic activities of TC74 and Th1 were lower than that of T22. The induction of chitinase (NAGase and endochitinase) was similar on the two substrates, while the induction of β-1,3-glucanase was higher in MM-PO. The higher induction of β-1,3-glucanase when the strains were grown in MM-PO could be explained by the fact that glucans are an important part of the cell wall of the *P. omnivora* sclerotium; this could have changed the response of the strains with respect to enzyme activity (Coley-Smith & Cooke, 1971). The results suggest that β-1,3-glucanase activity might play an important role in *P. omnivora* sclerotia parasitism by *T. asperellum*. When MM-PO was used as the growth substrate, the pattern of enzyme production in the two strains was very similar. Thus, it appears that the two evaluated strains of *T. asperellum* have the potential to biocontrol *P. omnivora* through sclerotia parasitism. When grown in MM-PO, the endochitinase and β-1,3-glucanase activity of the strains reached a peak on d 5 and 3 after inoculation of the media, respectively, whereas the activity of NAGase remained constant from the third day onward. Enzymatic activity appeared to be induced more rapidly in TC74 than in Th1, but the highest level of activity detected in the culture filtrates was similar in the two strains. The results of the enzymatic activity assays therefore indicate more rapid induction of chitinase and β-1,3-glucanase activities in TC74 than in Th1 but do not provide sufficient evidence to conclude that TC74 exhibits overall higher enzyme production than Th1.

The results of the gene expression analysis clearly demonstrate that mycoparasitism of *T. asperellum* on *P. omnivora* is closely related to the simultaneous expression of chitinase and β-1,3-glucanase activities. Our results also confirm the nature of the induced protein responsible for the activity observed in the chitinase and β-1,3-glucanase enzymatic activity assays. Thus, it can be concluded from our study that the parasitic interaction of *T. asperellum* and *P. omnivora* involves the expression of at least one of the following genes: endochitinase, N-acetylglucosaminidase, and β-1,3-exoglucanase. A mixture of hydrolytic enzymes with different and/or complementary modes of action is necessary for maximally efficient antifungal activity (Viterbo et al., 2002a), and synergistic antifungal activity has been observed for

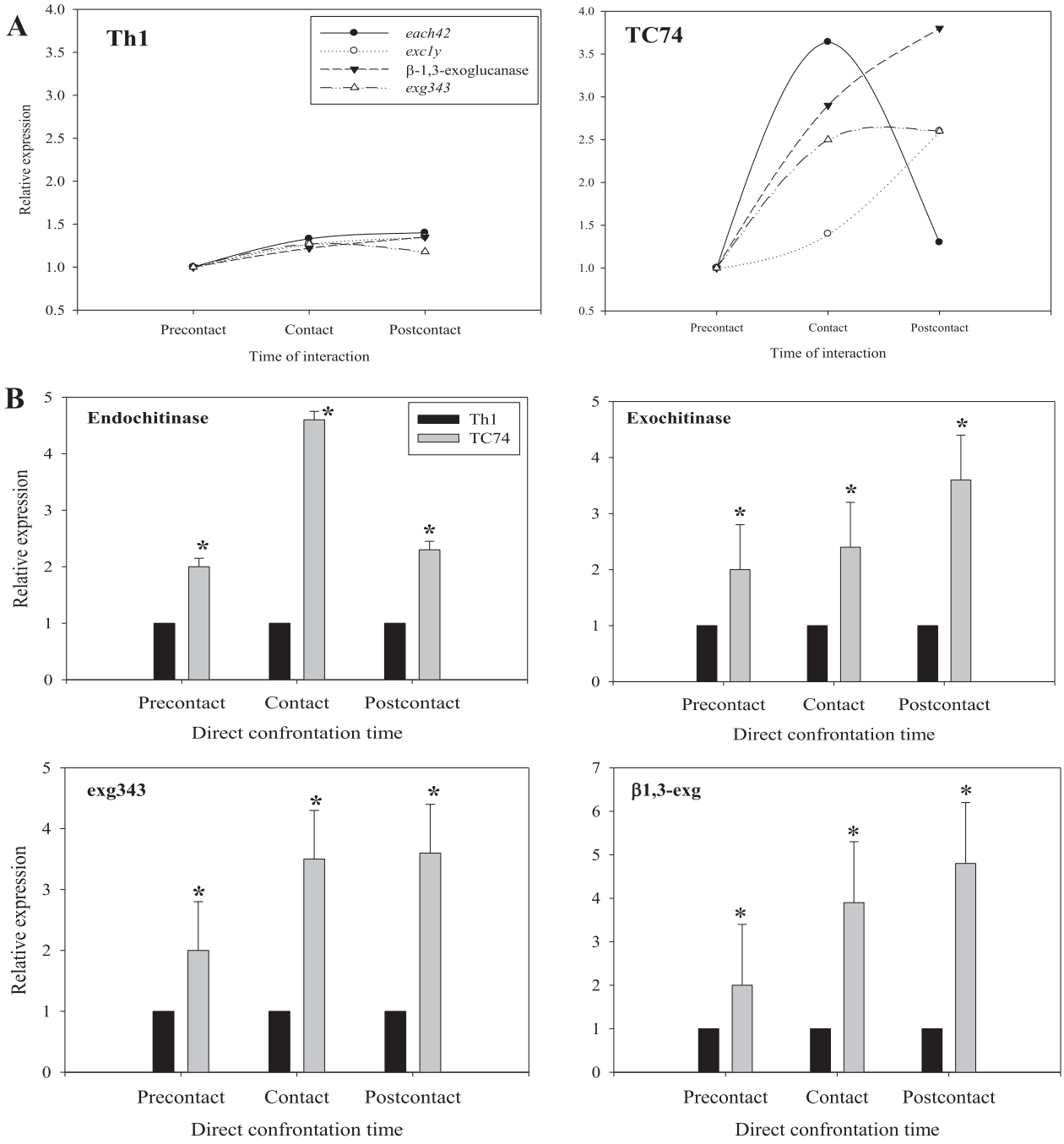

Fig 4 – Analysis of the chitinase and β-1,3-glucanase genes expression of *T. asperellum* strains during direct exposure to *P. omnivora* on solid plate MM supplemented with 0.3 % glucose. (A) Time-course of relative expression. (B) Relative expression of genes inTC74 regarding Th1. Stars indicate statistical significance (Tukey $P < 0.05$).

endochitinases in combination with NAGase (Lorito et al., 1993; Peterbauer et al., 1996), endochitinase and β-1,3-exoglucanase (El-Katatny et al., 2001) and NAGase and β-1,3-exoglucanase (Lorito et al., 1994).

Differences in the expression of hydrolytic enzymes, especially β-1,3-exoglucanases, were more easily observed during direct co-culture of the microorganisms on solid medium than in liquid medium. When grown in liquid medium, *T. asperellum* strains TC74 and Th1 were able to express their chitinases, both enq160 and exq290, in all carbon sources tested, including glucose. The levels of expression in the two strains were very similar. These strains also expressed their β-1,3-exoglucanases (exg290 and exg343) in all carbon sources tested, and the levels of expression in each strain showed minor variations that depended on the carbohydrate source used. These results differ from those previously reported for the expression of β-1,3-exoglucanases (Marcello et al., 2010). The latter authors observed differences in glucanase expression that they attributed to the levels of β-glucans present in the inducers. As is the case in most fungi, the *P. omnivora*

sclerotium cell wall primarily consists of β-1,3-glucans (Chet & Henis, 1975); therefore, the observed enzyme activity against dried powdered sclerotium (β-1,3-glucans), oat and barley β-glucans (β-1,3 and β-1,4-glucans) and laminarin (β-1,3 and β-1,6-glucans) indicates that the stimuli required for the induction of chitinases and exoglucanases in *T. asperellum* strains TC74 and Th1 are not highly specific. Likewise, no significant levels of transcription of exg290 were observed in the presence of glucose; however, the observed levels of transcription of exg343 were high. Although most β-1,3-glucanases produced by isolates of *T. harzianum* are repressed by glucose (De la Cruz et al., 1995; El-Katatny et al., 2001; Ramot et al., 2004), our results suggest that the synthesis of exg290, but not that of exg343, is controlled by carbon catabolic repression. The regulation of exg290 appears to be similar to that of *T. harzianum* and *T. asperellum* (El-Katatny et al., 2001; Ramot et al., 2004) and that of exg343 is similar to that of *Coniothyrium minitans* (Giczey et al., 2001).

During the direct co-culture of *T. asperellum* and *P. omnivora*, strains TC74 and Th1 expressed the same gene. This finding was confirmed when the strains were directly co-cultured against *P. omnivora*, *Rhizoctonia solani*, and *Botrytis cinerea*. However, significant differences were observed in the timing, intensity and kinetics of the gene expression. These differences appear to be related to the differences in parasitic capability between the two strains. Previous studies have shown that strain TC74 overgrows the mycelium and inhibits the growth of *P. omnivora*, *R. solani*, and *B. cinerea*, while Th1 is unable to do so (Guigón-López et al., 2010b).

At the pre-contact stage, chitinase and β-1,-exoglucanase expression was detected in both strains. This result indicates that chitinases and β-1,3-exoglucanase activities are triggered even before mycelium contact between an antagonist and its target occurs, a characteristic that gives *T. asperellum* a key advantage in parasitism of *P. omnivora*. The basal level of β-1,3-exoglucanase expression detected even before physical contact between host and parasite is consistent with the reported expression of the *tag83*, a β-1,3-exoglucanase gene of other *T. asperellum* strains (Marcello et al., 2010).

A characteristic of the Th1 response was that it was slow and weak. The kinetics of induction of the four genes was similar with the exception of exg343, which showed a slight variation. The response of TC74 was more intense, and this strain also exhibited similar induction kinetics for NAGase and β-1,3-exoglucanases; in contrast, for endochitinase the kinetics of gene expression were different, with a sharp decrease in gene expression after the mycelium contact stage. This reduction in gene induction might be due to a restricted concentration range over which the inducer is effective (Viterbo et al., 2002b). A large increase in the number of transcripts, occurring gradually over time, was detected in TC74 when an overgrown mycelium of *P. omnivora* was present. After physical contact between the two fungi occurred, TC74 β-1,3-exoglucanase was expressed at a level almost 5-fold higher than that in Th1, suggesting that this gene plays a key role in mycoparasitism.

The results presented here concerning endochitinase expression are in agreement with previous reports in which endochitinase activity was reported to be triggered before contact with the target fungus occurred (Cortes et al., 1998; Kullnig et al., 2000). However, our NAGase expression results differ from previously reported results because in those reports, NAGase was reported to be activated only after the mycoparasite came into contact with its host (Carsolio et al., 1994; Lorito et al., 1996; Mach et al., 1999). It is common that a pre-contact stage is required for expression of NAGase, which can be required to activate a chain reaction involving the expression other chitinases in *T. asperellum* (Mach et al., 1999) and other *Trichoderma* species (Harman et al., 2004). Nevertheless, the data suggest that when *T. asperellum* is challenged with *P. omnivora*, NAGase may play a more important role in mycoparasitism than endochitinase does. These results are also supported by the observed similarity in the increased expression of NAGase and β-1,3-exoglucanase in our experiments, which suggests synergistic activity of these two genes.

The expression of the chitinase and glucanase genes was similar in TC74 and Th1 grown in liquid medium, in agreement with the levels of enzymatic activity observed for the two strains in liquid MM-PO and MM-AF medium. However, the expression of genes encoding enzymes *in vitro* during direct interaction between *T. asperellum* and *P. omnivora* differed in the two strains, with TC74 displaying a faster and more intense response. This explains, in part, why TC74 is a more efficient antagonist than Th1. These results could indicate a loss of mycoparasitic capacity by Th1. Loss of mycoparasitic capacity was also reported by (Zeilinger et al., 2005). This finding can be attributed to the slow metabolism of Th1 results in slow recognition of the host; recognition of the host fungus is considered to play an important role in triggering the mycoparasitic response by *Trichoderma*.

This work establishes that different *Trichoderma* strains exhibit variability with respect to their biocontrol capability and host range. The approach used here was useful in demonstrating that TC74 is a more efficient strain than Th1 and that it more rapidly and strongly expresses and activates the enzyme arsenal required for mycoparasitism of *P. omnivora*.

Conclusions

The parasitic interaction between *Trichoderma asperellum* and *Phymatotrichopsis omnivora* involves the expression of the activities of at least one endochitinase, one N-acetylglucosaminidase and two β-1,3-exoglucanases. The expression of these enzymes is modulated during parasitism. Even when two strains express the same genes, better performance can occur due to a more timely response, a higher intensity of expression, and enhanced kinetics of gene expression.

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