Ciencia e Investigación AGRARIA

Cien. Inv. Agr. 42(2):243-250. 2015
www.rcia.uc.cl

CROP PROTECTION

DOI: 10.4067/S0718-16202015000200010

RESEARCH PAPER

Enhanced secretion of biocontrol enzymes by *Trichoderma harzianum* mutant strains in the presence of *Rhizoctonia solani* cell walls

Rubén Polanco[1], Constanza Pino[1], Ximena Besoain[2], Jaime Montealegre[3], and Luz M. Pérez[4]

[1]Laboratorio de Bioquímica, Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas, Universidad Andrés Bello, República 217, Santiago, Chile.
[2]Facultad de Agronomía, Pontificia Universidad Católica de Valparaíso, Casilla 4059, Valparaíso, Chile.
[3]Departamento de Sanidad Vegetal. Facultad de Ciencias Agronómicas, Universidad de Chile, Casilla 1004, Santiago, Chile.
[4]Asesorías e Inversiones Biostrategy Ltda., Hernando de Aguirre 1372, Santiago, Chile.

Abstract

R. Polanco, C. Pino, X. Besoain, J. Montealegre and L. M. Pérez. 2015. Enhanced secretion of biocontrol enzymes by *Trichoderma harzianum* mutant strains in the presence of *Rhizoctonia solani* cell walls. Cien. Inv. Agr. 42(2):243-250. The secretion of enzymes involved in biocontrol from the wild *T. harzianum* strains Th11 and Th12 and the mutants Th11A80.1, Th11C40.1 and Th12A10.1 was studied after their cultivation in the presence of *R. solani* cell walls as the sole carbon source. The results showed that endoprotease activity in the supernatants from Th11A80.1 and Th11C40.1 increased 2.77 and 4.79-fold, respectively, and the β-1,3-glucanase and β-1,4-chitinase activity from Th12A10.1 increased 1.86 and 1.54-fold, respectively, compared with the corresponding parental strains. The role of the three enzymes in the biocontrol of *R. solani* is discussed.

Key words: β-1,3-glucanases, endoproteases, β-1,4-chitinases.

Introduction

Several plant diseases are normally controlled by means of selected fungicides that contaminate the environment, destroy the soil microbiota, and the fungicides must be applied frequently because of the fast decrease in their activity. Due to this situation, several disease management practices have been proposed, including the use of *Trichoderma* spp. that have been proven useful in the control of phytopathogens affecting different crops (Benítez *et al*., 2004). Wild *T. harzianum* strains have already demonstrated their positive biocontrol effect on the tomato pathogens *Pyrenochaeta lycopersici* (Pérez *et al*., 2002; Besoain *et al*., 2003) and *Rhizoctonia solani* (Montealegre *et al*., 2005). However, the biocontrol effect could be improved by obtaining mutants with an improved biocontrol capacity. Some mutants obtained after the UV irradiation and protoplast fusion of *T. harzianum* (Besoain

Received September 9, 2014. Accepted June 22, 2015.
Corresponding author: jmonteal@uchile.cl

et al., 2007) have shown an improved biocontrol effect on *P. lycopersici* (Sánchez-Téllez *et al*., 2013). Additionally, some of these mutants have shown a biocontrol effect on *R. solani* (Montealegre *et al*., 2010). However, it is unknown whether these *T. harzianum* mutants have changed their ability to secrete extracellular enzymes involved in biocontrol such as β-1,3-glucanases, endoproteases and β-1,4-chitinases. The present work provides information on the secretion of these enzymes by selected wild and mutant *T. harzianum* strains and how their secretion is involved in the biocontrol effect on phytopathogens.

Materials and methods

Microorganisms

R. solani 618 (AG 4); *T. harzianum* Th11 and Th12 (wild strains); and ThA11.80.1, Th11C.40.1, and Th12A10.1 (mutant strains) were obtained from the laboratory collection (Laboratorio de Fitopatología y Control Biológico de Enfermedades).

Production of extracellular enzymes

The production of extracellular enzymes was induced in submerged cultures of each of the wild (Th11 and Th12) and mutant (ThA11.80.1, Th11C.40.1 and Th12A10.1) *T. harzianum* strains. The cultures were carried out in triplicate in conical flasks containing 200 mL of Mandels Salt Solution (Mandels *et al*., 1974) with the addition of 5 g L^{-1} of *R. solani* cell walls as the sole carbon source (Pérez *et al*., 2002). Briefly, cell walls were obtained from 50 mL of a five-day culture of *R. solani* in Mandels Salt Solution at 27 ºC and 120 rpm (orbital shaker). The suspension was filtered to eliminate conidia, and the non-filtered remainder was suspended in distilled autoclaved water, thoroughly stirred and centrifuged at 30,000 × *g* at 4 ºC. The pellet suspension, stirring and centrifugation was repeated until no residual glucose, protein or amino acids could be detected in the supernatant, as performed in Sivan and Chet (1989). The final pellet was freeze-dried and kept at -20 ºC until use. Each flask was inoculated with 5×10^6 conidia of *T. harzianum* strains. The cultures were incubated in an orbital shaker incubator at 28 °C and 200 rpm. After seven days, the cultures were centrifuged at 12,000 × *g* and 4 °C. The supernatants were assayed for protein quantification and enzymatic activity, and the mycelia were used for weight quantification. These parameters were used to compare the mutants with their corresponding wild parental strains.

Protein quantification

The proteins from the supernatants from each strain were quantified using the Bio-Rad Protein Assay following the instructions of the manufacturer. They are expressed as concentration (mg mL^{-1}) and total protein (mg or μg).

Enzymatic assays

β-1,3-glucanase was assayed using 0.1% AZCL-Pachyman in 100 mM sodium acetate with a pH of 6.0 and 10 μg of protein from the supernatants from each strain following the instructions of the manufacturer (Megazyme, Ireland). The enzyme unit was defined as the amount of enzyme necessary to produce 0.1ΔAbsorbance at 590 nm in a one-hour assay at 38 ºC. The assays were run in quadruplicate and repeated three times.

Endoprotease was assayed using 0.1% AZCL-Casein in 100 mM sodium phosphate with a pH of 7.0 and 10 μg of proteins from the supernatants from each strain following the instructions of the manufacturer (Megazyme, Ireland). The enzyme unit was defined as the amount of enzyme necessary to produce a 0.1ΔAbs at 590 nm in a one-hour assay at 38 ºC. The assays were run in quadruplicate and repeated three times.

β-1,4-chitinase was assayed in agarose gels as previously described (Pérez *et al*., 2002) using the supernatants from each strain as the enzyme source and a known activity of commercial β-1,4-endochitinase from *Serratia marcescens* (Sigma) as the internal standard. The Gel-Pro Analyzer Program (Install Shield Company) was used after developing the enzymatic activity as previously described (Pérez *et al*., 2002). The assays were run in quadruplicate and repeated three times. The enzyme unit was defined as the amount of enzyme necessary to release 1.0 mg of N-acetyl-D-glucosamine released from the chitin per hour at pH 6.0 at 25 °C in a 2-hour assay.

Quantification of mycelial dry weight

The mycelial from each strain were dried in an oven at 80 °C for 5 h and weighed on an analytical balance.

Analysis of data

The data on the protein concentration and enzyme activity found in the supernatants, both for the parental and mutant *T. harzianum* strains, were analyzed by ANOVA followed by Tukey's test ($P \leq 0.05$) using the Stadistica6 Program (Statsop).

Results and discussion

Mycelial dry weight of T. harzianum strains

The ability of the *T. harzianum* strains Th11, Th11A80.1, Th11C40.1, Th12 and Th12A10.1 to grow using *R. solani* cell walls as the sole carbon source was reflected in the corresponding mycelial dry weight after the incubation period, as well as on the ratio of the Strain/Parent mycelial dry weight (Table 1). The mycelial dry weight of the mutants from Th11 was significantly lower than that of the corresponding parental strain, suggesting that the UV-A or UV-C radiation previously used for obtaining the mutants (Besoain *et al*., 2007) may have affected their ability to grow at the same speed as the wild strains in the presence of *R. solani* cell walls as the sole carbon source. These lower dry weights and Strain/Parent ratios less than one reflect the lower amount of growth of the mutants in relation to Th11 in the same growing conditions, suggesting that proteolysis could have been enhanced or that the secretory pathway could have been affected (Flores *et al*., 1997). On the other hand, the mycelial dry weight from Th12 and Th12A10.1 did not significantly differ, which indicates that the UV-A radiation used for obtaining Th12A10.1 (Besoain *et al*., 2007) did not alter its ability to grow in the presence of *R. solani* cell walls. The use of different mutation strategies have been reported to induce the secretion of hydrolytic enzymes in *T. harzianum* strains (Vásquez-Garcidueñas *et al*., 1998; Rey *et al*., 2001; Pérez *et al*., 2007), although data on the mycelial dry weight are not always reported.

Proteins and enzymatic activity in the supernatants of T. harzianum strains

The total protein in the supernatants of the culture medium of the different *T. harzianum* strains grown in the presence of *R. solani* cell walls, expressed in mg and also based on the mycelial dry weight, is shown in Table 1. The protein concentration in the supernatants and the ratio of the Strain/Parent protein concentration are also shown in Table 1. Only Th11C40.1 showed a significant increase in secreted protein concentration. This is in contrast with its significant decrease in mycelial dry weight when compared with its parental strain Th11, reflected in the c.a. four-fold increase in its efficiency to secrete proteins based on the mycelial dry weight in comparison with Th11. The increase in secreted proteins by the *T. harzianum* mutant Th11C40.1 as a consequence of UV-C radiation could be related to post-translational events such as those related to secretory pathways and/or membrane permeation, as previously described (Rey *et al*., 2001). This latter group used a similar

Table 1. Mycelial dry weight and protein concentration in supernatants of different *T. harzianum* strains cultivated over seven days in the presence of *R. solani* cell walls as the sole carbon source.

	Type of strain	Mycelial dry weight (mg)	Ratio dry weight Strain/ Parent	Protein concentration in supernatants (mg mL^{-1})	Ratio protein concentration Strain/Parent	Total protein in the supernatant (mg)	Total protein based on mycelial dry weight (mg mg^{-1})
A. Series Th11							
				T. harzianum strains (series Th11)			
Th11	Parent	116.5 a	1.00	0.013 b	1.00	2.6	0.022
Th11A80.1	UV-A mutant	84.5 b	0.73	0.016 b	1.23	3.2	0.037
Th11C40.1	UV-C mutant	66.5 b	0.57	0.027 a	2.07	5.4	0.081
B. Series Th12							
				*T. harzianum strain*s (series Th12)			
Th12	Parent	91.25 a	1.00	0.022 a	1.00	4.4	0.048
Th12A10.1	UV-A mutant	95.00 a	1.04	0.025 a	1.14	5.0	0.052

Different letters in the columns indicate significant differences after analysis by ANOVA followed by the Tukey's test at $p<0.05$ within the same series.

approach when comparing a mutant of *T. harzianum* CECT 2413 that secretes more extracellular proteins with the corresponding parental strain (Rey *et al*., 2001).

The β-1,3-glucanase, endoprotease and β-1,4-chitinase activity in the supernatants of the culture medium of the different *T. harzianum* strains grown in the presence of *R. solani* cell walls, expressed as Units mL^{-1}, Units/mg proteins and Total Units, are shown in Table 2.

The *T. harzianum* mutant Th12A10.1 was the only strain that significantly increased its β-1,3-glucanase and β-1,4-chitinase activity as reflected in the ratios of total Strain/Parent activity (Table 2A and 2C). This mutant had previously shown an *in vitro* inhibitory effect on *R. solani* due to diffusible metabolites (Arias *et al*., 2006) and an *in vivo* 100% suppression of tomato plant mortality compared with the 20% suppression observed with Th12 (Montealegre *et al*., 2010). Thus, the increased secretion of β-1,3-glucanase and β-1,4-chitinase from this mutant could be related to the enhanced biocontrol effect on phytopathogens using cell walls containing both glucan and chitin (Bartnicki-García, 1968). However, this mutant did not show a significant increase in secreted proteins (Table 1); therefore, the increase in the secretion of these biocontrol enzymes could not be attributed to the changes in membrane permeation or in their secretory pathway (Rey *et al*., 2001), as has been suggested for the mutant PF1 of *T. harzianum* CECT 2413 where the secretion of proteins was enhanced three times the value of the wild type along with the increased activity of β-1,3-glucanase, β-1,6-glucanase and chitinase (Rey *et al*., 2001). In addition, the mutant PF1 of *T. harzianum* CECT 2413 produced 100% mortality in *R. solani*. Additionally, a simultaneous increase in β-1,4-chitinase and β-1,3-glucanase activity has been reported for the *T. harzianum* mutant Th650-NG-7, which also shows an improved biocontrol activity against *R. solani* (Pérez *et al*., 2007) and a 100% suppression of tomato plant mortality caused by the same pathogen (Montealegre *et al*., 2010). Thus, it appears that in terms of the specific *R. solani* cell wall degradation, the simultaneous action of these two enzymes is required (Potgieter and Alexander, 1966; Rey *et al*., 2001) due to its chitin-glucan composition (Bartnicki-García, 1968). However, different pathogens have different cell wall compositions; therefore, the composition must be taken into account when analyzing and selecting biocontrol microorganisms and their secreted enzymes.

Table 2. β-1,3-glucanase, endoprotease and β-1,4-chitinase activity in the supernatant from the *T. harzianum* strains grown in Mandels Salt Medium with the presence of *R. solani* cell walls as the sole carbon source.

	Type of strain	Enzymatic activity (U mL^{-1})	Specific activity (U $mgprot^{-1)}$	Total activity (U)	Ratio Total activity (Strain/Parent)
		T. harzianum strain			
A. β-1,3-glucanase					
Th 11	Parent	0.006 a	0.49 a	1.274 a	1.00
Th 11 A80.1	UV-A mutant	0.004 b	0.27 b	0.864 b	0.68
Th 11 C40.1	UV-B mutant	0.003 b	0.13 c	0.691 c	0.54
Th 12	Parent	0.003 b	0.14 b	0.607 b	1.00
Th 12 A10.1	UV-A mutant	0.006 a	0.23 a	1.130 a	1.86
		T. harzianum strain			
B. Endoprotease					
Th 11	Parent	0.004 c	0.34 b	0.887 c	1.00
Th 11 A80.1	UV-A mutant	0.012 b	0.77 a	2.454 b	2.77
Th 11 C40.1	UV-C mutant	0.021 a	0.79 a	4.250 a	4.79
Th 12	Parent	0.019 a	0.85 a	3.727 a	1.00
Th 12 A10.1	UV-A mutant	0.021 a	0.84 a	4.195 a	1.13
		T. harzianum strain			
C. β-1,4-chitinase					
Th 11	Parent	0.003 a	0.22 a	0.559 a	1.00
Th 11 A80.1	UV-A mutant	0.003 a	0.20 a	0.643 a	1.15
Th 11 C40.1	UV-B mutant	0.003 a	0.10 b	0.556 a	0.99
Th 12	Parent	0.003 b	0.16 b	0.695 b	1.00
Th 12 A10.1	UV-A mutant	0.005 a	0.21 a	1.070 a	1.54

Different letters in the columns indicate significant differences after analysis by ANOVA followed by the Tukey's test at $p<0.05$ within the same series.

The *T. harzianum* mutants Th11A80.1 and Th11C40.1 increased the secretion of endoprotease activity (Table 2B), maintained the secretion of β-1,4-chitinase (Table 2C) and decreased that of β-1,3-glucanase (Table 2A) when compared with the parental strain Th11. The increased secretion of endoproteases induced by the presence of the *R. solani* cell walls in these two mutants could explain their lower mycelial dry weight (Table 1). These two mutants have shown (Arias *et al*., 2006) the ability to secrete diffusible metabolites, which inhibited the *in vitro* growth of *R. solani* by 100% and 94.4%, respectively. They also suppress tomato plant mortality (Montealegre *et al*., 2010). The ability of *T. harzianum* to produce extracellular proteases has been known for a long time, and these enzymes have been involved in the biocontrol of phytopathogens (Kredics *et al*., 2005). For example, a *T. harzianum* (IMI 206040) strain that over-expressed the proteinase-encoding gene *prb1* (Flores *et al*., 1997) showed an improved biocontrol activity of *R. solani*. This *prb1* gene codifies for a basic proteinase (Prb1) in *T. harzianum*, which is induced by autoclaved *R. solani* mycelia, fungal cell wall preparations, or chitin (Geremia *et al*., 1993). Additionally, the UV protease-overproducing *T. harzianum* mutants (Szekeres *et al*., 2004) and *S. cerevisiae* expressing a subtilisin-like protease from *T. harzianum* (Yan and Qian, 2009) have shown increased control of *R. solani*. The decline or deactivation of plant pathogen enzymes and the increase in susceptibility

of the pathogen cell wall to hydrolytic enzymes secreted by *T. harzianum* are within the possible role of proteases in biocontrol by *Trichoderma* strains (Kredics *et al*., 2005). Thus, the increase in secreted endoproteases by the Th11-derived mutant strains Th11A80.1 and Th11C40.1 could account for the improved biocontrol activity based on the host lysis by attacking lipids and proteins, which are part of the cell wall skeleton of the phytopathogens, and/or by degrading enzymes used by the phytopathogens to penetrate the host plant.

Finally, the decrease in β-1,3-glucanase and the maintenance of β-1,4-chitinase activity by the Th11-derived mutants (Table 2A and 2C) suggest that the mutations produced by UV-A or UV-C radiation might have affected only the secretion of glucanases.

The enhanced activity of β-1,3-glucanase and β-1,4-chitinase in the *T. harzianum* mutant Th12A10.1 and of endoprotease in the *T. harzianum* mutants Th11A80.1 and Th11C40.1 in response to the presence of *R. solani* cell walls supports not only the already-tested improved biocontrol effect on this pathogen (Arias *et al*., 2006; Montealegre *et al*., 2010) but also the joint action of β-1,3-glucanase and β-1,4-chitinase and the independent effect of proteolytic activity for pathogen cell wall degradation. The characterization of each wild or mutant *T. harzianum* strain, in terms of the secreted enzyme activity, must be considered for the selection of biocontrol strains as along with the cell wall composition of the pathogen to be controlled. Mutations could enhance the secretion of biocontrol enzymes, and the resulting mutants could behave better, equal or worse than the corresponding parental strains. Thus, the selection of biocontrol strains will rely on the pathogen to be controlled and the enzyme systems that could provide the biocontrol agent for pathogen cell wall degradation and/or for the degradation/inactivation of the mechanisms used by the pathogen to cause disease.

Acknowledgements

This work was partially funded by FONDECYT (Fondo Nacional de Desarrollo Científico y Tecnológico, Chile) project 104053124 (Uso de bioantagonistas mejorados para el control de *Pyrenochaeta lycopersici*, *Phytophthora parasitica* y *Rhizoctonia solani* en el cultivo del tomate).

Resumen

R. Polanco, C. Pino, X. Besoain, J. Montealegre and L. M. Pérez. 2015. Aumento de secreción de enzimas de biocontrol en cepas mutantes de *Trichoderma harzianum* en presencia de paredes de *Rhizoctonia solani*. Cien. Inv. Agr. 42(2): 243-250. La secreción de enzimas involucradas en biocontrol por las cepas silvestres de *T. harzianum* Th11 y Th12, y de sus mutantes Th11A80.1, Th11C40.1 y Th12A10.1, se analizó luego de cultivarlas en medio líquido usando paredes celulares de *R. solani* como única fuente de carbono. Los resultados mostraron que Th11A80.1 y Th11C40.1 aumentaron la secreción de endoproteasas 2,77 y 4,79 veces, respectivamente; Th12A10.1 aumentó 1,86 veces la secreción de β-1,3-glucanasa y 1,54 veces la de β-1,4-quitinasa al compararlas con las cepas parentales. Se discute el rol de estas tres enzimas para el biocontrol de fitopatógenos.

Palabras clave: β-1,3-glucanasa, β-1,4-quitinasa, endoproteasas.

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