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ORIGINAL RESEARCH PAPER

Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production

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Abstract The genus *Trichoderma* is a potential biocontrol agent against several phytopathogenic fungi. One parameter for its successful use is an efficient coiling process followed by a substantial production of hydrolytic enzymes. The interaction between fifteen isolates of *Trichoderma harzianum* and the soil-borne plant pathogen, *Rhizoctonia solani*, was studied by light microscopy and transmission electron microscopy (TEM). Macroscopic observations of fungal growth in dual cultures revealed that growth inhibition of the pathogen occurred soon after contact with the antagonist. All *T. harzianum* isolates tested exhibited coiling around the hyphae of *R. solani*. The strains ALL23, ALL40, ALL41, ALL43 and ALL49 did not differ in coiling frequency and gave equal coiling performances. No correlation between coiling frequency and the production of cell wall-degrading chitinases, *N*-acetyl-β-D-glucosaminidase and β-1,3-glucanases, was found.

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Introduction

Several species of *Trichoderma* are used as biocontrol agent against several phytopathogens fungi, including *Rhizoctonia solani* (Howell 2003). *Trichoderma* spp. attach to the host hyphae via coiling, hooks and appressorium-like bodies, and penetrate the host cell wall by secreting lytic enzymes (Kubicek et al. 2001). Mycoparasites produce cell wall-degrading enzymes which allow them to bore holes into other fungi and extract nutrients for their own growth. Most phytopathogenic fungi have cell wall that contain chitin as a structural backbone arranged in regularly ordered layers and β-1,3-glucan as a filling material arranged in an amorphic manner. β-1,3-Glucanases and chitinases have been found to be directly involved in the mycoparasitism interaction between *Trichoderma* species and its hosts (Kubicek et al. 2001). The chitinolytic system of *Trichoderma* consists basically of two classes of enzymes: chitinases and *N*-acetyl-β-D-glucosaminidase (NAGase).

The *Trichoderma* mechanism of inducing chitinolytic and glucanolytic enzymes and their regulation is still a matter of speculation, despite many studies (Kubicek et al. 2001; Benítez et al. 2004). A considerable amount of research has been aimed at elucidating the β-1,3-glucanase and chitinase systems of *Trichoderma* species, mainly *T. harzianum*, during growth on different carbon sources (Kubicek et al. 2001; Benítez et al. 2004). While major advances have been made in our knowledge of chitinase and β-1,3-glucanase expression by *T. harzianum*, little is known regarding factors affecting production of these enzymes and the nature of the inducers and repressors (Kubicek et al. 2001; Benítez et al. 2004). During mycoparasitism the synthesized hydrolytic enzymes act synergistically. Thus, understanding the induction process from these enzymes is necessary in order to select the most efficient *Trichoderma* isolates for biocontrol.

Trichoderma species are readily isolated from Brazilian Cerrado soil by conventional methods and have been used for technological exploitation of enzyme production and biological control (Lima 2002). The main objective of this study was to investigate the coiling frequency of fifteen isolates of *T. harzianum* around and *R. solani* hyphae using both light microscopy and transmission electron microscopy (TEM) and the relative production of by these isolates of three cell-degrading enzymes.

Materials and methods

Culture conditions

Trichoderma harzianum (Enzymology Group Collection, UFG/ICB) and *Rhizoctonia solani* (EMBRAPA-CNPAF 03) were used.

Dual culture tests

The ability of *T. harzianum* strains to coil around *R. solani* hyphae was studied by using a dual culture technique. A disc of *T. harzianum* mycelia (5 mm diam) was taken from the edge of actively growing colony and placed on the surface of the PDA plate. Two days later, a disc of *R. solani* mycelia (5 mm diam) was placed 3 cm apart from *T. harzianum* on the same PDA plate. The plates were incubated at 28°C and mycelia samples from the interaction region were collected and examined by light microscopy. The coiling frequency was determined by counting loops in 10 squares with three repetitions.

Transmission electron microscopy (TEM)

Mycelial samples from the interaction region were fixed for 3 h at 28°C in 20 g glutaradehyde/l buffered with 0.1 M cacodylate buffer (pH 7.4) with 1 mM CaCl and 10 g sucrose/l. Specimens were washed with the same buffer and post-fixed with 10 g osmium tetroxide/l in the same buffer for 2 h at 28°C. After rinsing thoroughly with 0.1 M cacodylate buffer (pH 7.4), samples were dehydrated in a graded ethanol series. Fully dehydrated samples were moved from absolute ethanol through a 1:1 mixture of ethanol and propylene oxide to pure propylene oxide. Samples were infiltrated through a series of Epon-Araldit resin mixture in propylene oxide, followed by embedding in moulds with fresh 100% resin and polymerized at 65°C for 36 h. Ultrathin sections cut with a glass knife were collected on formvar-coated slot grids. After drying, the grids were stained with uranyl acetate and lead citrate and examined with an EM 10 CR electron microscope (Zeiss, Oberkochen, Germany) at 60 kV.

Enzyme production

For production of chitinases, *N*-acetyl-β-D-glucosaminidase and β-1,3-glucanases, a spore suspension (10^7 spores/ml) was inoculated into liquid medium (TLE) that contained: 1 g Bactopeptone/l, 0.3 g urea/l, 2 g KH_2PO_4/l, 14 g $(NH_4)_2SO_4$/l, 0.3 g $MgSO_4 \cdot 7H_2O$/l, 0.3 g glucose/l, 3.0 g/l (v/v) trace elements solution containing Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and 5 g/l of purified cell wall from *R. solani*. The cultures were grown in conical flasks with constant shaking (140 rpm) at 28°C for 72 h. The mycelium was harvested by filtration through filter paper, and the culture filtrate was dialyzed overnight against distilled water, freeze-dried, and used as source of enzymes. Purification of cell wall from *R. solani* was made by the method described by Mitchell and Taylor (1969).

Enzymes assay and protein determination

β-1,3-Glucanase activity was determined with a colorimetric method using laminarin as substrate (Noronha and Ulhoa 2000). One unit of β-1,3-glucanase activity was defined as the amount of enzyme that produced 1 μmol reducing sugar per min at 37°C.

N-Acetyl-*β*-D-glucosaminidase (NAGase) activity was determined with a colorimetric method using *p*-nitrophenyl-*β*-*N*-acetylglucosamine as substrate (Ulhoa and Peberdy 1992). One unit (U) of enzyme activity was defined as the amount of enzyme which releases 1 μmol *p*-nitrophenol in 1 min at 37°C.

Chitinase activity was determined with a colorimetric method using chitin as substrate (Ulhoa and Peberdy 1992). One unit (U) of enzyme activity was defined as the amount of enzyme which release 1 μmol *N*-acetylglucosamine in 1 h at 37°C.

Protein concentration was determined by the method of Bradford using bovine serum albumin as standard.

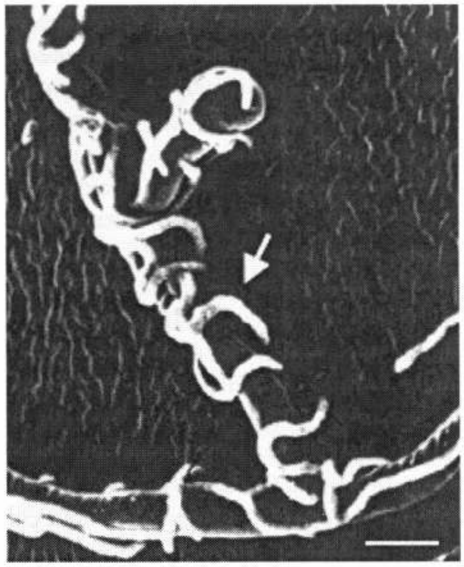

Fig. 1 Transmission electron microscopy of *T. harzianum* mycelium growing on *R. solani*. The arrow indicates the *T. harzianum* coiling. Bar = 10 μm

Results and discussion

Trichoderma species are widely used in agriculture as biocontrol agents. Although much research has been done, our understanding of mycoparasitism is still incomplete (Kubicek et al. 2001; Benítez et al. 2004). *Trichoderma* attaches to the pathogen via cell-wall carbohydrates. Once it is attached, it coils around the pathogen and forms the appresoria. The next steps consist of the production of cell wall-degrading enzymes (CWDE) and peptaibols (Benítez et al. 2004). As the coiling capacity is an important condition for effective biocontrol using *Trichoderma* species, here we first investigated the coiling capacity of fifteen *T. harzianum* strains isolated from the Cerrado soil of Brazil against *R. solani*.

The first apparent physical contact between *T. harzianum* and its host, *R. solani*, occurred within 2–3 days after inoculation, followed by growth inhibition. All 15 *T. harzianum* isolates produced dense coils around the hyphae of *R. solani*, followed by penetration of host hyphae (Fig. 1). Similar results have been found for *T. harzianum* against *Crinipellis perniciosa* (De Marco et al. 2000), *Sclerotium rolfsii* (El-Katany et al. 2001) and *Rhizoctonia cerealis* (Innocenti et al. 2003). The coiling behaviour of the isolates is shown in Fig 2. Strains ALL23, ALL40, ALL41, ALL43 and ALL49 had the highest coiling frequency. Interestingly, the latter four were all isolated from the same habitat.

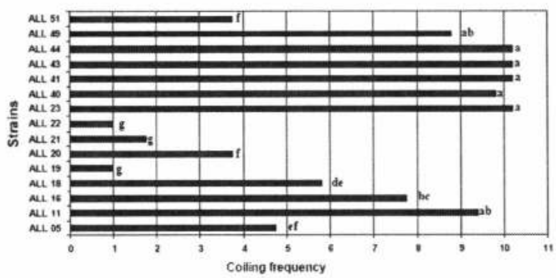

Fig. 2 Coiling frequency of different *T. harzianum* strains on *R. solani*. The coiling frequency was evaluated by microscopy from analyze of 10 squares in interaction zone between antagonist/pathogen with three repetition. The averages followed by different letters means statistic difference determined by Tukey HSD test ($P = 0.05$)

The coiling process is more complex than we suppose. Many reports suggest the involvement of signal transduction pathways components such as G proteins, cAMP and MAP kinase, which control extracellular enzyme and coiling around host hyphae (Mukherjee et al. 2003; Silva et al. 2004). In *Trichoderma*, there is a biochemical evidence for participation of G-α in coiling since an increase in coiling around nylon fibers was detected after addition of activators of G-protein (Omero et al. 1999).

Recently a considerable amount of research has been aimed at elucidating the β-1,3-glucanase system of *Trichoderma* species, mainly *T. harzianum* (Benítez et al. 2004). Some β-1,3-glucanases have been purified and their physicochemical and molecular properties determined (Vazquez-Garciduenas 1998; Noronha and Ulhoa 2000). Here we demonstrated the synergistic action between chitinases and β-1,3-glucanase. In general, all strains showed a relatively high β-1,3-glucanase activity (Fig. 3), with isolate ALL44 the most active. However, we cannot correlate high coiling capacity with β-1,3-glucanase activity because isolates, such as ALL19, ALL21 and ALL22, showed low coiling capacity but high β-1,3-glucanase specific activity. Despite this finding, *Trichoderma* strains that over-produce both chitinase and β-1,3-glucanase showed a good biocontrol against *Botrytis cinerea* and *Rhizoctonia meloni* (Benitez et al. 2004).

All the *Trichoderma* isolates produced NAGase and chitinase (Fig. 4, 5). The highest NAGase activity was found in ALL40 (Fig 4). Interestingly, a positive correlation between high coiling efficiency and high NAGase activity was observed only for the ALL40 isolate. This suggests that these two processes can be independent in most strains. The highest chitinase activities were found in the ALL11, ALL18, ALL22, ALL23, ALL40, ALL43 and ALL44 isolates (see Fig. 5). However, we cannot correlate high coiling capacity with chitinase activity because isolates, such as ALL22 and ALL19, had a low coiling capacity but high chitinase activity. We also found no correlation between high NAGase and high chitinase activity. There is evidence that the expression of *nag1* gene is essential for chitinase *chit42* gene expression (Zeilinger et al. 1999). However, our data suggest that this may

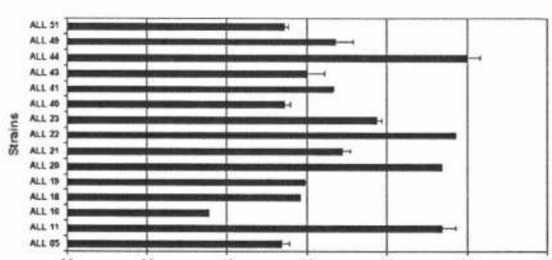

Fig. 3 β-1,3-Glucanase activity from different *T. harzianum* strains during growth in purified cell wall of *R. solani*. Enzyme activity was determined with a colorimetric method using laminarin as substrate

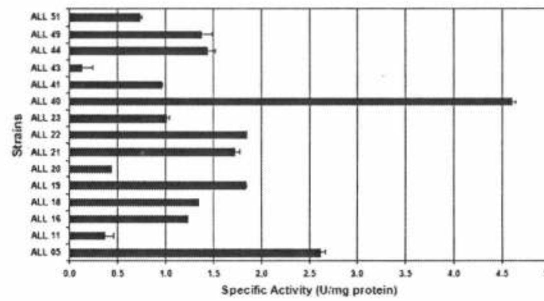

Fig. 4 *N*-Acetyl-β-D-glucosaminidase activity from different *T. harzianum* strains during growth in purified cell wall of *R. solani*. Enzyme activity was determined with a colorimetric method using *p*-nitrophenyl-β-*N*-acetylglucosamine as substrate

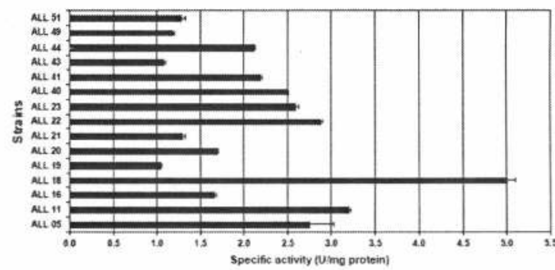

Fig. 5 Chitinase activity from different *T. harzianum* strains during growth in purified cell wall of *R. solani*. Enzyme activity was determined with a colorimetric method using chitin as substrate

not be so for all strains. Isolate ALL40 had the highest NAGase activity but this did not guarantee a high specific chitinase activity.

Further analyses of coiling capacity and specific hydrolytic enzymes activities together with analyses of such factors as temperature tolerance, viability under stress conditions, growth rate and formulation selection, will allow the production of better *Trichoderma* biocontrol agent.

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References

Benitez T, Rincon AM, Limon MC, Codon AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol 7(4):249–260

De Marco JL, Lima LHC, Valle de Souza M, Felix CR (2000) A *Trichoderma harzianum* chitinase destroys the cell wall of the phytopathogen *Crinipellis perniciosa*, the causal agent of witches broom disease of cocoa. World J Microbiol Biotecnol 16:383–386

El-Katany MH, Gudelj M, Robra K-H, Enaghy MA, Gubitz GM (2001) Characterization of a chitinase and an endo-1,3-glucanase from *Trichoderma harzianum* Rifae T24 involved in control of the phytopathogen *Sclerotium rolfsii*. Appl Microbiol Biotechnol 56:137–143

Howell CR (2003) Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis 87:4–10

Innocenti G, Roberti R, Montanari M, Zakrissom E (2003) Efficacy of microorganisms antagonistic to *Rhizoctonia cerealis* and their cell degrading enzymatic activities. Mycol Res 107:421–427

Kubicek CP, Mach RL, Peterbauer CK, Lorito M (2001) *Trichoderma*: from genes to biocontrol. J Plant Pathol 83:11–24

Lima AL (2002) Molecular and biochemical characterization of *Trichoderma* isolated from Brazilian cerrado soil. PhD Thesis. Universidade de Brasília, Brasilia, Brazil

Mukherjee PK, Latha J, Hadar R, Horwitz BA (2003) TmkA, a mitogen-activated protein kinase of *Trichoderma virens*, is involved in biocontrol properties and repression of conidiation in the dark. Eukaryotic Cell 2(3):446–455

Noronha EF, Ulhoa CJ (2000) Characterization of a 29-KDa β-1,3-glucanase from *Trichoderma harzianum*. FEMS Lett 183:119–123

Omero C, Inbar J, Rocha-Ramirez V, Herrera-Estrela A, Chet I, Horwitz BA (1999) G proteins activators and cAMP promote mycoparasitic behavior in *Trichoderma harzianum*. Mycol Res 103(12):1637–1642

Silva RN , Silva SP, Brandão RL, Ulhoa CJ (2004) Regulation of *N*-acetyl-β-D-glucosaminidase produced by *Trichoderma harzianum*: evidence that cAMP controls its expression. Res Microbiol 155:667–671

Ulhoa CJ, Peberdy JF (1992) Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum*. Enz Microbial Technol 14(3):236–240

Vazquez-Garciduenas S, Leal-Morales CA, Herrera-Estrella A (1998) Analysis of the b-1,3-glucanolytic system of the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol 64(4):1442–1446

Zeilinger S, Galhaup C, Payer K, Woo SL, Mach RL, Fekete C, Lorito M, Kubicek CP (1999) Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genet Biol 26(2):131–140