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Methodology article

Characterization of a protease produced by a *Trichoderma harzianum* isolate which controls cocoa plant witches' broom disease

Janice L De Marco* and Carlos Roberto Felix

Address: Departamento de Biologia Celular, Universidade de Brasília. 70910-900, Brasília, Brazil

E-mail: Janice L De Marco* - jdemarco@uol.com.br; Carlos Roberto Felix - carlosrf@unb.br

*Corresponding author

Abstract

Background: Several *Trichoderma* strains have been reported to be effective in controlling plant diseases, and the action of fungal hydrolytic enzymes has been considered as the main mechanism involved in the antagonistic process. However, although *Trichoderma* strains were found to impair development of *Crinipellis perniciosa,* the causal agent of cocoa plant witches' broom disease, no fungal strain is available for effective control of this disease. We have then undertaken a program of construction of hydrolytic enzyme-overproducing *Trichoderma* strains aiming improvement of the fungal antagonistic capacity. The protease of an indian *Trichoderma* isolate showing antagonistic activity against *C. perniciosa* was purified to homogeneity and characterized for its kinetic properties and action on the phytopathogen cell wall.

Results: A protease produced by the *Trichoderma harzianum* isolate 1051 was purified to homogeneity by precipitation with ammonium sulfate followed by hydrophobic chromatography. The molecular mass of this protease as determined by SDS-polyacrylamide gel electrophoresis was about 18.8 kDa. Its N-terminal amino acid sequence shares no homology with any other protease. The purified enzyme substantially affected the cell wall of the phytopathogen *C. perniciosa.* Western-blotting analysis showed that the enzyme was present in the culture supernatant 24 h after the *Trichoderma* started to grow in casein-containing liquid medium.

Conclusions: The capacity of the *Trichoderma harzianum* protease to hydrolyze the cell wall of *C. perniciosa* indicates that this enzyme may be actually involved in the antagonistic process between the two fungi. This fact strongly suggest that hydrolytic enzyme over-producing transgenic fungi may show superior biocontrol capacity.

Background

Since the pioneering work of Weindling and Fawcett on the use of *Trichoderma* strains to control damping-off caused by *Rhizoctonia solani* Kühn in citrus, considerable attention has been focused over the past 20 years on the isolation of fungal antagonist that could be as effective as pesticides in the repression of fungal pathogens [1]. However, the molecular basis of biological control is not clearly understood. Proposed mechanisms resulting in biocontrol are competition for the substrate [2], the ability to colonize the niche favored by the pathogen, antagonism by antibiotics [3,4] and the action of cell wall-

degrading enzymes [5]. It has been claimed that a battery of antibiotic substances produced by *Trichoderma* spp. are responsible for their antagonistic properties [3]. On the other hand, chitinolytic enzymes produced by *Trichoderma harzianum* Rifai have been suggested as being responsible for the observed antifungal activity [6,7]. Indirect evidence suggested that *T. harzianum* antagonize first and foremost by antibiosis leading to cell death, followed by degradation of the cell wall by chitinolytic enzymes [8]. In addition, *T. harzianum* growing in liquid medium containing *Botrytis cinerea* cell wall produced considerable proteolytic activity [4], which was suggested to be involved in the antagonistic process. This possibility is further supported by the finding that an alkaline protease (prb1) is also induced in the presence of *Rhizoctonia solani* cell wall [9].

Cocoa plant witches' broom is a serious disease caused by the hemibiotrophic fungus *Crinipellis perniciosa*. This fungus is currently causing severe damage to many cocoa plantations in South America. Phytosanitation and, to a lesser extent, chemical control are recommended in certain situations but are not effective and/or practical in all circumstances. Nevertheless, some *Trichoderma* spp isolates were previously found to control the development of *C. perniciosa* in field conditions and, consequently, to control the disease (José L. Bezerra, *personal comm.*). Production of hydrolytic enzymes by two of these fungal isolates (1051 and TVC) in liquid medium was then studied [10]. Both fungi were found to produce substantial amounts of chitinases, N-acetylglucosaminidase, β-glucanases, cellulases, amylases and proteases. One chitinase and one amylase produced by the isolate 1051 were purified and characterized [10,11]. The chitinase drastically affected the *C. perniciosa* cell wall *in vitro*[10], indicating that this enzyme may play an important role during the antagonistic process between *T. harzianum* and the phytopathogen *C. perniciosa*. Nevertheless, substantial information provides support for the concept that the extraordinary capacity of *T. harzianum* to attack the structures of phytopathogens is related to the synergistic action of hydrolytic enzymes including chitinases, glucanases, lipases and proteases [1]. The properties of several hydrolases produced by fungi showing antagonistic capacity against the causal agent of cocoa plant witches' broom disease are now being investigated in this laboratory [10–13]. Here, we report the purification and some properties of one protease produced by *T. harzianum* 1051 grown in liquid medium containing casein as carbon source.

Materials and methods
Microorganisms
Trichoderma harzianum, isolate 1051, was obtained from the collection of the Centro Nacional de Pesquisa de Monitoramento e Avaliação de Impacto Ambiental (CNPMA/

EMBRAPA, Jaguariuna, SP, Brasil). The phytopathogen *Crinipellis perniciosa* was kindly provided by Dr. José Luiz Bezerra from CEPLAC/Ilheus, BA, Brasil. Both fungi were maintained by serial passages in Bacto-dextrose-agar medium.

Enzyme production and assay
For enzyme production, *T. harzianum* was grown in TLE liquid medium as previously described [10], for 72 h at 28°C. Protease activity was assayed using a reaction system containing 500 μL of a 1% solution of hammarstein casein dissolved in 0.1 M HEPES buffer, pH 8.0, 250 μL of the HEPES buffer, and 0–250 μL of enzyme solution. Reaction was conducted for 20 min at 37°C and stopped by addition of 1.25 mL of 10% TCA, incubation (40 min) on ice and centrifugation (10 min, 5,000 rpm). The absorbance of the supernatant was determined at 280 nm. One unit of proteolytic activity corresponds to the amount of enzyme required to cause an increase of 1.0 A_{280} nm unit in the absorbance of the supernatant within 20 min. All enzyme assays were carried out in triplicate. In all cases, the standard deviation values were smaller than 3% of the mean values.

Protease purification
Four 1 liter-erlenmeyer flasks, each containing 500 mL of TLE liquid culture medium [0.1 % bactopeptone; 0.03 % urea; 0.2 % KH_2PO_4; 0.14 % $(NH_4)_2SO_4$; 0.03 % $MgSO_4.7H_2O$; 0.03 % $CaCl_2.6H_2O$; 1 mL of 0.01 % trace elements solution (Fe^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+}), 0.02 % glucose, pH 5.5) containing 0.5% casein] were inoculated with 2×10^7 spores of *T. harzianum* 1051 and the culture incubated for 72 h with agitation (120 rpm) at 28°C. The culture supernatant were then collected by filtration on filter paper and the proteins precipitated with 20% ammonium sulfate. This was followed by centrifugation at 28.800 g for 30 min, and reprecipitation of the supernatant with 60% ammonium sulfate. The resulting pellet (hereafter called crude enzyme extract) was ressuspended in 50 mM sodium phosphate buffer, pH 5.0, and loaded on a Phenyl-Sepharose column (10×1.8 cm) equilibrated with the phosphate buffer. Proteins were then eluted with a negative linear gradient formed with 50 mL of 50 mM sodium phosphate buffer, pH 5.0, containing 1.0 M ammonium sulfate, and 50 ml of the same buffer. Samples of 3.0 ml were collected at a flow rate of 54 mL/h.

SDS-PAGE and immunoblotting analysis
Protease samples were submitted to electrophoresis in polyacrylamide gels under denaturing conditions, according to Laemmli [14]. Proteins present in the gels were stained with silver reagent according to the method of Blum *et al.*[15]. For immunoblotting analysis, proteins present in the SDS-polyacrylamide gels were transferred to a nitrocellulose membrane [16] using a LKB Multiphor II

(Pharmacia). The membranes were then incubated over-
night with 2 % skimmed milk solution at 4°C, and the
proteins immunodetected using the polyclonal mouse an-
tibody specific for the *T. harzianum* 1051 protease, alka-
line phosphatase-linked mouse anti-IgG antibody (Sigma
Chem.Co) and color development reagent (132 μL of ni-
tro blue tetrazolium + 66 μL of 5-bromo-4-chloro-3-in-
dolyl dissolved in 20 mL of phosphate buffered saline).

For production of the mouse polyclonal antibody against
the *T. harzianum* 1051 protease, three mices received intra-
peritoneally at 20-day intervals three doses (30 μg each)
of the purified protease. The first injection was given with
complete Freund's adjuvant, the second with incomplete
Freund's adjuvant and the third without any adjuvant. The
animals were bled 45 days after the first injection and se-
rum was collected by centrifugation after blood clotting.

Protein sequencing
The N-terminal amino acid sequence of the *Trichoderma*
1051 protease was determined by the Edman degradation
method using an automatic sequencer (Applied Biosys-
tems 477A Automatic Protein Sequencer) fitted on line to
a 120 A PTH-analyzer. Comparison of the protease amino
acid sequence with other proteins was done using the
Swiss-Prot databank.

Enzymatic digestion of Crinipellis perniciosa mycelium and scanning electron microscopy (SEM) analysis
Disks (5 mm) of solid culture medium containing *C. per-
niciosa* mycelium were placed in microcentrifuge tubes
containing 1 mL of either *T. harzianum* 1051 culture su-
pernatant showing proteolytic activity (220 U/mL) or the
corresponding purified protease (75 U/mL). After incuba-
tion for 0, 24 and 48 h at 37°C, the supernatants were
used for determination of reducing sugars by the dinitro-
salicylic acid method (DNS assay) according to Miller
[18], glucose by the glucose oxidase method [17] and pro-
tein by a simplification of the method of Lowry *et al.*[19].
The *C. perniciosa* mycelium disks submitted to the action
of the protease samples were then fixed for 2 h at 4°C in
2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde
buffered with 0.05% sodium cacodylate buffer, pH 7.2,
and postfixed in 1% (w/v) osmium tetroxide in the same
buffer. The specimens were washed with buffer and dehy-
drated in 30–100% (v/v) acetone, critical-point dried in
CO_2 and sputter-coated with gold. Materials were exam-
ined microscopically at an accelerating voltage of 10.0 kV.

Results and discussion
T. harzianum 1051 growing in casein-containing liquid
medium produces substantial proteolytic activity as previ-
ously reported [20]. The supernatant of *T. harzianum* 1051
cultures were then used to purify the protein showing pro-
teolytic activity against casein. Upon freeze-drying of the
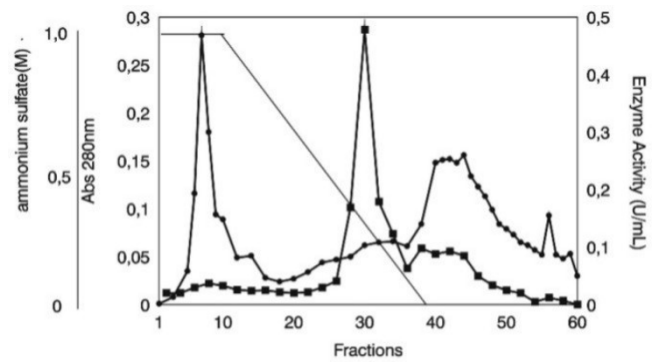
Figure 1
Chromatography of the crude enzyme preparation of *Tri-
choderma harzianum* 1051 on a Phenyl-Sepharose column. ■
protease activity; ● Absorbance at 280 nm; — $(NH_4)_2SO_4$
negative gradient.
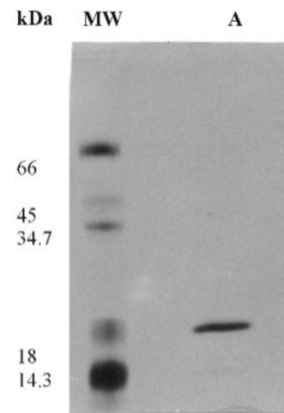
Figure 2
SDS-PAGE of the protease from *Trichoderma harzianum* 1051
purified on a Phenyl-Sepharose column. Mw, molecular
weight standards; A, purified protease. Proteins were silver
stained.

culture supernatant, there was no significant loss of en-
zyme activity. The proteases present in the freeze-dried
culture supernatant reconstituted with water were only
slightly precipitated with ammonium sulfate at the con-
centration of 20%, but was fully precipitated with this salt
at the final concentration of 60 % at pH 5.0. We then used
the enzyme sample obtained by precipitation with 20 %
and 60 % ammonium sulfate for further purification of
the enzyme using a Phenyl-Sepharose column (Fig. 1).
The proteins were eluted as several peaks both before and
after the ammonium sulfate gradient was started. Never-
theless, the bulk proteolytic activity was eluted as a single
peak, i.e., only the proteins eluted with an ammonium
sulfate concentration of 0.23–0.16 M (fractions 28–37)
were active against casein. The enzyme sample resulting

Table 1: Summary of the purification protocol of the protease produced by *Trichoderma harzianum* 1051.

Purification Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield(%)
*Freeze-dryed culture medium	274	5.0	55	-	100
** crude enzyme	220	7.5	29.3	1	80
***phenyl-Sepharose eluate	75	0.63	119	4.06	27

*Freeze-dried culture medium ressuspended with 20 ml of 50 mM phosphate buffer, pH 5.0; **pellet resulting from precipitation of the freeze-dried material, with 60% $(NH_4)_2$ SO_4 and resuspended with 3 ml of the phosphate buffer; *** Protease sample (Fig. 1, fractions 30–37) resulting from chromatography of the crude enzyme on a phenyl-Sepharose column.

Table 2: Amounts of reducing sugar, glucose and protein in the reaction medium containing *Crinipellis perniciosa* mycelium and crude enzyme extract, native and heat-denatured *Trichoderma harzianum* 1051 purified protease (75 U), or *Trichoderma harzianum* 1051 culture medium containing proteolytic activity (220 U). Hydrolysis was conducted for 48 hours. For assay of reducing sugar, glucose and protein see Materials and Methods.

|	Treatment with		
Compound	crude enzyme extract	native purified protease	heat-denatured purified protease
Reducing sugar	0.06*	0	0
Glucose	0.037**	0	0
Protein	2.23***	0.202***	0

*μg of reducing sugar produced in 1 hour, **μg of glucose produced in 1 hour, ***μg of protein produced in 1 hour.

from pooling of fractions 30–37 contained a single protein as demonstrated by SDS-PAGE [15] analysis (Fig. 2). The protein fractions eluted from the column after the ammonium sulfate gradient showed very low proteolytic activity and several protein species, as analyzed by SDS-PAGE (not shown). The summary of the purification protocol indicated that the enzyme was purified 4.06-fold (Tab. 1). The higher amount of total protein in the pellet resulting from precipitation of the freeze-dried culture supernatant with ammonium sulfate must be resulting from artifact, since this salt may react with Lowry reagents. The molecular mass of the enzyme was estimated to be 18.8 kDa (Fig. 2).

The effect of the pH on the protease activity was determined at the pH range of 7.0–10.0. At this range, maximal activity was determined at the pH values of 7.0 and 8.0, but substantial proteolytic activities were encountered at pH 9.0 and 10.0 (Fig. 3A). The effect of acidic pH (<7.0) could not be determined, since the casein precipitates at acid pH conditions. This fact has made it difficult to determine the optimal pH for the purified protease, and the

presented data do not exclude the possibility that the enzyme is mostly active at pH values lower than 7.0. The optimal pH value determined for the proteolytic activity produced by *T. viride* in mixed culture with *Sclerotium rolfsii* in soil, was 6.0 [21], this value being lower than the optimal pH determined for the proteolytic activities from *Trichoderma virens*[22], and for the extracellular proteases from *Streptomyces* spp. G157 [23] and *S. pactum*[24]. The *T. harzianum* 1051 enzyme was mostly active at 37°C (Fig. 3B). This optimal temperature value is very close to the optimal temperatures determined for the proteases from *Streptomyces* spp. G157 [23], and *S. pactum*[24]. The purified enzyme was only fairly stable at 37°C and pH 8.0 (not shown). It lost about 50% of its original activity after preincubation for 15 min at this temperature and pH, followed by the incubation with the substrate for 20 min at 37°C, as stated in materials and methods.
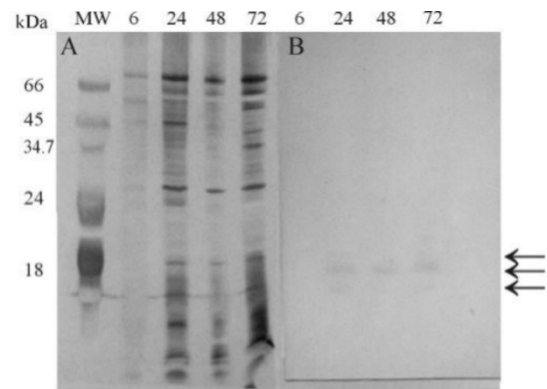
SDS-PAGE analysis revealed that several proteins were present in the culture supernatant of *Trichoderma harzianum* cultures grown on casein-containing medium (Fig. 4A). As casein has a molecular mass of about 23 kDa, the
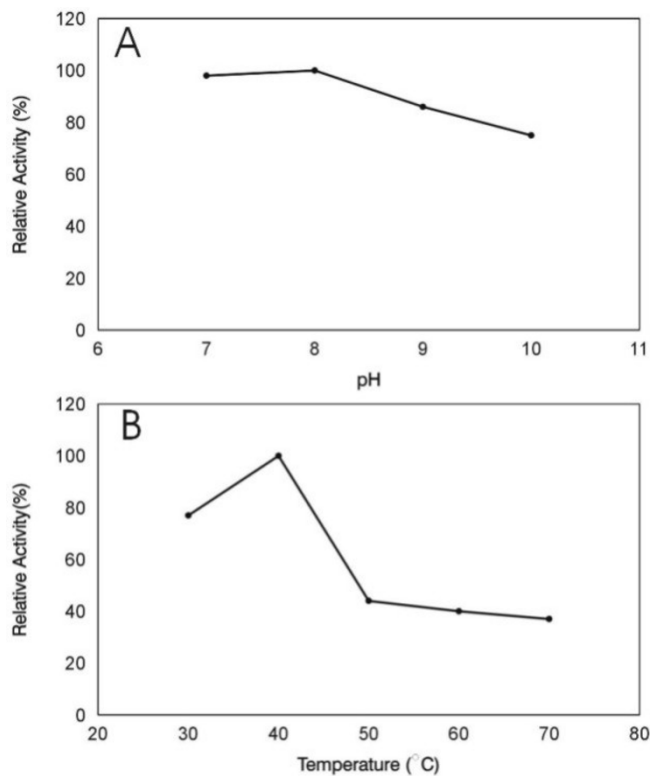
Figure 3
The effect of pH (A) and temperature (B) on the activity of the purified protease from *Trichoderma harzianum* 1051.

Figure 4
SDS-PAGE (A) and Western blot (B) analysis of the proteins present in the culture supernatant of *Trichoderma harzianum* 1051 grown in liquid medium containing 0.5% casein, for different growing times: 6 h, 24 h, 48 h, and, 72 h. Mw, molecular mass standards. Samples of culture medium containing about 100 µg of proteins were diluted in sample buffer and applied in the gel. Proteins in the SDS-PAGE were silver stained. The nitrocellulose membrane was developed with the polyclonal antibodies raised in mice against the *Trichoderma harzianum* 1051 purified protease, and alkaline phosphatase-conjugated anti-mouse IgG and the color developing reagents nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl. The arrows indicate position of the 16, 17, and, 18.8 kDa proteins.

possibility that most of those proteins resulted from hydrolysis of the substrate present in the culture can be excluded, since most proteins have molecular mass values higher than 23 kDa. Western-blotting analysis using anti-protease antibodies raised in mice against the purified *T. harzianum* 1051 protease revealed that the antibodies recognized three proteins, indicating that the *T. harzianum* 1051 produces and secretes at least three proteases, one with a molecular mass of 18 kDa, and the other two with molecular masses of about 16–17 kDa (Fig. 4B). The 18.8 kDa protein recognized by the antibody corresponds to the protein purified and analyzed as above (Fig. 4B) corresponds to the purified protease as demonstrated in Fig. 2. Nevertheless, the possibility that the 17–18 kDa proteases result from proteolysis of the 18 kDa protein can not be excluded, since both proteins were present in the culture medium already 24 h after the beginning of culture. However, there is still the possibility that these two smaller proteins were recognized unspecifically by the anti-protease antibody.

The N-terminal amino acid sequence of the *T. harzianum* 1051 18.8 kDa protease (VPSTVWTVCP10) showed no homology with any other protease. It did, however, show homology of about 54%–75% with the amino acid sequences of proteins produced by *Ectatomma tuberculatum*[25], *Ascaris lumbricoides*[26], *Escherichia coli*[27], and *Rhizobium meliloti*[28].

The effect of the proteolytic activity-exhibiting *T. harzianum* 1051 culture supernatant and of the corresponding purified protease (Fig. 1 and Fig. 2) on the *C. perniciosa* cell wall was tested by measuring the amounts of reducing sugar and protein produced during incubation of the enzyme samples with the phytopathogen mycelium (Tab. 2). As much as 0.202 µg of protein/µL of reaction mixture, as measured by the method of Lowry *et al.*[19] were present in the reaction mixture 48 h after the beginning of the period of incubation of the purified native enzyme with the *Crinipellis* mycelium (Tab. 2). At the same time, no protein was detected in the reaction mixture containing heat-denatured enzyme. The purified native and the denatured enzyme produced nor reducing sugar neither glucose. However, these products were present in the reaction mixture containing crude enzyme extract and the phytopathogen mycelium. This is probably due to the presence of other enzymes besides the protease. The action of the protease on the *C. perniciosa* mycelium was further confirmed by SEM analyses which revealed that the
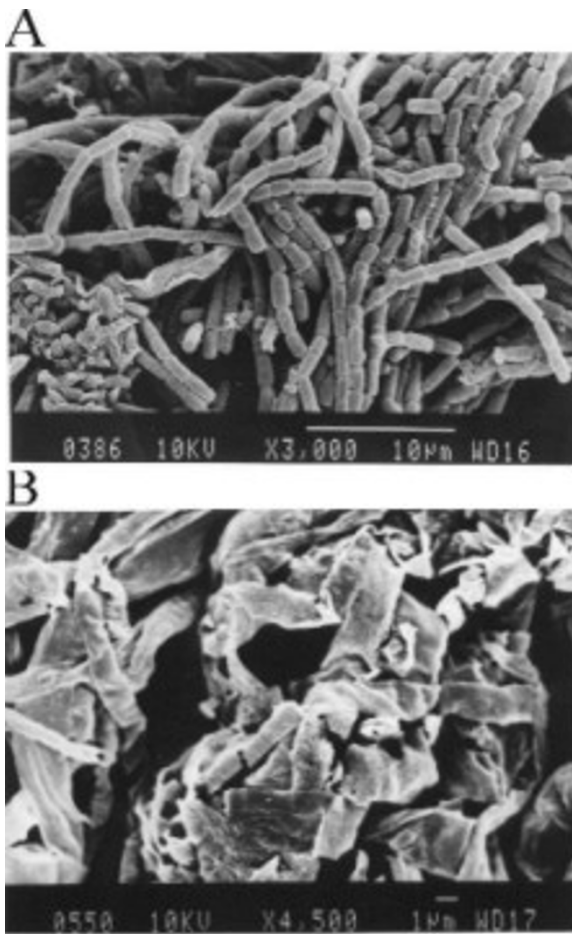
Figure 5
Scanning electron microscopy of the *Crinipellis perniciosa* mycelium treated for 48 h with native (A), and heat-denatured (B) *Trichoderma harzianum* 1051 purified protease.

purified enzyme could hydrolyze the cell wall of the phytopathogenic fungus *C. perniciosa* as soon as 48 h after contact (Fig. 5B). As expected, no hydrolytic effect was observed for the denatured enzyme on the phytopathogen mycelium (Fig. 5A). This finding supports previous observation that the *T. harzianum* isolate 1051 controls witches' broom disease in the field (J. L. Bezerra, *personal comm.*) and is able to impair the growth of *C. perniciosa* when both fungi are allowed to grow on the same plate containing solid medium (dual culture). The most common event involved in the antagonistic process is perforation of the phytopathogen cell wall by lytic enzymes produced by the antagonist [29]. The effects of purified *Trichoderma* chitinase on the cell wall of *Rhizoctonia solani* and *Sclerotium rolfsii*[12] and on *C. perniciosa* mycelium [10] were reported before supporting the involvement of hydrolytic enzymes in the antagonistic process. As a consequence, it has been claimed that hydrolytic enzymes and the genes en-

coding them may be useful for production of transgenic microorganisms with superior biocontrol capability and the development of transgenic plants with high resistance to pathogens [30]. Further studies on molecular characterization of the protease produced by witches' broom disease-controlling *T. harzianum*, and studies about its possible participation in the antagonistic process are being carried out.

Conclusions

The *Trichoderma harzianum* isolate 1051 which was previously shown to control cocoa plant witches' broom disease in the field, impairs growth of the disease agent, *Crinipellis perniciosa*, in solid medium. One protein with molecular mass of 18.8 kDa may account for the bulk of the proteolytic activity secreted by the *Trichoderma* during its growth in culture medium containing casein. Mouse polyclonal antibody produced against the 18.8 kDa protease recognizes two other proteins with molecular masses of 16–17 kDa. The 18.8 kDa purified protease was able to hydrolyze the phytophatogen *C. perniciosa* cell wall *in vitro*, indicating that this enzyme may play a role in the antagonistic interaction between the *Trichoderma* and the phytopathogen.

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