

PRODUCTION OF HYDROLYTIC ENZYMES BY TRICHODERMA ISOLATES WITH ANTAGONISTIC ACTIVITY AGAINST *CRINIPELLIS PERNICIOSA*, THE CAUSAL AGENT OF WITCHES' BROOM OF COCOA

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ABSTRACT

Two isolates of *Trichoderma*, which reduce the incidence of witches' broom disease caused in cocoa by *Crinipellis pernicioso*, were evaluated for their potential to produce hydrolases in liquid medium. Very low or no hydrolytic activity was produced in the absence of any substrate. The activities of chitinase, N-acetylglucosaminidase, β -1,3-glucanase, total cellulase, endoglucanase, aryl- β -glucosidase, β -glucosidase, protease and amylase increased dramatically within 72-120 h of growth in the presence of specific substrates. Except for N-acetylglucosaminidase and β -glucosidase *Trichoderma harzianum* isolate 1051 produced the largest amounts of hydrolases. The possible involvement of these enzymes in the antagonistic interaction between *Trichoderma* and *C. pernicioso* is discussed.

Key words: *Trichoderma*, *Crinipellis pernicioso*, hydrolases, antagonistic interaction

INTRODUCTION

The basidiomycete fungus *Crinipellis pernicioso* causes witches' broom disease of cocoa (*Theobroma cacao*) (2). This fungus is a very destructive pathogen which has a hemibiotrophic life-style, is a species-specific pathogen of cocoa (16) and causes major crop losses in South American and Caribbean plantations (13). Infection is initiated when germ tubes from *C. pernicioso* basidiospores penetrate meristematic tissue within growing apical buds. The host response is localized at the point of infection, where there is considerable tissue enlargement (25) and development of lateral shoots (green brooms) (22). The fungus remains in the primary (biotrophic, monokaryotic) phase for some time within the enlarging green broom, but after 6-9 weeks the green brooms begin to show necrosis. The change is associated with dikaryotization of the fungus to a secondary (saprotrophic, dikaryotic) phase. Subsequently basidiocarps are formed on the dead brooms (22). Breeding for resistance, phytosanitation and the application of

fungicides are still the main tools for controlling this disease (5). Nevertheless, fungicides have many undesirable attributes. Novel biocontrol agents have therefore been looked for (7), and *Trichoderma* isolates have been the preferred choice. Besides other modes of action, cell wall-degrading enzymes such as chitinases (10,11,19,20) and β -1,3 glucanases (21,24,28), have been associated with the ability of *Trichoderma* spp to control plant pathogens (26). In fact, a strain of *Trichoderma viride* offered prospects for biological control of *Crinipellis pernicioso* (3). Brooms, once colonized, never produced basidiocarps and the contents of cellulose and hemicellulose in brooms infected by the *Trichoderma* were lower than in uninfected brooms (6). This finding has been correlated with the capacity of *Trichoderma* to grow faster than *C. pernicioso* in dual culture (4), and to produce extracellular enzymes on solid media such as cellulase, amylase and proteases (5). Recently, two strains of *Trichoderma* were found to impair the progress of witches' broom disease in field conditions caused in cocoa by *C. pernicioso* (José L. Bezerra, personal

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communication). We have therefore undertaken a project on evaluation at the potential of these two *Trichoderma* strains to produce hydrolytic enzymes. Here we report on production of chitinase, N-acetylglucosaminidase, β -1,3-glucanase, protease, cellulases, and amylase in the presence of the corresponding substrate.

MATERIALS AND METHODS

Microorganisms and enzyme production

T. harzianum 39.1 was obtained from the collection of the Microbial Genetics and Biochemistry Group of the University of Nottingham (UK). The other *Trichoderma* isolates 1051 (*Trichoderma harzianum* 1051) and TVC (*Trichoderma* sp) were kindly provided by Dr. Itamar Soares de Melo from the Centro Nacional de Pesquisa de Monitoramento e Avaliação de Impacto Ambiental (CNPMA/EMBRAPA). All microorganisms were maintained on agar medium. For enzyme production, about 3×10^7 spores (in one mL of saline) were transferred to 25 mL of autoclaved TLE culture medium (19) containing 1% of either chitin, potato starch, microcrystalline cellulose or casein, and pH adjusted to 5.5. Cultures were then incubated with shaking (120 rpm) at 28°C. After several time periods, culture supernatants were filtered (Whatman N° 2) and stored at -20°C until use for determination of enzyme activities.

Enzyme assays

Chitinase activity (EC 3.2.1.14) was assayed at 37°C monitoring the amount of reducing sugar formed from acetylated chitin. N-acetylglucosaminidase activity (EC 3.2.1.30) was measured at 37°C using *p*-nitrophenyl- β -N-acetylglucosaminide (*p*-PGlcNAc) as the substrate. β -1,3-glucanase (EC 3.2.1.39) was assayed based on the release of reducing sugar from laminarin. Reactions were carried out at 37°C. The reaction systems used were as described previously (19). One unit of enzyme was defined as the amount of enzyme necessary to produce 1 μ mol of reducing sugar in one minute (chitinase and β -1,3-glucanase) or 1 μ mol of *p*-nitrophenol in one minute (chitobiosidase).

Total cellulase activity was determined by measuring the amount of reducing sugar formed from filter paper. Reactions were conducted for 60 min at 50°C (29). One unit of total cellulase activity correspond to the amount of reducing sugar produced in one minute.

Endoglucanase activity (EC 3.2.1.4) was assayed by measuring the amount of reducing sugar formed from carboxymethyl-cellulose (CM-cellulose). The reaction system contained 0-250 μ L of enzyme solution and 500 μ L of sodium citrate buffer (50 mol/L), pH 6.0, containing 1% CM-cellulose (low viscosity). Reactions were allowed to proceed for 60 min at 50°C and stopped with 1.5 mL of dinitrosalicilate reagent. One unit of endoglucanase activity was defined as the amount of protein necessary to produce one mmol of reducing sugar in one minute.

β -glucosidase activity (cellobiosidase, EC 3.2.1.21) was assayed by measuring production of glucose from cellobiose. Reactions were conducted for 30 min at 50°C. Aryl- β -glucosidase (EC 3.2.1.2) was assayed by monitoring the amount of *p*-nitrophenol formed in 10 min at 42°C from *p*-nitrophenol- β -D glucopyranoside. The reaction systems were as previously described (29). One unit of cellobiosidase or aryl- β -glucosidase activity was defined as to the amount of protein necessary to produce 1 mmol of glucose or *p*-nitrophenol respectively, in one minute.

Protease (EC 3.4.) was assayed using “Hammarsten” casein as described by Santos *et al.* (27). One unit of protease activity was defined as the amount of enzyme necessary to cause a change of one unit in the absorbance of the supernatant in 20 min.

Amylase dextrinizing activity was assayed by measuring the change of blue values of amylose-iodine complexes. The reaction was allowed to proceed for 30 min at 40°C (9). One unit of dextrinizing activity was defined as the amount of enzyme which hydrolyses 0.1 mg of soluble starch in one minute.

RESULTS AND DISCUSSION

Trichoderma harzianum isolate 1051 and *Trichoderma* sp. isolate TVC were previously shown to effectively control witches’broom disease of cocoa caused by *C. pernicioso* in field conditions (José L. Bezerra, personal communication). This is in agreement with the report that *T. viride* decreased the incidence of fruits infected by *C. pernicioso* (5,6). *Trichoderma* spp are able to parasitise a wide range of fungal hosts of varied cell wall structure and composition. Microscopic examination revealed formation of hyphal coiling and formation of short lateral branches by *T. viride* when associated with *C. pernicioso* (5). Additional studies revealed that lytic enzymes such as proteases, amylases, and cellulases, but not antibiotics, were produced by the antagonist. Therefore, the killing of the *C. pernicioso* in culture, and in dry brooms of cocoa, coupled with the absence of an active antibiotic support the idea that the most important mechanism involved in this host-parasite interaction seems to be mycoparasitism.

Both *Trichoderma* isolates tested produced and secreted on induction substantial amounts of chitinolytic and glucanolytic enzymes, in comparison to *T. harzianum* strain 39.1 under the same growth conditions. The chitinase increased within 72 h. Maximal activity (0.39 U/mL) was produced by isolate 1051. This activity was 45-fold and 13-fold higher than those determined for the TVC (0.085 U/mL) and 39.1 (0.013 U/mL) strains, respectively (Fig. 1A). The highest N-acetyl-glucosaminidase activities were produced by isolate 1051 (0.41 U/mL) and strain 39.1 (0.47 U/mL) grown for 72 h. The activity produced by strain TVC (0.01 U/mL) at 72 h was 47-fold smaller (Fig. 1B). The chitinolytic activity induced by chitin in *T. harzianum* comprises

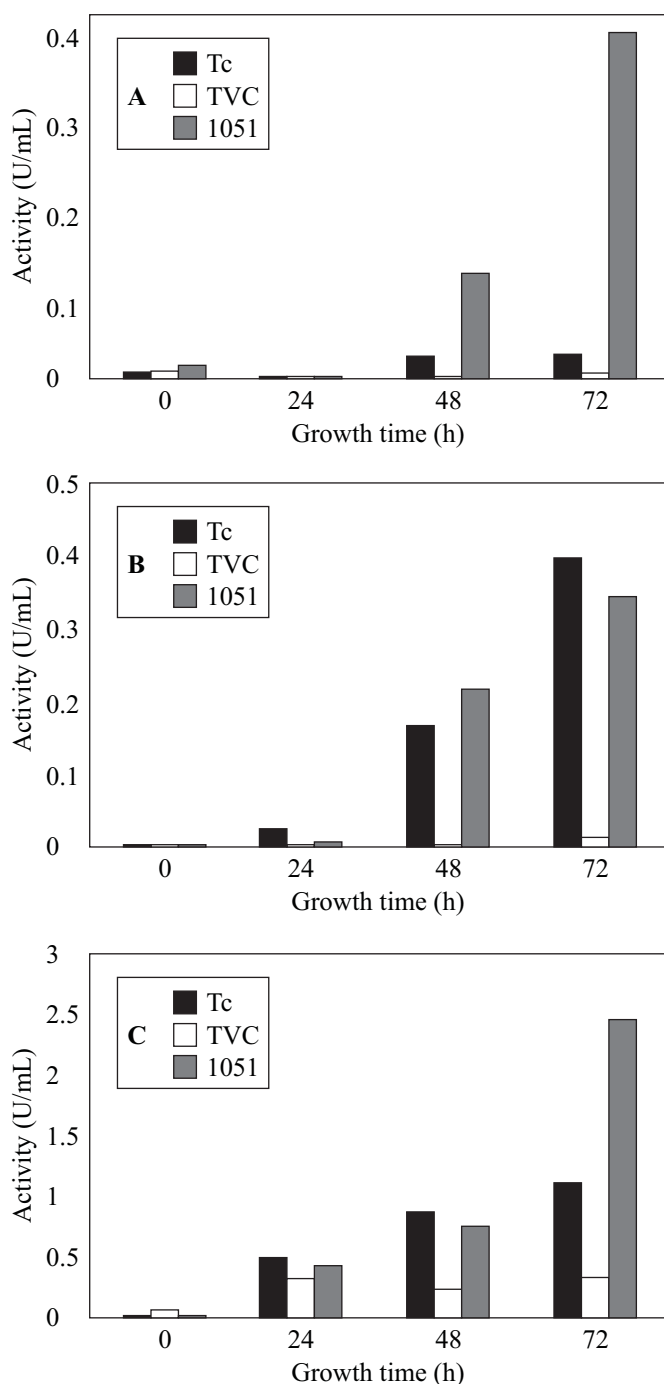


Figure 1. Chitinase (A), N-acetylglucosaminidase (B) and 1,3-glucanase (C) activities in the culture medium of *Trichoderma harzianum* isolate 1051, *Trichoderma* sp. isolate TVC and *Trichoderma harzianum* 39.1 (Tc).

at least four endochitinases, two N-acetyl-glucosaminidase and one exochitinase (17). The highest β -glucanase activity (2.8 U/mL) was also produced by *T. harzianum* isolate 1051 after 72h of

growth. This activity was 7.2-fold and 2.2-fold higher than the activity produced by *T. harzianum* Tc (1.27 U/mL) and isolate TVC (0.38 U/mL), respectively (Fig. 1C). The enzyme activities found in non-induced cultures (not shown) were very low or absent. Chitinolytic enzymes and β -glucanases are thought to play an important role in the mycoparasitism between *Trichoderma* spp and phytopathogens. We have recently demonstrated that a 46-kDa chitinase (CHIT 46) drastically affects *in vitro*, the cell walls of the phytopathogens *Sclerotium rolfsii* and *Rhizoctonia solani* (19). This CHIT 46 chitinase was shown to be secreted into the culture medium as soon as 2 h after the fungus comes into contact with chitin (20). Fungal cell walls are complex structures constituted almost exclusively of polysaccharides such as β -glucan (cellulose, β -1,3- and β -1, 6-glucan) and chitin (12). These polysaccharides may well act as inducers of hydrolytic enzymes during the antagonistic interaction of *Trichoderma* with *C. pernicioso*. In fact, the purified chitinase produced by *Trichoderma harzianum* isolate 1051 considered in this work was shown to effect “in vitro” the *C. pernicioso* cell wall (10).

Trichoderma spp. are known to produce cellulases (23) which hydrolyse β -1,4-glucans. In accordance, all three *Trichoderma* isolates were found to produce substantial amounts of these enzymes. The highest total cellulase (Fig. 2A) and endoglucanase (Fig. 2B) activities were produced by isolate 1051 after 120 h of growth. However, although the cellobiase activity (0.503 U/mL) produced by isolate 1051 after 120 h of growth was 2.64 -fold higher than that produced by isolate TVC (0.19 U/mL) after the same time of growth, this activity was 1.22-fold lower than that (0.616 U/mL) produced by strain 39.1 (Fig. 3A). The aryl- β -glucosidase activity produced by both isolates 1051 and TVC were smaller than that of produced by 39.1 (Fig. 3B). Basidiomycetes belong to a group of fungi which have chitin and glucans (probably β -1,3 and β -1,6-glucans) as main components of their cell walls (8). The β -1,4-glucanolytic enzymes produced by *Trichoderma* may well be involved in hydrolysis of the *C. pernicioso* cell wall glucans during antagonism. Alternatively, hydrolysis of the cellulose present elsewhere by cellulases would attend the *Trichoderma* parasite metabolic needs.

Filamentous fungal cell walls also contains lipids and proteins (18). It therefore expected that antagonistic fungi synthesize proteases which may act on the host cell-wall. The *Trichoderma* isolates 1051 and TVC secreted equivalent amounts (about 1.41 U/mL) of proteolytic activity after 72 h of growth. These activities were significantly higher than that (0.86 U/mL) produced by *T. harzianum* strain 39.1 under the same conditions (Fig. 4). An alkaline protease (Prb1) from *T. harzianum* strain IMI which was induced by chitin, autoclaved mycelia and fungal cell-walls was suggested to be involved in mycoparasitism (15). This assumption was further supported by the fact that direct confrontation assays between *T.*

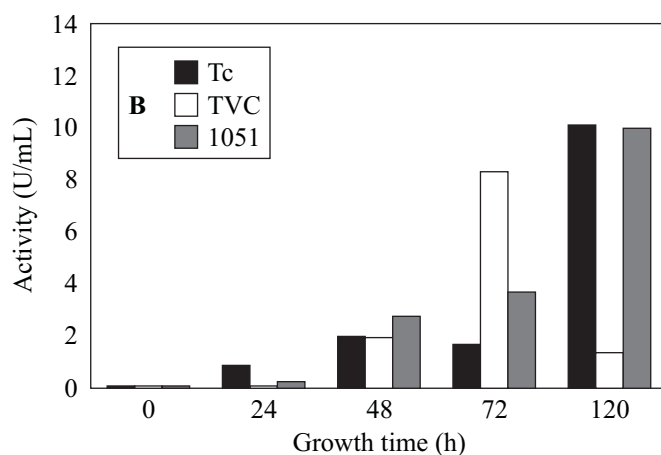
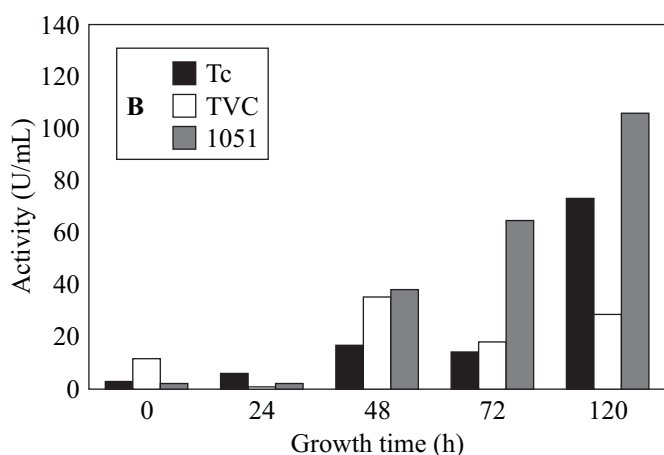
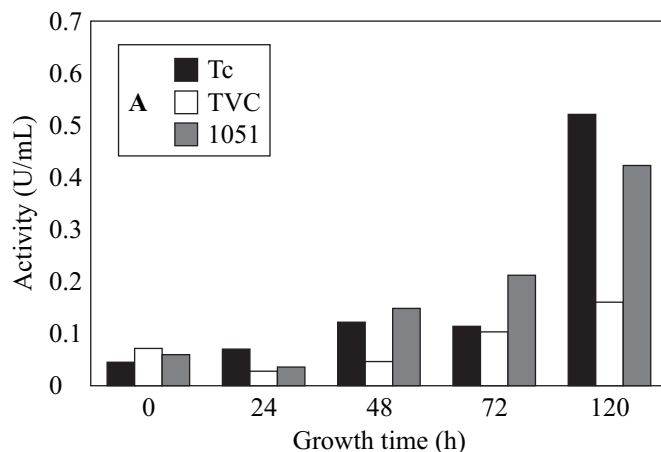
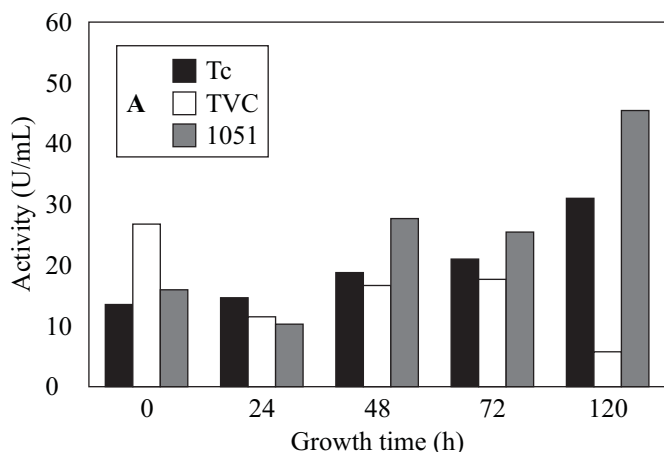


Figure 2. Total cellulase (A) and endoglucanase (B) activities in the culture medium of *Trichoderma harzianum* isolate 1051, *Trichoderma* sp. isolate TVC and *Trichoderma harzianum* 39.1 (Tc).

Figure 3. Glucosidase (A) and aryl-D-glucosidase (B) activities in the culture medium of *Trichoderma harzianum* isolate 1051, *Trichoderma* sp. isolate TVC and *Trichoderma harzianum* 39.1 (Tc).

harzianum strain IMI 206040 and *Rhizoctonia solani* resulted in induction of Pbr1 mRNA synthesis (14). The protease produced by the *T. harzianum* isolate 1051 was purified and tested for its ability to hydrolysis the *C. perniciosa* cell wall. It does affect the phytopathogen “in vitro” (11).

The *Trichoderma* isolate 1051 and strain 39.1, also produced amylolytic dextrinizing activity (2.66 U/mL) which were about 221-fold higher than that produced by isolate TVC within 60 h of growth (Fig. 5). In all cases, the activity produced in the absence of starch was insignificant, thereby indicating an inductive process. There is no report of the presence of starch in fungal cell walls. However, glycogen which is a closely related polysaccharide is present in the cell walls of some fungi (8). The Basidiomycetes group, which includes *C. perniciosa*, doesn't belongs to this particular class of fungi. In this case, the amylases could act non-specifically on the *C. perniciosa* cell wall. However, electron microscopy studies using the *T.*

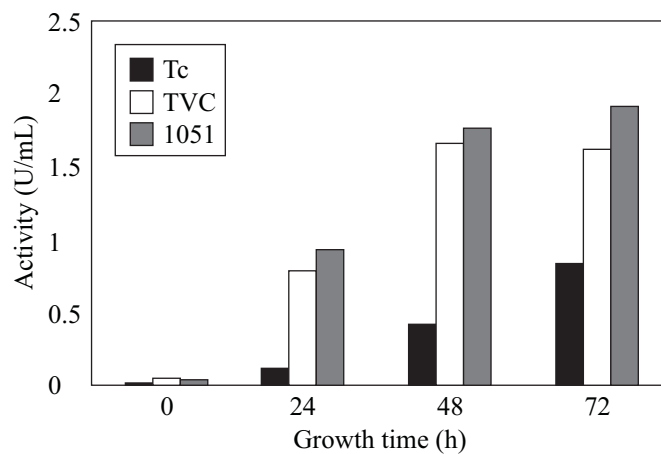


Figure 4. Protease activity in the culture medium of *Trichoderma harzianum* isolate 1051, *Trichoderma* sp. isolate TVC and *Trichoderma harzianum* 39.1 (Tc).

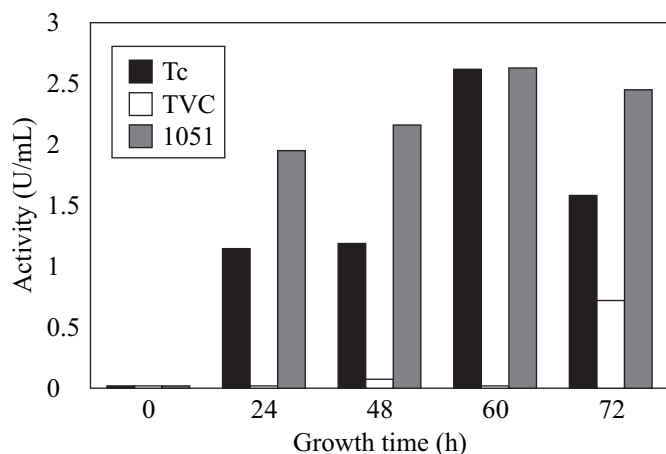


Figure 5. Amylase dextrinizing activity in the culture medium of *Trichoderma harzianum* isolate 1051, *Trichoderma* sp. isolate TVC and *Trichoderma harzianum* 39.1 (Tc).

harzianum isolate 1051 purified amylase showed that this enzyme has no action on the phytopathogen cell wall (1). Alternatively, as starch is widely distributed in nature, these enzymes could assist saprophytic survival by degrading starch present elsewhere.

RESUMO

Produção de enzimas hidrolíticas por cepas de *Trichoderma*, com atividade antagonista contra *Crinipellis pernicioso*, agente causador da “vassoura-de-bruxa” no cacau

Dois isolados de *Trichoderma* os quais reduzem a incidência da doença “vassoura-de-bruxa”, causada em plantações de cacau pelo fungo *Crinipellis pernicioso*, foram avaliados quanto ao seu potencial de produção de hidrolases em meio líquido. Na ausência de qualquer substrato nenhuma atividade hidrolítica foi produzida. As atividades de quitinase, N-acetilglicosaminidase, β -1,3-glucanase, celulase total, endoglucanase, aril- β -glucosidase, β -glucosidase, protease e amilase aumentaram significativamente durante o crescimento dos isolados em meio líquido contendo os respectivos substratos. As maiores atividades foram encontradas nos tempos de 72 a 120 horas de crescimento. Com exceção das enzimas N-acetilglicosaminidase e β -glucosidase, os maiores níveis de hidrolases foram encontrados nas culturas do isolado 1051 de *Trichoderma harzianum*. O possível envolvimento destas enzimas na interação antagonística entre *Trichoderma* e *C. pernicioso* é discutido.

Palavras-chave: *Trichoderma*, *Crinipellis pernicioso*, hidrolases, interação antagonística.

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