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BRIEF COMMUNICATION

Chitinases of *Coffea arabica* genotypes resistant to orange rust *Hemileia vastatrix*

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Abstract

Two *Coffea arabica* - *Hemileia vastatrix* incompatible interactions (I_1: coffee cv. Caturra - rust race VI and I_2: coffee cv S4 Agaro - rust race II) and a compatible interaction (coffee cv. Caturra - rust race II) were compared in relation to the infection process and chitinase activity. In the two incompatible interactions the fungus ceased growth in the early infection stages, while in the compatible interaction no fungus growth inhibition was observed. A high constitutive level of chitinase activity was detected in the intercellular fluid of healthy leaves. Upon infection, chitinase isoforms were more abundant in incompatible interactions than in the compatible interaction. Immunodetection showed that class I chitinases are particularly relevant in the incompatible interactions and might participate in the defence response of the coffee plants.

Additional key words: immunodetection, coffee-orange rust interactions, intercellular fluid, resistance response.

Orange rust, caused by *Hemileia vastatrix,* is the most widespread disease of *Coffea arabica*, causing premature leaf fall, yield losses and plant death during severe attacks. Resistance of coffee plants to orange rust is, according to the gene-for-gene interaction (Flor 1942), conditioned by at least nine dominant genes (S_H1 - S_H9) single or associated (Rodrigues *et al.* 1975). This resistance is often characterised by restricted fungal growth associated with host cell death at the infection sites (the hypersensitive reaction; HR) (Rijo *et al.* 1991, Coutinho *et al.* 1993, Martins and Moraes 1996, Silva *et al.* 2002). The HR appears to be an efficient response, particularly against biotrophic pathogens like rusts, which depend on the living host cells for survival (Mansfield *et al.* 1997, Heath 2000, Mendgen *et al.* 2000). Other defence responses are also rapidly induced, as for instance the synthesis of enzymes of the phenylpropanoid pathway and the antifungal pathogenesis-related (PR) proteins (Bowles 1990). A general role for the PR proteins in adaptation to various stress conditions, including resistance against pathogen, is well documented (Stintzi *et al.* 1993, Van Loon 1997). One family of the most well characterized PR-proteins is that of the chitinases (PR-3, PR-4, PR-8 and PR-11), enzymes that catalyse the hydrolysis of chitin, the predominant constituent of fungal cell walls (Boller 1988). Indeed, using cytochemical localization, chitin was revealed in the walls of intercellular hyphae and haustoria of coffee orange rust (Silva *et al.* 1999). Chitinases, like other PR proteins, attain high levels in the apoplast (Regalado and Ricardo 1996) where they protect the plants against extracellular growth of fungi due to the capacity to degrade fungal cell walls, leading to the inhibition of fungal growth (Salzer *et al.* 2000).

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Abbreviations: HR - hypersensitive reaction; IF - intercellular fluids; PR - pathogenesis-related.
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Changes in chitinases and glucanases of crude leaf extracts were detected in incompatible and compatible C*offea arabica* - *H. vastatrix* interactions (Maxemiuc-Naccache *et al.* 1992). These researchers observed an increase in the activities of the enzymes during the first days after inoculation, in the incompatible interactions but not in the compatible ones. Expressed sequence tags (ESTs) analysis in incompatible coffee - orange rust interactions, showed that 25 % of the ESTs have similarity with proteins involved in plant cell defence reactions, including chitinases (Fernandez *et al.* 2004). Although chitinase activity has often been associated with plant resistance to fungi the role of this enzyme in coffee resistance is not fully understood. In the present study we investigated histopathology of orange rust development and the accompanying expression of chitinase isoforms in intercellular fluids (IF) of two *Coffea arabica* genotypes differing by one gene of resistance to *H. vastatrix*.

Five years old coffee (*Coffea arabica* L.) genotypes S_H5 (Caturra) and S_H4S_H5 (S4 Agaro) were grown in a greenhouse (night/day temperature 18/25 ºC) and inoculated with fresh urediospores of *Hemileia vastatrix,* races II (v_5) and VI (unknown genotype for virulence), according to D´Oliveira (1954). These plant genotypes and fungal races establish two incompatible interactions, I_1 (*C. arabica* Caturra - *H. vastatrix* race VI) and I_2 (*C. arabica* S4 Agaro - *H. vastatrix* race II) and one compatible interaction, C (*C. arabica* Caturra - *H. vastatrix* race II).

Light microscope observations of infected leaves were performed according to Rijo and Rodrigues (1977) to evaluate the fungal development inside the leaf tissues, at 1, 2, 3, 4 and 7 d after inoculation. Chitinase was extracted from IF of inoculated and non-inoculated fresh leaf tissues as described by Parent and Asselin (1984), using 100 mM Tris-HCl buffer (pH 7.6) with 50 mM L-ascorbic acid, 500 mM KCl and 25 mM 2-mercapto-ethanol as infiltration solution. The IF fraction was desalted, concentrated on centrifugal filter (*Centricon YM-10, Millipore,* MA, USA) and stored at -80 ºC and protein content subsequently determined using the commercial *Bio-Rad* (Hercules, USA) protein assay kit. Total chitinase activity of IF was determined by the colorimetric method of Wirth and Wolf (1990) at 550 nm. Triplicate samples of leaf IFs were collected at 1, 2, 3, 4 and 7 d after inoculation and experiments were repeated at least twice.

Determination of chitinase activity directly on native gels electrophoresis (PAGE) was performed according to Minic *et al.* (1998). Ten µg of IF proteins were loaded per well and separated at pH 8.9 on a 10 % polyacrylamide gel containing 0.04 % of glycol-chitin. After running the electrophoresis, gels were incubated for 2 h at 40 ºC in 100 mM sodium acetate buffer pH 5, and then for 10 min at room temperature in 500 mM Tris-HCl buffer (pH 8.9) containing 0.01 % (m/v) fluorescent brightener 28 (*Sigma-Aldrich,* Steinheim, Germany), and subsequently washed several times with distilled water. The lytic zones, appearing as dark bands on a fluorescing background, were detected by UV radiation. Amount of chitinase in bands on zymograms were evaluated using the *ImageQuant*™ TL image analysis software (*GE LifeSciences*).

For chitinases immunodetection IF proteins were separated by electrophoresis under denaturing conditions, performed in 12 % SDS-polyacrylamide gels according to Laemmli (1970). After SDS-PAGE, the separated proteins were transferred onto polyvinylidene difluoride membranes and probed with polyclonal antibodies raised against class I chitinase from potato (E. Kombrinck, Max Plank Institute, Leipzig, Germany), class I/II chitinase from barley (N.P. Olivia, International Rice Research Institute, Manila, Philippines), class III chiti-nase from lupin (Regalado and Ricardo 1996) and class V chitinase from tobacco (D. Gillham, *Syngenta*, Reading, UK). Immunodetection was performed using a chemiluminescent substrate for horseradish peroxidase secondary antibody according to Pierce's Western blot protocol (goat anti-rabbit IgG horseradish peroxidase conjugated; *SuperSignal West Femto*, Pierce, USA).

As previously described (Rijo and Rodrigues 1977, Silva *et al.* 1999, 2002) urediospores of *H. vastatrix* germinate on the lower epidermis of coffee leaves, producing a germ tube that subsequently differentiates an appressorium on stomata. Urediospore germination and appressoria formation over stomata appeared to be readily accomplished by both orange rust races (II and VI). In both incompatible interactions, the fungus ceased its growth in the early stages of the infection process, either before haustorium formation (I_1 interaction) showing a pre-haustorial resistance, or after the formation of a reduced number of haustoria (I_2 interaction), post-haustorial resistance (Fig. 1). On the contrary, in the compatible interaction (C) the fungus grew in the majority of infection sites with no apparent inhibition (Fig. 1).

Using the Wirth and Wolf (1990) method, total chitinase activity was detected in leaves of healthy plants and it was slightly increased in the incompatible interactions I_1 and I_2 (data not shown). In fact, when using native PAGE gels, all chitinase isoforms bands were already present in healthy tissues. However, when comparing the densitometric volumes of the chitinase bands in native PAGE gels, differences between incompatible I_1, I_2 and C were noticeable, particularly in the early stage of the infection process. In the I_1, the intensity of bands with Rf 0.15, 0.19, 0.24, 0.29, 0.45 and 0.70 was higher than in the C and in the healthy leaves (H_1), during the first 1 - 3 d after inoculation (Fig. 2). For the I_2 the intensity of the bands with Rf 0.23, 0.27 and 0.62 was higher then in healthy leaves (H_2) since the first day after inoculation. On the contrary, bands with Rf 0.32, 0.83 and 0.90 only increased by 4 - 7 d after inoculation (Fig. 2). For C interaction the intensity of bands with Rf 0.29, 0.45, 0.52 and 0.66 increased only in a later stage of the infection process (4 - 7 d after

inoculation) (Fig. 2).

In order to get additional information on the nature of the detected chitinases we used several antibodies corresponding to different classes of chitinases (I, I/II, III and V). The Western blots gave negative results with antibodies from classes I/II and V (data not shown) and positive results for classes I and III (Fig. 3). Class I antibody detected a band of 36 - 37 kDa and class III antibody a band of 37 - 38 kDa, in both I_1 and C interactions (Fig. 3). For the I_2 the class I antibody detected bands of 33, 34, 37 and 38 kDa and class III antibody detected bands of 30, 31, 36 and 38 kDa (Fig. 3). Furthermore in I_2 it was noted that in early stages of infection (1 - 3 d after inoculation) the level of detection of class I antibody was higher than in the healthy leaves (H_2). For I_1, since the fungus dies very early (before haustorium formation), the infection sites are quite restricted and so chitinase activity can not be followed for a long period.

Constitutive forms of plant chitinases (vacuolar or apoplastic) have been associated with embryogenesis (Domon *et al.* 2000, Rojas-Herrera and Loyola-Vargas 2002) and other developmental processes, and also with different stress conditions, including defence responses (Reiss and Bryngelsson 1996, Kombrink and Somssich 1997, Regalado *et al.* 2000, Profotová *et al.* 2007, Serrano *et al.* 2007). Most of the chitinases are capable of degrading fungal cell walls *in vitro*, especially at the hyphal tips and, thus, have a protective function against fungal colonization of plants (Boller 1988, Neuhaus 1999).

We also have detected a high constitutive level of chitinase activity in the IF of healthy *C. arabica* leaves, but we observed in the early stages of the I_1 and I_2 an increase in chitinase activity, in association with restriction of fungal growth. As previously shown, this is coincident with cell death (HR) (Rijo *et al.* 1991, Coutinho *et al.* 1993, Martins and Moraes 1996, Silva *et al.* 2002), suggesting that chitinases take part in this response. Indeed, chitinases have been implicated in the regulation of events leading to program cell death (PCD) (Passarinho *et al.* 2001), which appear to be associated with the HR (De Wit 1997, Heath 2000).

As a further step in the study of the coffee chitinases we described for the first time the diversity of chitinase isoforms, and analyse their association with the infection process. As concerns the first stages of coffee-rust interaction, the chitinases isoforms showed higher activity in I_1 and I_2 than in C, suggesting they have a role in the resistance response. Although an increase of activity of some chitinase isoforms was also detected in C, this was only observed in a later stage of the infection when the fungus was already established in the tissues. The high chitinase activity detected in I_2 was accompanied by an increased expression of class I chitinases. These chitinases are of basic nature, contain a cysteine-rich N-terminal domain with putative chitin-binding properties, and have antifungal activity (Neuhaus 1999).

As a general conclusion it can be said that infection by an avirulent pathogen leads to a faster and higher induction of chitinase accumulation, while the attack by a virulent pathogen induces chitinases to a lesser extent and more slowly (Kasprzewska 2003). It appears that there exists some correlation between the early expression of chitinases in the incompatible coffee-orange rust interactions with the restriction of fungal growth and the hypersensitive response. However, the exact function of these chitinase isoforms in resistance of coffee plants against orange rust needs further study.

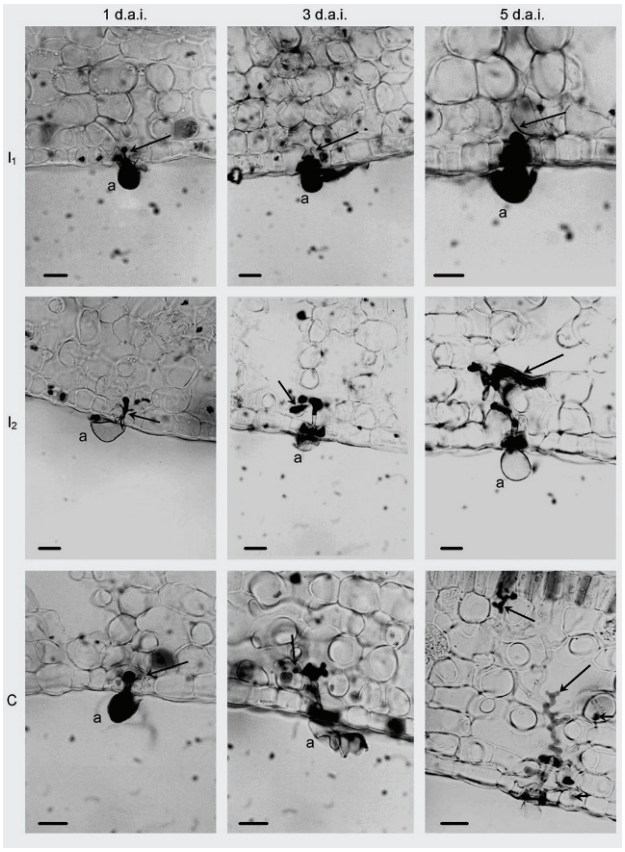

Fig. 1. Colonization of leaf tissues by the fungus in different compatible (C) and incompatible (I_1 and I_2) coffee-rust interactions, 1, 3 and 7 d after inoculation (d.a.i.). Light microscope observations, blue lactophenol staining. I_1 - appressorium (a) over stomata and penetration hypha (*arrow*), *scale bars* = 15 µm (1, 3 and 7 d.a.i.); I_2 - appressorium (a) over stomata and penetration hypha (*arrow*), *bar* = 13 µm appressorium (a) over stomata, intercellular hypha with a haustorium (*arrow*) in the mesophyll cell, *bar* = 17 µm (3 d.a.i.), appressorium (a) over stomata, and senescent hypha, (*arrow*), *bar* = 14 µm (7 d.a.i.); C - appressorium (a) over stomata, penetration hypha (*arrow*), *bar* = 14 µm (1 d.a.i.), appressorium (a) over stomata, intercellular hypha with an haustorium (*arrow*) in the mesophyll cell, *bar* = 17 µm (3 d.a.i.), intercellular hyphae in the mesophyll cells (*arrows*) and haustoria (*arrowheads*), *bar* = 17 µm (7 d.a.i.).

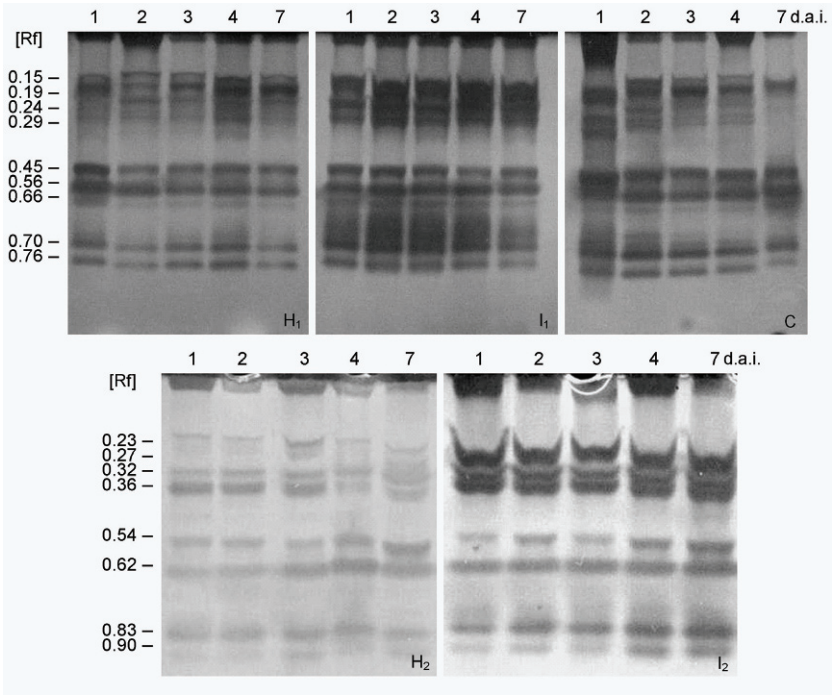

Fig. 2. Native PAGE analysis of chitinase activity of leaf IF proteins from coffee-orange rust interactions. Gels were stained with glycol-chitin as chitinase substrate. Healthy leaves (H_1 and H_2), compatible (C) and incompatible (I_1 and I_2) interactions at 1, 2, 3, 4 and 7 d after inoculation (d.a.i.). The scale indicates relative mobility (Rf) of chitinase isoenzymes. Changes shown were consistently observed in at least 3 independent experiments.

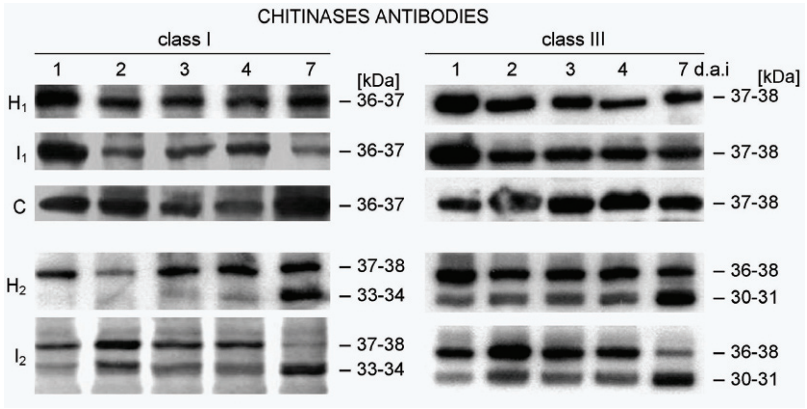

Fig. 3. Western blot analysis of leaf IFs proteins from coffee-orange rust interactions. Polyclonal antibodies raised against chitinases from class I and III, were used at 5000-fold dilution to detect coffee antigens. Healthy leaves (H_1 and H_2) compatible (C) and incompatible (I_1 and I_2) coffee-orange rust interactions at 1, 2, 3, 4 and 7 d after inoculation (d.a.i.). Changes shown were consistently observed in at least 3 independent experiments.

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