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Research Article

Expression of Hydrolytic Enzymes During Interaction of *Moniliophthora roreri*, Causal Agent of Frosty Pod Rot and *Theobroma cacao* Pods

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Abstract

Moniliophthora roreri is a hemibiotrophic fungus causal agent of Frosty Pod Rot (FPR) in cacao (*Theobroma cacao*) pods. The *M. roreri* causes one of the most economically important diseases of *Theobroma cacao* in producing areas in Mexico, Central and South America. The hemibiotrophic fungi have genes that allow them to infect pods by penetration through the surfaces of the cacao pod. Until now, it is not yet clear what is the mechanism used by *M. roreri* to penetrate the cacao pod. In this study the induction of the genes related to this phenomenon was evaluated in order to propose a mechanism of infection. Production of chitinases, chitosanases, glucanases, proteases, lipases and cutinases were induced *in vitro* by growing *M. roreri* 111A in 1% (w/v) of colloidal chitin, chitosan, laminarin, casein, tween 20™, apple cutin and cocoa pod shell. Cacao pod shell used as substrate induced the highest enzyme specific activities of chitinases (1.338), chitosanases (0.430), glucanases (0.430) and cutinases (0.167) compared to chitin (1.010), chitosan (0.316), laminarin (0.301) and apple cutin (0.040) in 12 h. The effect of carbon source on enzyme activity in a "Cross-inducted" way was evaluated. Tween 20 induced higher specific activities of chitinases (10.31), chitosanases (9.38), glucanases (7.89), lipases (4.66) and cutinases (5.32). Conversely casein did not increase the proteolytic activity (3.27) as did laminarin (4.45), chitin (4.28) and glucose (3.90). The levels of gene induction obtained by RT-PCR cultured with cocoa pod shell at 12 h showed that a chitinase, a lipase and a cutinase had significant differences ($p = 0.05$) compared to a basal expression with glucose. The data suggest that these enzymes may be involved in the early stages of the infection process by *M. roreri*.

Key words: *Moniliophthora roreri, Theobroma cacao*, frosty pod rot, biotrophic pathogen

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Theobroma cacao is a tropical tree whose fruits are the source of seeds that are converted into cacao products, such cocoa pozwder and cocoa butter. The tree grows around the world in tropical climates with sufficient moisture (Schwan and Wheals, 2004). However, the high relative humidities in which cacao trees grow favor the development of fungi that cause diseases that threaten production. Cacao production has been severely limited by fungal diseases throughout Central and South America. These diseases include *Moniliophthora perniciosa* (Stahel), which causes Witches Broom Disease (WBD) and *Moniliophthora roreri* (Evans, 2016) that causes FPR. The *M. perniciosa* infects all cacao meristematic tissues and *M. roreri* attacks only the *T. cacao* pod (Marelli *et al*., 2009). These pathogens cause two of the most economically important diseases of *T. cacao* in Central and South America (Meinhardt and Bailey, 2016).

An important aspect to understand the FPR disease is the expression of genes during the interaction between *M. roreri* and *T. cacao* pod. The *M. roreri* is a hemibiotrophic basidiomycete who initiates infection with a period of biotrophy, followed by a necrotrophic phase. At the initial stages of biotrophy *M. roreri* causes only minor responses from the plant and appear to evade plant defenses with stealthy methods (Mendgen and Hahn, 2002; Fujikawa *et al*., 2012). In the necrotrophic phase *M. roreri* causes rapid cell death in hosts elicits major molecular responses from the plant and overwhelms the plant defenses. Pods develop malformations, secrete copious amounts of lytic enzymes and toxins and sporulation and rot occurs. The research of how *M. roreri* interacts with *T. cacao* pods is derived from these two extremes.

Recently, Meinhardt *et al*. (2014) sequenced the *M. roreri* genome and the predicted open reading frames were validated by RNA-Seq. In order to find out genes related to each stage, the differential gene expression was estimated for the biotrophic (30 days post infection) and necrotrophic (60 days post infection) phases. However, the mechanism of infection in the 1st h, such as recognition, sensing and adhesion of *M. roreri* to *T. cacao* pod must be critical to develop the disease. To the best of our knowledge, there are no reports of the genes and enzymatic activity of *M. roreri* in the very early stages of contact to *T. cacao* pods.

Infection and penetration strategies are diverse, ranging from mechanical penetration of the cuticle, usage of natural or artificial breaches like stomatas or wounds and enzymatic degradation of plant cuticle and aerial surfaces. The aim of this research was to study the *in vitro* expression of some enzyme genes involved in the early stages of the infection of *T. cacao* pod by *M. roreri*. Additionally, we tested *in vitro* ability of *M. roreri* to induce hydrolytic enzymes related to cacao pod infection.

MATERIALS AND METHODS

Fungal strain isolation: The strain used was isolated from FPR-infected cocoa pods collected from Union Miramar farm in Chiapas (Latitude 27.625, Longitude 100.791), Mexico. Briefly, pods were superficially disinfected by immersion in a 2% sodium hypochlorite solution for 10 min and rinsed 3 times with sterile water. A cork borer was inserted in the pod to obtain a cylindrical sample. A portion of this cylinder 5 mm below the pod surface was cut and placed on Potato Dextrose Agar (PDA) and incubated for 4-7 days at 25°C. The growing colonies were selected by their appearance, cut using a scalpel and transferred to a new PDA plate for incubation under the same conditions.

DNA extraction, PCR and sequencing: DNA extraction of *M. roreri*, PCR and sequencing were carried out according to Cuervo-Parra *et al*. (2011).

Molecular identification: The DNA sequence was submitted to homology search in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and best hit sequences were downloaded. A phylogenetic tree was constructed using the Mega 6 software (Tamura *et al*., 2013) by Neighbor-joining with 500 bootstrap replicates and it was supported by the bootstrap method.

Production of hydrolytic enzymes by *M. roreri*: Flasks containing 40 mL of PDB were inoculated with mycelial plugs of *M. roreri* grown previously 7 days on PDA. Flasks were incubated for 4 days at 25°C to allow mycelial development. The exhausted media were removed by decantation in aseptic conditions and fungal pellets were rinsed with sterile water in order to remove remaining media. About 40 mL of culture medium of induction were added to each flask. The medium consisted of KNO_3 6.0, K_2HPO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.002, $ZnSO_4 \cdot 7H_2O$ 0.002, $MgCl_2 \cdot 7H_2O$ 0.002 and carbon source 10 expressed as g L^{-1} in distilled water and pH was adjusted to 5.0. The carbon sources used were glucose, chitin, chitosan, laminarin, casein, tween 20, apple cutin and cocoa pod shell (freeze-dried and ground). Flasks in duplicate were then incubated at 25°C and 2 mL samples were collected at 0, 1, 2, 4, 8 and 12 h after inoculation. Samples were centrifuged at 5000 g for 5 min at 4°C and the

supernatants from each sample were used for the determination of enzyme activity. Mycelial pellets were used for RNA isolation.

Enzyme activity assays and protein determination: Chitinase, chitosanase and glucanase activities were determined according to the DNS method (Miller, 1959). One unit of chitinase (or chitosanase) activity was defined as the amount of enzyme needed to liberate 1 mmol of N-acetylglucosamine from colloidal chitin (or chitosan) in a minute. One unit of glucanase activity was defined as the amount of enzyme needed to release 1 mmol of glucose from 1% laminarin suspension in a minute (De Marco *et al*., 2003). Proteolytic activity was determined incubating at 60°C for 30 min a mixture of supernatant (0.2 mL), 0.4 mL of casein (Sigma), 0.5% (w/v) in distilled water and 0.4 mL 0.2 M acetate buffer, pH 5.5. One unit of protease activity was defined as the amount of enzyme needed to increase 0.1 unit the absorbance at 280 nm of the sample measured under the assays conditions (Merheb *et al*., 2007). Lipolytic activity was determined incubating a mixture of supernatant (0.1 mL), 0.1 mL 0.01 M of p-nitrophenil-laurate (pNPL) in ethanol at 60°C for 30 min. After 0.25 mL of 0.1 M Na_2CO_3 were added and the mixture was centrifuged (16,000×g for15 min at 25°C), the absorbance at 410 nm was measured. One unit of lipase activity was defined as the amount of enzyme needed to release 1 mmol of p-pnitrophenol from pNPL under the assay conditions (Nawani *et al*., 1998). Cutinase activity was determined by a spectrophotometric assay (405 nm) using p.nitrophenil-butirate (PNPB) as substrate. Culture supernatant (100 mL) was added to a mixture of 900 mL (100 mL PNPB 50 mM dissolved in 800 mL 50 mM phosphate buffer pH 7.0, 0.2% triton X-100 and 0.43 M tetrahydrofuran). The mixture was incubated for 1 min against a blank solution. One unit of cutinolytic activity was defined as the amount of cutinase required to release 1 mmol of p-nitrophenol under the specified conditions (Calado *et al*., 2002). Apple cutin was prepared according to Baker and Bateman (1978). Protein determination was made according to Bradford (1976).

PCR amplification of hydrolytic enzymes-encoding genes: The six pairs of primers shown in the Table 1 were designed based on sequences of the enzymes of *Moniliophthora perniciosa*. PrimerBlast online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used and the primers were synthesized at the IBT (Instituto de Biotecnología, UNAM, Cuernavaca, Mexico). The DNA extraction of *M. roreri* was performed according to Cuervo-Parra *et al*. (2011) and PCR according to the manufacturer's instructions (Promega Kit). Reactions were performed in a thermocycler (Bio Rad Model Gene Cycler™) and PCR reactions were performed according to the following setting: One cycle of 2 min for initial denaturation at 95°C, 35 cycles of three stages: Denaturation for 45 sec at 95°C, primer annealing for 45 sec at 55°C and extension for 1 min at 72°C and a final extension step of 7 min at 72°C. Amplicons were purified with the Geneclean® kit (Hercules, CA) and sequenced by the IBT. Sequences were deposited in the NCBI GenBank.

Determination of the expression level by qRT-PCR: Samples of *M. roreri* mycelium were recovered at 0 and 12 h in duplicate after inoculation from flasks containing culture media with glucose and cocoa shell as carbon source. About 100 mg of freeze-dried ground mycelium was used for RNA isolation according to the trizol reagent® protocol (Invitrogen, USA). The RNA was suspended in nuclease-free water and its concentration was determined by spectrophotometry. To perform the qRT-PCR the Power Sybr® Green RNA-to-CT 1-step kit (Applied Biosystems, USA) was used. Oligonucleotides were designed and synthetized from the DNA sequence of the amplicons obtained previously (Table 2). The reaction mixture (50 µL) contained 25 µL of Power Sybr® Green RNA mix (2X), 1 µL (10 µM) of each primer, 0.125 µL RT enzyme mix, 1 µL of RNA template. The reaction consisted of reverse transcription step (48°C for 30 min) and the PCR: 95°C for 10 min and 40 cycles of denaturation at 95°C for 15 sec and alignment and extension at 60°C for 1 min. Reactions were carried out in a StepOne plus® thermal cycler (Applied Biosystems, USA). Data obtained were analyzed using the software StepOne™ (Applied Biosystems, Carlsbad, CA, USA) applying the Ct method to find the fold value for each sample (Schmittgen and Livak, 2008). The GAPDH gene was used as control. The RNA obtained from 0 h on glucose medium was used as reference. The qRT-PCR reactions were carried out in duplicate and the C_T (cycle treshold) values were obtained.

Table 1: Primers for enzyme genomic search by PCR

Gene	Code	Forward primer	Reverse primer
Chitinase	Chis1	5′-CAC AAG CAC CAA ACA AGA AC-3′	5′-AAA GAA GCG CGA GAC TCT A-3′
Chitosanase	Chit	5′-AGC GAG CAG ACT CCA TTG-3′	5′-CAG CTT GCG AGT AAG CAT C-3′
Endoglucanase	Gluc	5′-GCG TCA TCA TTC GCA CTA-3′	5′-TCT TAC TCT TAT CCC AAG ACA G-3′
Serin carboxipeptidase	SCP	5′-CTC GTT GGA ATG CTC GCT C-3′	5′-CTG CGA AAA CCC AGT TCC AA-3′
Lipase	Lip	5′-TGT AAA GGA CCT GTT GAT GC-3′	5′-TTA GTT GAA TTC CCC GTC AA-3′
Cutinase	Cut	5′-TTG GAA AGA ACG AAT TGG GC-3′	5′-TCC TCG TCC CTG CTT TG-3′

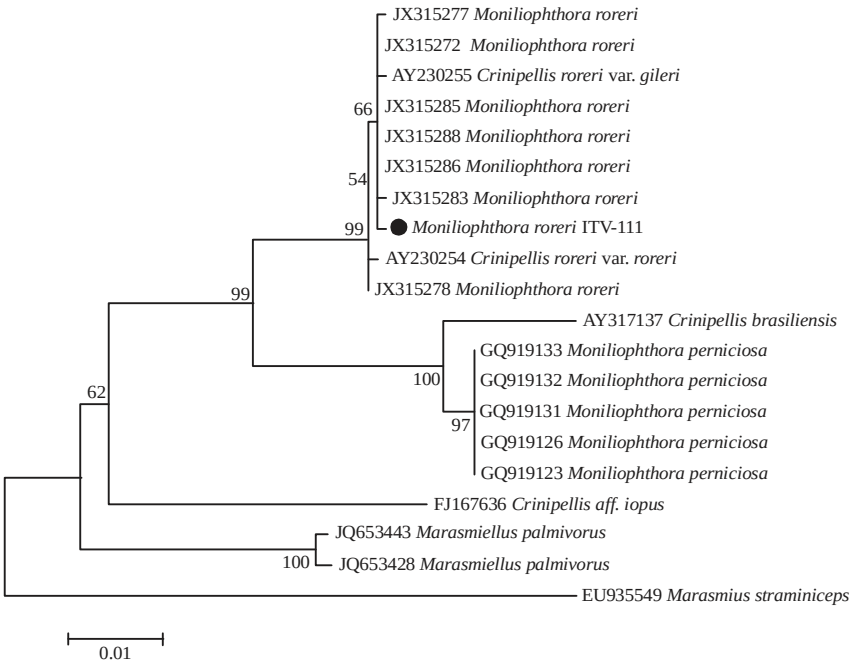

Fig. 1: Evolutionary relationships of taxa, the evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.20200893 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 567 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6

Table 2: Oligonucleotides for quantitative SYBRGreen™ RT-PCR

Gene	Code	Forward primer	Reverse primer	bp	Gene coverage
Chitinase	Chis1RT	5′-TCC GTT ACT GCT TCA GTG GT-3′	5′-AAA GAA GCG CGA GAC TCT A-3′	126	886-1012
Chitosanase	ChitRT	5′-AGC GAG CAG ACT CCA TTG-3′	5′-GAT GCC AGG CGC ATA CCT-3′	103	1-103
Endoglucanase	GlucRT	5′-GTC CCA TTG GGG TAC ATC C-3′	5′-TCT TAC TCT TAT CCC AAG ACA G-3′	92	605-697
Serincarboxipeptidase	SCPRT	5′-CTC GTT GGA ATG CTC GCT C-3′	5′-CTG TCG AGC GTG TAA TTC GTT-3′	102	1-102
Lipase	LipRT	5′-ACT CCA CCT CCC CTT ACC AT-3′	5′-ACC TTG TGG AAT GGG GTC TG-3′	94	23-116
Cutinase	CutRT	5′-TTG GAA AGA ACG AAT TGG GC-3′	5′-CCG TTA CTG GCA TCC CCT C-3′	140	1-140
Glyceraldehyde-phosphate Dehydrogenase*	GPDH*	5′-GGA TCT GTC GGT GCT CAC TA-3′	5′-AAC GTA CAT GGG TGC ATC AG-3′	--	--

*GPDH is used as reference gene (Rincones *et al*., 2008)

Average C_T for each gene studied was subtracted from the average C_T values for the reference gene (GPDH) and in this way the normalized C_T value for each gene was obtained. Normalized C_T value of each gene at 0 h of incubation was subtracted from the values of normalized C_T of the same gene at 12 h, thus obtaining the ΔC_T for each gene. By substracting the ΔC_T of a gene in glucose medium from that in cocoa pod medium was obtained $\Delta\Delta C_T$, which represents the change in gene expression during growth on glucose medium respect medium cocoa pod. Quantitation Relative Value (RQ) is calculated by the formula "$2^{-\Delta\Delta C_T}$".

RESULTS AND DISCUSSION

Identification of *M. roreri* ITV111 based on ITS sequences: Molecular identification of *M. roreri* was carried out by sequencing Internal Transcribed Spacer (ITS) regions. The *M. roreri* ITV111 ITS regions was 99% similar to *M. roreri* ITS sequences of *M. roreri* strains (GenBank numbers DQ-222923, DQ-222925 and DQ-222927). Next *M. roreri* ITS sequences were obtained from the NCBI database and used to construct a phylogenetic tree (Fig. 1). The *M. roreri* ITV-111 is phylogenetically related to the isolates of *M. roreri*

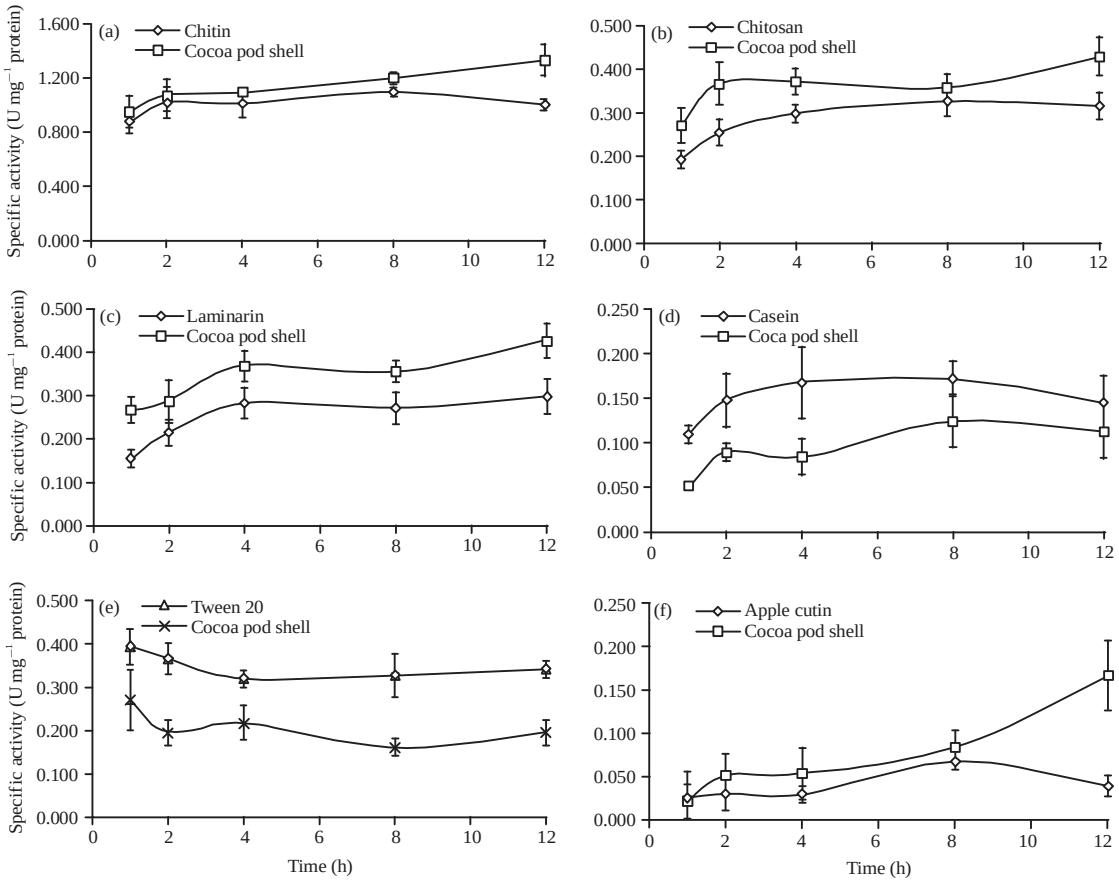

Fig. 2(a-f): Enzymatic activity in culture supernatants from *Moniliophthora roreri* 111A growing for 12 h on minimal medium supplemented with 1% (w/v) of (a) Colloidal chitin, (b) Chitosan, (c) Laminarin, (d) Casein, (e) tween 20™ and (f) Apple cutin or cocoa pod shell, lyophillized and powdered as carbon source. The standard errors for each sample mean are indicated by the vertical bars

C6, C13, C14 and C15 that belongs to the subgroup IV of *M. roreri*, which are found mainly in eastern Colombia and Central America (Phillips-Mora *et al*., 2007). This cluster is well separated from other isolates of *M. roreri.*

***M. roreri* ITV111 induce hydrolytic enzymes with cocoa pod shell as carbon source:** Figure 2 shows the differences in the production of extracellular enzymes among the carbon sources (chitin, chitosan, laminarin, apple cutin, casein and tween 20) and cocoa pod shell. Enzyme activities were significantly greater in cocoa pod shell medium than in media containing chitin, chitosan, laminarin and apple cutin as the carbon source. In contrast, proteolytic and lipolytic activities were significantly low in cocoa pod shell medium in comparison with casein or tween 20 media. Next the effect of carbon source was evaluated on enzyme activity in a "Cross-inducted" way. In this study it is found that *M. roreri* ITV111 produced varied amounts of enzymes and responded differently according to the substrate added to the medium (Table 3). Surprisingly tween 20 induced higher specific activities of chitinases (10.31), chitosanases (9.38), glucanases (7.89), lipases (4.66) and cutinases (5.32) (U mg^{-1} protein). Rubio *et al.* (2008) found that olive oil as carbon source induced high levels of expression of cutinases in comparison with cutin or pectin in *T. harzianum*. Pio and Macedo (2007) found that in *Fusarium oxysporum*, using flaxseed as carbon source induced higher amounts of cutinases (15.2 U mL^{-1}) in comparison with cutin (11.2 U mL^{-1}). Cutin is a non-soluble biopolyester composed of fatty acids and is a structural component of the cuticle that covers the cocoa pod shell (Pio and Macedo, 2007). These results also showed that casein did not increase the proteolytic activity (3.27) as did laminarin (4.45), chitin (4.28) and glucose (3.90). Chitinase activity (U mg^{-1} protein) was also detected in a medium

Table 3: Enzymatic activities of culture supernatant evaluated at 8 h growth

	Enzymatic activity (U mg^{-1} protein)					
Substrate	Chitinase	Chitosanase	Glucanase	Protease	Lipase	Cutinase
Glucose	2.355±0.59	2.141±0.536	3.458±1.208	3.899±2.420	0.557±0.431	ND
Colloidal chitin	7.668±0.427	6.971±0.388	4.112±4.134	4.283±1.362	ND	ND
Chitosan	7.400±1.647	6.727±1.497	8.448±4.569	2.450±1.647	0.572±0.844	1.076±2.535
Laminarin	5.891±2.466	5.355±2.242	7.732±3.065	4.454±1.613	2.898±3.116	ND
Casein	4.524±2.797	4.113±2.543	5.510±0.771	3.272±0.519	1.408±0.525	0.303±1.696
Tween 20	6.980±0.824	6.346±0.749	4.433±2.776	2.111±0.823	3.224±1.243	4.167±0.505
Apple cutin	10.314±2.543	9.377±2.312	7.891±2.623	2.875±0.415	4.659±1.31	5.317±1.098
Cocoa pod shell	8.908±2.060	8.098±1.873	8.060±1.453	2.241±0.861	0.589±0.398	0.905±1.049

supplemented with apple cutin (8.91), colloidal chitin (7.67) and chitosan (7.40) and cutinase activity in the medium supplemented with cocoa pod shell (1.8), chitosan (1.08) and apple cutin (9.91). Moreira *et al*. (2005) showed that the pathogenic fungus *Myrothecium verrucaria* produced greater protease activities (U mL^{-1}) in xylose (45.9), glucose (81.4), maltose (58.1), lactose (89.0) and olive oil (69.55) than in casein (33.4) or ovoalbumin (43.4). Conversely Esteves *et al*. (2009) reported that *P. chlamydosporia* induced greater proteolytic activity in minimal medium than in a medium containing gelatin. According to our data *M. roreri* ITV111 not only had the ability to induce enzymatic activities when grown in an appropriate polysaccharide, but also induced other enzymatic activities in a "Cross-induction way". These results showed that this type of induction could play an important role in the infection process of *M. roreri*. This behavior of cross-induction has been previously reported in *T. harzianum* (Rubio *et al*., 2008), *Fusarium oxysporum* (Pio and Macedo, 2008), *Venturia inaequalis* (Koller *et al*., 1991) and *Myrothecium verrucaria* (Moreira *et al*., 2005).

This study also shows the expression of chitinases (2.36) chitosanases (2.14), glucanases (3.46), proteases (3.90) and lipases (0.56) with glucose as carbon source. These results are opposite to those reported by Moreira *et al*. (2005) who found that "Polysaccharidases" were repressed by glucose. Enzyme induction of polysaccharidases, proteases and lipases in the presence of glucose may indicate a more permanent role for these enzymes rather than just degradation (Koller *et al*., 1991) and presumably play an important role in the phytopathogenicty of *M. roreri*.

In the biotrophic stage of infection of *Theobroma cacao* pods by *M. roreri*, the production of the fungus cell wall glycosyl hydrolases is inducted within the 30 days post infection (dpi). These genes were primarily related to processes of nutrition and modification of plant and fungal cell walls (Meinhardt *et al*., 2014) but there are not reports yet wether the fungus is able to produce degradative enzymes *in vitro*. According to the results obtained in this study, *M. roreri* was not only capable of *in vitro* expressing the enzymes: Chitinase chitosanase, glucanase, serine protease, lipase and cutinase when grown in suitable polysaccharides for the expression of particular hydrolases, but also expressed other activities in a "Cross-inducted" way.

PCR amplification of hydrolytic enzymes-encoding genes: Chitinase, chitosanase, endoglucacanase, serincarbopeptidase, lipase and cutinase *M. roreri* ITV111 genes were amplified and sequenced. Their molecular size were 667, 631, 533, 540, 492 and 571 bp. The sequences were subjected to homology search using the NCBI BLAST and the results showed that they were similar to mRNA of *M. roreri* strain 2997: 98% to chitinase (XM_007853204), 100% to chitosanase (XM_007847502), 97% to endoglucanase (XM_007859339), 98% to serincarbopeptidase (XM_007854997), 98% to lipase (XM_007852651) and 99% to cutinase (XM_007844694). The sequences obtained were employed to design new primers which were then used to carry out the qRT-PCR reactions (Table 2).

Expression level by qRT-PCR: Figure 3 shows the relative gene expression levels (DDC_T) of the six genes during growth of *M. roreri* ITV111 on minimal medium suplemented with cocoa pod shell or glucose as carbon source. Three of the genes (CHIT, LIP and CUT) showed significant differences ($p = 0.05$) when the expression was compared during growth on glucose or *M. roreri* cocoa pod shell. It has already been widely reported the existence of fungal cutinases, however, to date, it has not been clearly established the role of this enzyme in the phytopathogenicity of fungi. It has been shown that the hydrolysis products of the cuticle by cutinases: 10, 16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid induces the germination process in *Fusarium solani* spores probably because they indicate contact with the plant surface (Podila *et al*., 1988). The knockout of certain cutinases in this same fungus prevents the formation of penetration structures. A signaling system based on cutinases has been proposed (Skamnioti and Gurr 2008; Rogers *et al*., 1994). In other cases, disruption of cutinase genes decreased pathogenicity but not germination

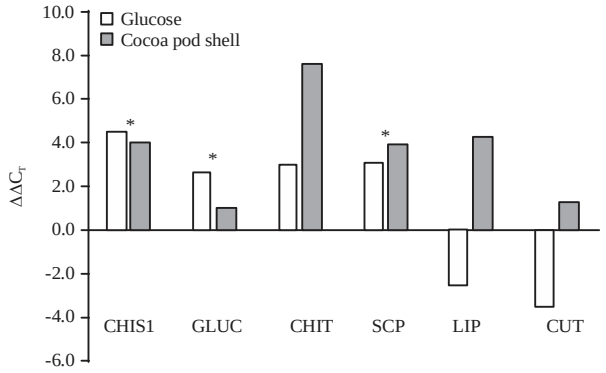

Fig. 3: Relative expression levels of chitinase (Chis1), glucanase (Gluc), chitosanase (Chit), protease (SCP), lipase (LIP) and cutinase (CUT) of *Moniliophthora roreri* 111A growing 12 h on minimal media supplemented with 1% glucose and cocoa pod shell. The C_T values were obtained for each gen at 0 and 12 h after inoculation (hai). Normalization were made using GPDH as reference gene. Normalized C_T values differences were calculated for each gene from 0-12 hai. The RQ was calculated using this value of each substrate, *No Significantly difference according to Tukey's test (p = 0.05)

as in *Pyrenopeziza brassicae* (Davies *et al*., 2000) and *Fusarium solani* (Chassot and Metraux, 2005; Shaykh *et al*., 1977). Some authors studied the rol of cutinase as degrading enzyme in fungi (Rubio *et al*., 2008). In *M. roreri*, a cutinase was expressed during the necrotrophic phase of the infection of cocoa pods, suggesting that this could be the role for this enzyme in *M. roreri*. However, further research is required to determine the role of this enzyme. Other enzymes expressed by plant pathogenic fungi are lipases, that are used for membrane degradation and reserve lipids of plants as carbon source. Disruption of genes of lipase in *F. graminearum* resulted in loss of phytopathogenicity (Williams *et al*., 2014). There are not reports so far on lipase expression during interaction of cocoa pods and *M. roreri* or *M. perniciosa*. However, it has been found in *Ustilago maydis*, a basidiomycete that infects corn cobs. The possible role of this enzyme is the degradation of plant material (Meinhardt *et al*., 2014). Reports of expression of chitosanases suggest that it is used to modify the surface chitin of the tips of the hyphae to avoid the degradative effect of plant chitinases (Thadathil and Velappan, 2014). Meinhardt *et al*. (2014) found in *M. roreri* that this enzyme was expressed during biotrophic phase of infection indicating that may be related to the recognition of the plant surface. There is, however, other theory which proposes that plants accumulate chitosan as an antifungal agent and chitosanases induced by the fungus are used to degrade this compound and allow the fungus to infect the plant (Vargas *et al*., 2012).

Although, enzymatic activity is seen in the 1st h of fermentation (Fig. 2), no increase was observed in the gene expression levels determined by RT-PCR for chitinases, glucanases and proteases. This may be attributed to the fact that the highest expression level of these enzymes is in the necrotrophic stage (King *et al*., 2011; Divon and Fluhr, 2007).

CONCLUSION

The conclusion of this work suggest that *M. roreri* express the hydrolytic enzymes chitosanases, lipases and cutinases during the early stages of the interaction with *Theobroma cacao* pods.

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