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A potential role for an extracellular methanol oxidase secreted by *Moniliophthora perniciosa* in Witches' broom disease in cacao

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ABSTRACT

The hemibiotrophic basidiomycete fungus *Moniliophthora perniciosa*, the causal agent of Witches' broom disease (WBD) in cacao, is able to grow on methanol as the sole carbon source. In plants, one of the main sources of methanol is the pectin present in the structure of cell walls. Pectin is composed of highly methylesterified chains of galacturonic acid. The hydrolysis between the methyl radicals and galacturonic acid in esterified pectin, mediated by a pectin methylesterase (PME), releases methanol, which may be decomposed by a methanol oxidase (MOX). The analysis of the *M. pernciosa* genome revealed putative *mox* and *pme* genes. Real-time quantitative RT-PCR performed with RNA from mycelia grown in the presence of methanol or pectin as the sole carbon source and with RNA from infected cacao seedlings in different stages of the progression of WBD indicate that the two genes are coregulated, suggesting that the fungus may be metabolizing the methanol released from pectin. Moreover, immunolocalization of homogalacturonan, the main pectic domain that constitutes the primary cell wall matrix, shows a reduction in the level of pectin methyl esterification in infected cacao seedlings. Although MOX has been classically classified as a peroxisomal enzyme, *M. perniciosa* presents an extracellular methanol oxidase. Its activity was detected in the fungus culture supernatants, and mass spectrometry analysis indicated the presence of this enzyme in the fungus secretome. Because *M. pernciosa* possesses all genes classically related to methanol metabolism, we propose a peroxisome-independent model for the utilization of methanol by this fungus, which begins with the extracellular oxidation of methanol derived from the demethylation of pectin and finishes in the cytosol.

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1. Introduction

The basidiomycete fungus *Moniliophthora perniciosa* (Aime and Phillips-Mora, 2005) is the causal agent of Witches' broom disease (WBD) in cacao (*Theobroma cacao*), one of the most devastating diseases of cacao in the Americas (Griffith et al., 2003).

M. perniciosa is classified as a hemibiotrophic pathogen and presents two morphologically distinct life phases, biotrophic and necrotrophic, that are correlated with different symptoms during the progression of WBD (Evans, 1980). The biotrophic mycelium is monokaryotic, with no clamp connections, and infects flower cushions, developing fruit, and meristematic tissues. These hyphae inhabit the intercellular space, where they grow slowly at a low density, causing hypertrophy and hyperplasia of the infected branches, which are the main symptoms of the green broom stage (Evans, 1978; Penman et al., 2000; Silva, 1999). These symptoms are caused by a drastic biochemical change during the development of the green brooms (Scarpari et al., 2005). Very little is known about the characteristics of this monokaryotic mycelium *ex planta* because its cultivation is difficult due to its instability; a glycerol-based culture media for the cultivation of a monokaryotic and biotrophic-like mycelia was developed only recently (Meinhardt et al., 2006).

Five to eight weeks after the start of the infection, a dikaryotization process occurs, and the mycelia become necrotrophic, presenting two nuclei per cell that are connected by typical basidiomycete clamp connections. The necrotrophic mycelia rapidly invade the host plant cells, completely destroying the infected tissues and causing the extensive degradation symptoms of the dry

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broom phase (Delgado and Cook, 1976; Evans and Bastos, 1980; Frias et al., 1991; Griffith and Hedger, 1994). Although the mechanisms that trigger the dikaryotization process in *M. perniciosa* are not yet completely elucidated, recently a study published by our group reported that an alternative oxidase plays a role in the biotrophic development of *M. perniciosa* and regulates the transition to its necrotrophic stage (Thomazella et al., 2012).

The introduction of WBD in Bahia, the main Brazilian cacao-producing state, occurred in 1989 (Pereira et al., 1989). Since then, cacao production has decreased drastically and Brazil has become a net importer of cacao in order to supply the national chocolate industry. Due to the extreme losses in cacao production, in the early 2000s, the Witches' broom Genome Project was initiated to decode the *M. perniciosa* genome and, based on the data acquired, select genes that could be relevant during the progression of WBD for characterization. In 2008, a draft genome analysis and an EST and microarray-based transcriptome study were published (Mondego et al., 2008; Rincones et al., 2008). These studies revealed the presence of sequences similar to methanol oxidase (*mox*), an enzyme that could be related to the previously reported ability of *M. perniciosa* to grow on methanol as the sole carbon source (Mondego et al., 2008), as shown in Fig. 1.

MOX is the key enzyme in methanol metabolism in methylotrophic yeasts and other methanol-degrading organisms. It consists of an octameric flavoprotein that oxidizes methanol to formaldehyde and hydrogen peroxide (Ozimek et al., 2005). The peroxide generated is decomposed by a catalase, and the formaldehyde is subjected either to the action of a dihydroxyacetone synthase (DHAS) or to direct oxidation by the enzymes formaldehyde dehydrogenase (FMDH) and formate dehydrogenase (FDH) (Kaszycki and Koloczek, 2000).

In almost all methanol-degrading organisms, such as the methylotrophic yeasts *Pichia angusta* and *Pichia pastoris* and the ascomycete phytopathogen *Cladosporium fulvum*, MOX is a peroxisomal enzyme (Ozimek et al., 2005; Segers et al., 2001). It is synthesized in the cytosol and imported into the peroxisomes, triggered by a peroxisomal targeting signal (PTS1) that is located in the C-terminal part of the enzyme (Ozimek et al., 2006). To date, the only known exception concerns the MOX produced by the basidiomycete *Gloeophyllum trabeum*, which is secreted into the extracellular space, despite the absence of a clear secretion signal in its sequence (Daniel et al., 2007).

In plants, one of the main sources of methanol is the pectin present in the plant cell walls (Nemecek-Marshall et al., 1995). Pectins are one of the major components of the middle lamellae and primary plant cell walls in dicotyledonous species, where they compose 30–35% of the cell wall dry weight (Pelloux et al., 2007). Pectins are highly complex polysaccharides that are rich in calcium ions, and are formed by a range of different domains, two of which may be distinguished: homogalacturonan and xylogalacturonan. Homogalacturonans are linear chains of α-(1-4)-linked D-galacturonic acid, which can be either methylesterified or acetylesterified. Xylogalacturonan is a homogalacturonan with (1,3)-β-D-xylopyranoside side chains, which like homogalacturonan, can be methylesterified (Willats et al., 2001).

The degradation of the pectin present in plant cell walls is a strategy used by many phytopathogenic fungi to invade the host tissues and establish infection. The fungi *Sclerotinia sclerotiorum* (Guimaraes and Stotz, 2004), *Botrytis cinerea* (Han et al., 2007), and *M. perniciosa* (Rio et al., 2008) produce oxalate, which removes calcium ions bound to pectin to produce calcium oxalate crystals, thus exposing the host cell walls to plant cell wall-degrading enzymes (PCWD) of fungal origin. One of the main PCWDs is pectin methylesterase (PME), an enzyme that removes the methyl ester radicals present in the galacturonic acid backbone in esterified pectin, releasing methanol (Sakai et al., 1993). PMEs are enzymes produced by plants that are related to growth, development, and defense against pathogens (Pelloux et al., 2007), though many

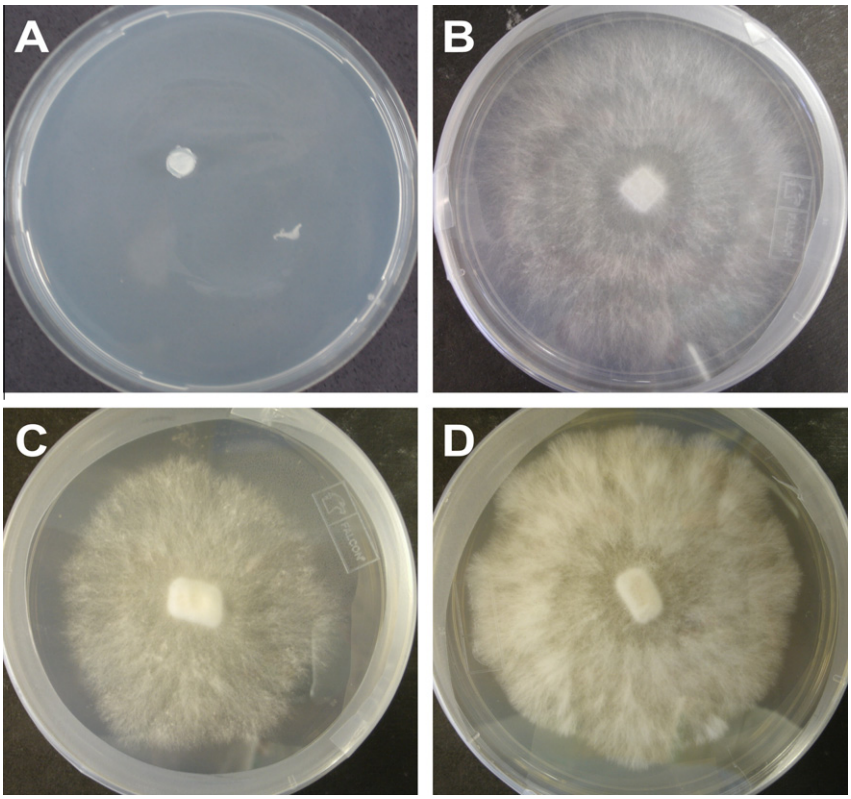

Fig. 1. *M. perniciosa* is able to grow on methanol as the sole carbon source. Minimal medium without any carbon source, which cannot support *M. perniciosa* growth (A, control) and the same media supplemented with glucose (B), methanol (C) and cacao extract (D), at final concentrations of 1% (v/v).

phytopathogenic fungal species secret PMEs as pathogenicity factors. For example, the disruption of the *B. cinerea* pectin methylesterase gene Bcpme1 reduces virulence in several host plants, including apple fruits, grapevines, and *Arabidopsis thaliana* leaves (Valette-Collet et al., 2003). The analysis of *M. perniciosás* genomic and expressed sequences revealed the presence of a sequence similar to a pectin methylesterase.

Although it has been demonstrated that methylotrophic yeasts, such as *Pichia methanolica* (Nakagawa et al., 2005) and *Candida boidinii* (Yurimoto et al., 2000), are able to utilize the methanol released from pectin, very little is known about the correlation between pectin and methanol metabolism among the phytopathogenic fungal species.

In this study, we show that *M. perniciosa* produces a methanol oxidase that is secreted into the extracellular space. Its relative expression *in vitro* and *in planta* is associated with PME expression levels and with the reduction in the level of methyl esterification observed in infected cacao seedlings, suggesting that this fungus metabolizes the methanol generated by pectin degradation. Moreover, we show that *M. perniciosa* possesses all genes related to methanol utilization, and their expression indicates that methanol can be a relevant energy source for this fungus.

2. Material and methods

2.1. Biological material and growth conditions

The isolate FA553 of *M. perniciosa* was used for all experiments in this work (Mondego et al., 2008). In our laboratory, necrotrophic mycelia of this isolate were maintained on plates of Malt Yeast Extract Agar (Difco) at 28 °C. The spores were obtained from necrotrophic mycelia according to the protocol developed by Teixeira et al. (in preparation). Biotrophic-like mycelia were obtained from spore germination in biotrophic maintenance media (Meinhardt et al., 2006).

For all *in vitro* gene expression experiments, both biotrophic-like and necrotrophic mycelia were grown for 7 days at 28 °C, under constant agitation at 120 rpm, in biotrophic liquid media, which contains glycerol as the sole carbon source (Meinhardt et al., 2006); the same media was used for the biotrophic-like and necrotrophic mycelia growth to avoid gene expression variations associated with different growth conditions. After the 7-day growth period, the mycelia were separated from the media by filtration, washed twice in sterile distilled water and inoculated in new aliquots of the same media supplemented with the following sole carbon sources at final concentrations of 1% (w/v): glycerol, glucose, methanol, non-esterified pectin from citrus (Sigma, polygalacturonate) and esterified pectin from citrus (Sigma, degree of esterification, DE $\geqslant$ 85%). After a 24-h period of incubation, the mycelia were separated from the media by filtration, washed twice in sterile distilled water, frozen in liquid nitrogen, and stored at −80 °C for further RNA isolation.

T. cacao L. variety (Catongo) was used for the *M perniciosa* gene expression assays *in planta*. Plants were grown for approximately 3 months in a greenhouse under controlled temperature (26 °C) and humidity (>80%) at a photoperiod of 12 h. After this period of growth, the apical meristems of the plants were removed 2 weeks before the infection with the fungal spores to stimulate growth of the lateral meristems. One day before the infection, the plants were transferred to a high-humidity chamber to induce stomatal opening. We used 30 µl of a solution containing approximately 10^5 spores/ml to infect each plant. Symptoms of WDB were observed, and the plants were harvested based on the progression of symptoms of the disease: asymptomatic, green broom 1, green broom 2 and necrosis; the plants were collected in triplicate for each stage analyzed. As the experimental control, non-infected plants of the same age were collected. All collected plants were frozen in liquid nitrogen and stored at −80 °C freezer for further RNA isolation. For the immunolocalization assays, seedlings from asymptomatic and necrosis stages and their respective non-infected controls of the same age were harvested and fixed in Karnovsky solution overnight at room temperature and under vacuum (Karnovsky, 1965).

2.2. RNA isolation

Mycelia of *M. perniciosa* grown *in vitro* were processed for RNA extraction using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA from infected and non-infected plant material was isolated according to a protocol developed in our laboratory (Vidal et al., 2010). All isolated RNA was qualitatively analyzed in 1% denaturing formaldehyde/agarose gel electrophoresis and quantified using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific).

2.3. Real-time PCR

One microgram of total RNA from *in vitro* cultures and plant material was treated with RQ1 RNAse–free DNAse (Promega) and used for cDNA synthesis with SuperscriptII® Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The online program Primer3 (Rozen and Skaletsky, 2000) was used to design all PCR primers based on the sequences obtained from the Witches' broom Genome Project database (Table 1). The optimal primer annealing temperature was set to 55 °C, and the amplicon size varied from 100 to 110 bp. Quantitative PCR was performed using the SYBRGreen MasterMix (Applied Biosystems), and the fluorescence was detected with the Step One Plus platform (Applied Biosystems). A 16 µl reaction was set with 8 µl of the SYBRGreen MasterMix, 40 ng of cDNA and 100 nM of each primer. The thermal cycling conditions were 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 20 s and 60 °C for 1 min. After amplification, a melt-curve step consisting of one cycle of 94 °C for 15 s and a ramp varying from 60 °C to 94 °C in intervals of 0.3 °C was added to inspect the reactions for the formation of primer dimers and unspecific amplicons. The melting temperatures of the fragments were determined according to the manufacturer's protocol (Table 1). No-template reactions (water) were included as negative controls in every plate for all of the primers used; for plant material analyses, non-infected plants were also assayed to ensure that the fungal primers were not amplifying cacao genetic material.

The data analysis was performed using a mathematical method described previously (Pfaffl, 2001), using the C_t (cycle threshold) mean of biological triplicates. Standard curves for each primer pair were generated by serial cDNA dilutions. The housekeeping genes β-actin and α-tubulin (Table 1) were used to normalize the qPCR reactions for *in vitro* and infected plant material, respectively.

2.4. Methanol oxidase assay

Supernatants of liquid *M. perniciosa* cultures were separated from the mycelia by filtration through several layers of filter paper

Table 1
Primers and melting temperatures (MT) for quantitative SYBRGreen real-time PCR.

	Forward	Reverse	MT (°C)
PME	TTTTGTACTCAAACTTGCATCACC	CTGAAGGAAGATTAGACAGACACG	75.6
MOX	CTGTCGGCGTAGCCTATGTT	TCCACTGCTCAGAACGACAC	82.3
ACT	CCCTTCTATCGTCGGTCGT	AGGATACCACGCTTGGATTG	80.7
TUB	GACCAACAGCTTGTCTTTGC	GACATTGCAAACATCGAGGA	81.0

(Whatman No. 1). The secretome samples were then concentrated 100-fold using an Amicon Ultra-15 Centrifugal Filter Unit with an Ultracel-10 membrane (Millipore) and quantified by the Bradford assay using bovine serum albumin as the standard.

The concentrated supernatants of liquid *M. perniciosa* cultures were used to detect methanol oxidase activity. MOX activity was assayed spectrophotometrically at 405 nm through the oxidation of 2,2-azino-bis(3-ethylbenzo-6-thiazoline sulfonic acid) (ABTS) (Sahm and Wagner, 1973). The reactions were set to a final volume of 2 ml, containing 100 mM of the substrate (methanol, ethanol and 2-propanol), 2 μmol of ABTS, 40 μg of horseradish peroxidase (Sigma), and approximately 10 μg of total protein from the culture supernatants. The reaction was performed at 25 °C in an air-saturated 100 mM potassium phosphate buffer at pH 7.5. The oxidation of ABTS was detected by observing the appearance of a green coloration. The 405 nm absorbance was measured at 1 min intervals. The K_m for methanol and ethanol was calculated as described previously (Segers et al., 2001; Van der Klei et al., 1990).

2.5. Enzymatic in-gel digestion and mass spectrometry analysis

Concentrated supernatants of liquid *M. perniciosa* cultures, obtained as described in Section 2.4, were used in mass spectrometry analysis. SDS–PAGE electrophoresis (12.5%) was used to separate 20 μg of the concentrated protein mixture. The bands corresponding to the molecular mass range from 70 to 75 kDa, as predicted for MOX, were excised from the gel and subjected to in-gel trypsin digestion, which was performed as described previously (Hanna et al., 2000), with modifications. As a negative control, we performed the same preparation with a cell-free culture media.

The resulting peptide solution was dried in a SpeedVac concentrator, resuspended in 20 μl of 0.1% formic acid and an aliquot of 4.5 μl was analyzed in a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) connected to a nanoflow liquid chromatography (LC–MS/MS) by an EASY-nLC system (Proxeon Biosystem) through a Proxeon nanoelectrospray ion source. Peptides were separated on a 2–90% acetonitrile gradient in 0.1% formic acid using a pre-column EASY-Column (2 cm × id 100 μm, 5 μm particle size) and an analytical column EASY-Column (10 cm × id 75 μm, 3 μm particle size) at a flow rate of 300 nl/min over 45 min. The nanoelectrospray voltage was set to 1.7 kV, and the source temperature was 275 °C. All instrument methods for the Orbitrap Velos were set up in the data-dependent acquisition mode. The full scan MS spectra (m/z 300–2000) were acquired in the Orbitrap analyzer after accumulation to a target value of $1e^6$. The resolution was set to $r = 60{,}000$ and the 20 most intense peptide ions with charge states $\geqslant 2$ were sequentially isolated to a target value of 5000 and fragmented in the linear ion trap by low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 1000 counts. Dynamic exclusion was enabled with an exclusion size list of 500, exclusion duration of 60 s, and repeat count of 1. An activation $q = 0.25$ and activation time of 10 ms were used (de Souza et al., 2012).

Peak lists (msf) were generated from the raw data files using the software Proteome Discoverer 1.3 (Thermo Fisher Scientific) with Sequest search engine and they were searched against *M. perniciosás* predicted protein database (17,012 sequences) with carbamidomethylation (+57.021 Da) as a fixed modification, oxidation of methionine (+15.995 Da) as a variable modification, one trypsin missed cleavage and a tolerance of 10 ppm for the precursor and of 1 Da for fragment ions, filtered using xcorr cutoffs (+1 > 1.8, +2 > 2.2, +3 > 2.5 and +4 > 3.25) and false discovery rate of 0.01, performed using a reverse *M. perniciosás* predicted protein database.

2.6. Immunofluorescence detection of the level of methyl esterification in infected and non-infected cacao seedlings

The immunolocalization of the methylesterified and non-methylesterified domains of homogalacturonan present in infected and non-infected cacao seedlings was performed as described elsewhere (Buckeridge and Reid, 1994; Orfila and Knox, 2000). The primary antibodies JIM5 and JIM7, which recognize homogalacturonan epitopes with low (0–40%) and high (15–80%) levels of methyl esterification (0–40%), respectively, were used (Willats et al., 2000).

The fixed samples (Section 2.1) were dehydrated, cleared in xylene and infiltrated in Paraplast® Plus (McCormick™). Twelve-micrometer transverse sections of stems from asymptomatic and necrotic infected cacao plants and non-infected controls of the same age were dewaxed with butyl acetate, hydrated and incubated in blocking solution (3% whole milk diluted in 0.01 M saline phosphate buffer pH 7.1) for 30 min. Subsequently, the samples were separately incubated with primary antibodies (1:5 dilution) for 3 h, washed three times with PBS buffer and incubated with Goat Anti-Mouse IgG, (H + L) FITC-conjugated secondary antibody for 1 h at room temperature in the dark. Control samples were incubated only with the secondary antibody for 1 h at room temperature in the dark. The samples were immediately analyzed with an Olympus BX51 epifluorescence microscope. At least 10 different sections of each plant were analyzed.

2.7. Transcriptomic analysis using RNA-seq

The RNA isolated from *M. perniciosa* necrotrophic mycelia induced in glycerol or methanol (Section 2.1) was processed for global transcriptome analysis by large scale mRNA sequencing (RNA-seq). Libraries were prepared according the manufacturer's instructions (Illumina). The mRNA was purified from 10 μg of total RNA using Sera-Mag Magnetic Oligo(dT) Beads (Thermo Scientific), and fragmented in the presence of divalent cations under high temperatures. The fragmented mRNA was used to synthesize the cDNA. The first strand was synthesized using Superscript II Reverse Transcriptase and random hexamers. Double-stranded cDNA was then produced using the enzymes DNA polymerase I and RNAse H. Subsequently, abrupt extremities were obtained by treatment with the enzymes T4 DNA polymerase and Klenow DNA polymerase; a 3′-adenine was added by the enzyme Klenow exo-Adapters with an overhang thymine were added, and non-ligated adapters were purified in gel. Finally, the libraries were enriched through 15 cycles of amplification using primers that anneal to the adaptors. The libraries were quantitative and qualitatively assayed with the Qubit fluorometer (Invitrogen) and the Experion capillary electrophoresis system (Bio-RAD). Each library was subjected to a 36-cycle single end sequencing in the Genome Analyzer II_x platform (Illumina).

To identify expressed genes, the reads obtained from large scale sequencing were mapped against the *M. perniciosa* genomic database using the mapping tool Bowtie (Langmead, 2010), allowing one mismatch. Ambiguous reads that mapped with similar scores to several sites were discarded. The measure of gene expression was calculated in terms of RPKM (reads per kilobase per million mapped reads) (Mortazavi et al., 2008).

3. Results

3.1. Sequence analysis of M. perniciosa methanol oxidase

The *M. perniciosa* predicted protein database (17,012 sequences) was analyzed, and three sequences with the potential to encode

methanol oxidase were initially identified. These sequences were compared to MOX sequences from other organisms by the Blast algorithm against the NR database and subjected to a search of conserved domains and classical MOX motifs, using the CDD and Pfam databases. Fig. 2A shows a scheme of the three predicted MOX sequences. All described MOX from fungal species contain two GMC-oxidoreductase (GMC, glucose–methanol–choline) domains and a conserved ADP-binding β–α–β motif on the N-terminal portion of the protein (Ozimek et al., 2005). As shown in Fig. 2A, the sequences 2 and 3 lack the N-terminal β–α–β motif and sequence 3 presents an incomplete N-terminal GMC-oxidoreductase domain; moreover, these sequences are shorter than sequence 1 and MOX sequences from other organisms, in which protein size varies from 650 to 670 amino acids. Because sequence 1 is the only sequence that contains both complete GMC-oxidoreductase domains and the classical β–α–β motif and because it was the only sequence previously identified in cacao-extract-induced EST libraries (Rincones et al., 2008), we focused our work on this gene. This sequence will be referred to as *Mp_mox*.

The *Mp_mox* genomic (GenBank ID: JX024739) sequence and complete CDS present 3490 and 1953 nucleotides, respectively, containing 29 exons and 28 introns. The predicted protein contains 650 amino acids and has a molecular weight of approximately 72 kDa. Fig. 2B shows an alignment of the C-terminal part of the *M. perniciosa* MOX with sequences from the methylotrophic yeasts *P. pastoris* and *P. methanolica*, the ascomycete fungus *C. fulvum* and the basidiomycete species *Coprinopsis cinerea* and *G. trabeum*, and the predicted sequences 2 and 3. *Mp-mox* shares 89% identity with *C. cinerea* and *G. trabeum* sequences compared with its 52% identity with the other species mentioned above. Sequence analysis with the program InterPro Scan Sequence Search (http://www.ebi.ac.uk/Tools/pfa/iprscan) showed the existence of two conserved GMC-oxidoreductase domains: an N-terminal domain corresponding to the amino acids 7–313 and a C-terminal domain that spans from amino acid 427 to 614. Residues 13–18 contain a putative flavin adenine dinucleotide (FAD) binding site (GGGPAG) within the predicted ADP-binding β–α–β motif, which is present in most FAD-binding proteins (Ozimek et al., 2005; Wierenga et al., 1986). An N-glycosylation site at the residue 323 was predicted with a score of 0.5229 by the software NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc).

One of the main characteristics of MOX from most organisms is the presence of a peroxisomal targeting signal, PTS1, at the C-terminal region of the protein. The (S/A/C)-(K/R/H)-(L/A) tripeptide interacts with the Pex5p receptor protein, leading to the importation of the protein by the peroxisomes (de Hoop and Ab, 1992). As shown in Fig. 2B, the *M. perniciosa* MOX sequence lacks PTS1. Otherwise, the last 26 amino acids (marked in gray) show a high similarity with the terminal residues of the *G. trabeum* and *C. cinerea* sequences; this sequence appears to be a particular characteristic of MOX from basidiomycete species as it differs substantially from the C-terminal sequences of other fungal species.

3.2. M. perniciosa MOX is a secreted protein

One hundred-fold concentrated culture supernatants (secretome) from *M. perniciosa* were assayed for MOX activity. As described in Section 2.4, MOX activity was assayed by following the oxidation of ABTS at 405 nm with a spectrophotometer. Table 2 shows MOX relative activity and K_m values in different substrates. As shown in Table 2, the preferred substrate for MOX is methanol, but it also shows a high activity when the substrate is ethanol.

LC–MS/MS analysis on an LTQ Orbitrap-Velos mass spectrometer was used to confirm the presence of methanol oxidase in the extracellular extracts of *M. perniciosa* cultures. The concentrated fungal secretome was electrophoretically separated in a 12.5% one dimensional SDS–PAGE gel. As MOX is predicted to have a molecular mass of approximately 72 kDa, the bands corresponding to the molecular mass range of 70–75 kDa were excised from the gel (Fig. 3A) and analyzed in the LTQ-Orbitrap-Velos mass spectrometer. A total of 2722 spectral counts were assigned to 205 proteins of the *M. perniciosa* predicted protein database. As shown in Fig. 3B, three unique MOX peptides were identified, totaling 7% of protein coverage. This finding confirms that *M. pernciosa* secretes this enzyme into the extracellular space, despite the absence of a predicted secretory signal peptide in its

Table 2
Relative activity and K_m values for MOX in different substrates.

Substrate	Relative activity (%)[a]	K_m (mM)
Methanol	100	17.3
Ethanol	88	21.2
2-Propanol	7	–

[a] Activities are given relative to methanol.

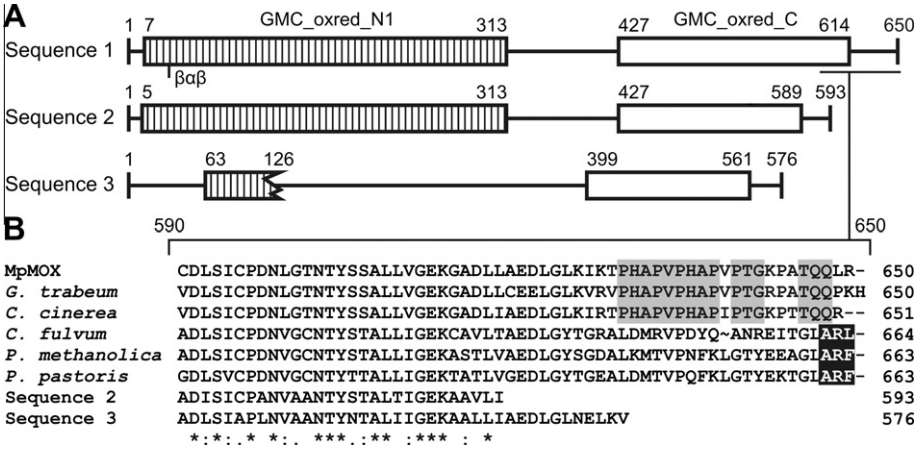

Fig. 2. (A) Schematic alignment of the three sequences identified with the potential to encode methanol oxidase in the *M. perniciosa* genome, showing their amino acid length and the presence of conserved domains. (B) Alignment of the C-terminal sequence of Mp-mox from *M. perniciosa* (GenBank ID: JX024739) with MOX from *Gloeophyllum trabeum* (ABI14440.1), *Coprinopsis cinerea* (XP_001838223.2), *Cladosporium fulvum* (AAF82788.1), *Pichia methanolica* (AAF02494.1) and *Pichia pastoris* (AAB57850.1) and the other two predicted sequences from *M. perniciosa*. The identical amino acid residues are indicated with asterisks. The specific C-terminal sequence present in MOX from the basidiomycete species is labeled in gray, and the C-terminal peroxisomal targeting signal (PTS1) present in MOX from the ascomycetes and methylotrophic yeasts is labeled in black.

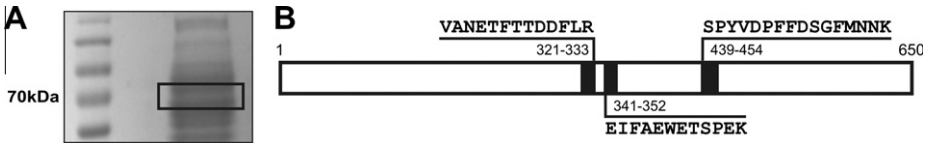

Fig. 3. MOX from *M. perniciosa* is an extracellular enzyme. (A) SDS–PAGE from *M. perniciosa* secretome indicating a 70–75 kDa band that was excised from the gel and subjected to mass spectrometry analysis. (B) Mass spectrometry analysis identified three unique MOX peptides.

Table 3
Peptide sequences identified by mass spectrometry analysis.

Peptide sequence	Spectral counts	Charge	*m*/*z*	MH + [Da]
VANETFTTDDFLR	1	+2	764.8710	1528.7348
SPSPYVDPFFDSGFMNNK	1	+2	1032.9590	2064.9107
EIFAEWETSPEK	1	+2	733.3496	1465.6919

M – oxidation of methionine.

sequence. The peptide sequences, the number of spectral counts, and their *m*/*z* are detailed in Table 3.

3.3. Mp-mox is upregulated in the presence of methanol and esterified pectin

The relative expression of *Mp-mox* and *Mp-pme* transcripts was measured by Real-time PCR in both biotrophic-like and necrotrophic mycelia grown *in vitro* in the presence of glycerol, glucose, methanol, polygalacturonate or pectin with 90% esterification. As shown in Fig. 4, there was a 12- and 9-fold increase in the expression of *Mp-mox* in the biotrophic-like and necrotrophic mycelia, respectively, in the presence of methanol when compared with glycerol as the carbon source. This is consistent with the fact that methanol is a substrate for MOX and a known inducer of its expression in other organisms (Ozimek et al., 2005). In the presence of esterified pectin, both *Mp-mox* and *Mp-pme* genes were upregulated. The expression of *Mp-mox* and *Mp-pme* was increased approximately 10 and 9 times, respectively, in both types of mycelium. This result suggests that the methanol released during the demethylation of pectin by the enzyme PME is a substrate for MOX, which shows an increased level of expression. Interestingly, in the presence of non-esterified pectin, neither gene was upregulated, thus reinforcing the hypothesis that the demethylation of pectin is a key process for *Mp-mox* and *Mp-pme* upregulation. The lack of variation in gene expression between the biotrophic-like and necrotrophic mycelia show that, although these mycelia produce distinct symptoms in the progression of WBD, they may exhibit similar characteristics when grown under the same conditions *in vitro*. The expression of these genes in the presence of glucose did not show any difference when compared to growth in glycerol.

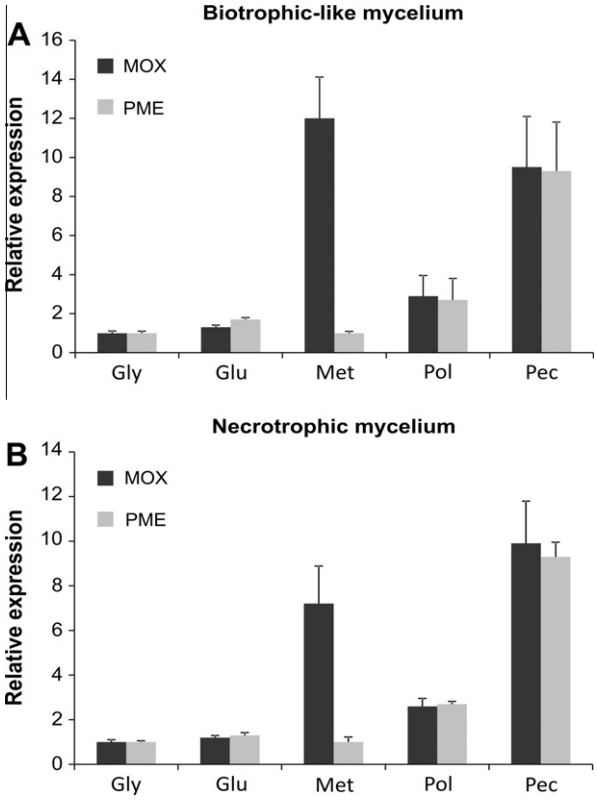

Fig. 4. *Mp-mox* and *Mp-pme* relative expression in the biotrophic-like and necrotrophic mycelium from *M. perniciosa* grown *in vitro* and supplemented with different sole carbon sources. Gly: glycerol; Glu, glucose; Met: methanol; Pol: polygalacturonate (pectin DE 0%); Pec: pectin, DE > 85%. The values are means of biological triplicates and the bars indicate the standard error of the mean. The data were normalized to the β-actin gene and the condition Gly was considered as the control of the experiment and was used to normalize the other conditions expression values.

3.4. The reduction of the level of methyl esterification in infected cacao seedlings is correlated with the higher levels of M. perniciosa Mp-pme relative gene expression in planta

Mp-mox and *Mp-pme* relative expression in infected cacao plants was measured by Real-time quantitative PCR. As described in Section 2.1, 3-month-old cacao seedlings were infected with *M. perniciosa* spores. The symptoms of WBD were observed and the plants were harvested according to the progression of the disease: asymptomatic, green broom 1, green broom 2 and necrosis. As shown in Fig. 5A, *Mp-mox* and *Mp-pme* are coregulated during the progression of WBD in the stages analyzed. The relative expression of both genes is reduced in the green broom phase compared with the asymptomatic stage and increases during the necrosis stage, when the expression of both genes reaches their highest level.

Spores of *M. perniciosa* infect cacao plants as biotrophic mycelia, which grow slowly and at low densities in the infected plants. As the disease progresses, the amount of fungal material *in planta* increases, reaching a maximum at the late stages of the dry broom phase when the necrotrophic mycelia completely colonizes the infected tissues (Evans, 1978; Frias et al., 1991; Griffith and Hedger, 1994). Fig. 5B shows the amplification plots and Ct, with a threshold of 0.1, for the housekeeping gene α-tubulin in the four stages of WBD analyzed. The C_t difference for the α-tubulin gene between the asymptomatic ($C_t = 30$) and the necrosis phase ($C_t = 23$) indicates that there is approximately 100 times more fungal material in the infected plants during the necrosis phase than in the previous stages. The relatively higher *Mp-mox* and *Mp-pme* expression levels in the necrosis phase, in conjunction with the higher number of mycelia infecting the plants during this phase, led us to hypothesize that the final quantity of the transcripts of these genes and,

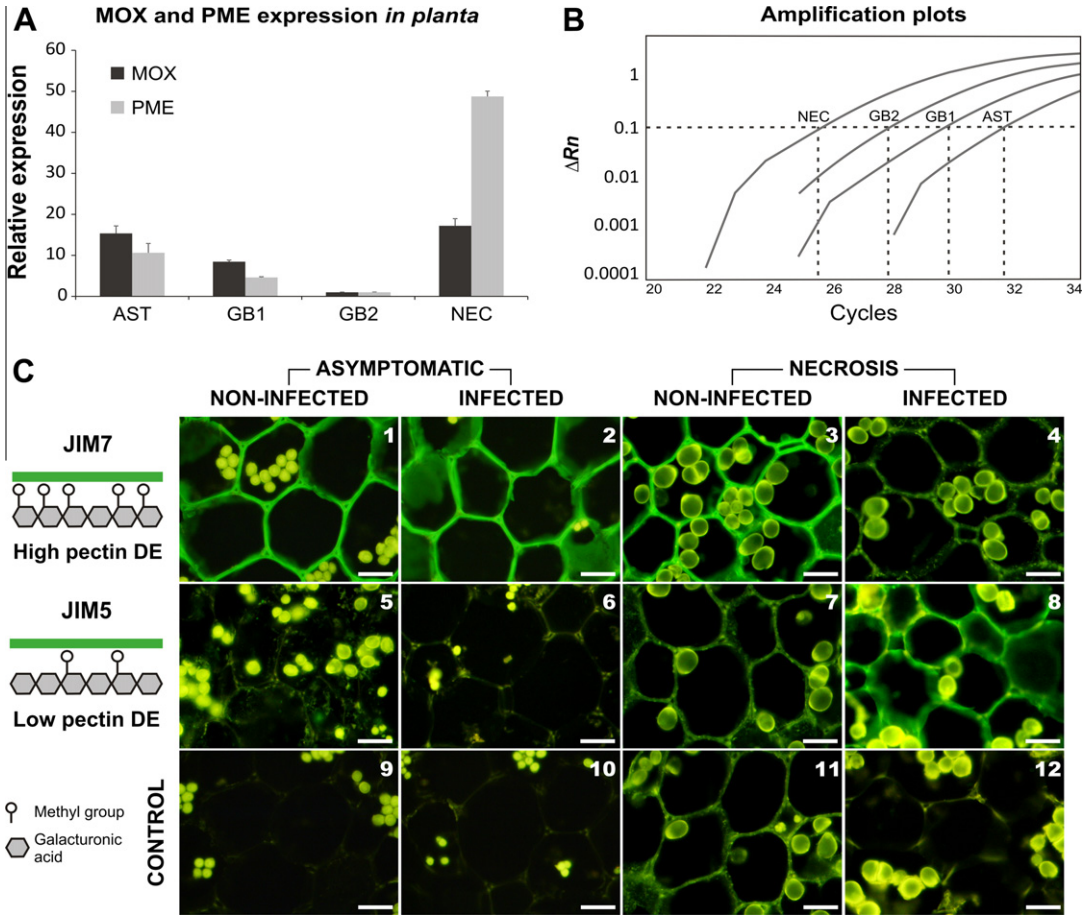

Fig. 5. (A) *Mp-mox* and *Mp-pme* relative expression *in planta* in different stages of the progression of WBD: AST, asymptomatic; GB1: green broom 1; GB2, green broom 2 and NEC: necrosis. The values are means of biological triplicates and the bars indicate the standard error of the mean. The data were normalized to the α-tubulin gene and the condition GB2 was used to normalize the other conditions expression values. (B) Amplification plots of the α-tubulin gene in the four stages of the progression of WBD analyzed, with a threshold of 0.1, showing an increase of the amount of fungal material infecting cacao in the late stages of the disease; (C) Immunofluorescence detection of the pectic homogalacturonan domain in infected and non-infected cacao seedlings (stem tissues in transverse section). Infected asymptomatic plants (2, 6 and 10) and same age non-infected plants (1, 5 and 9); infected necrotic plants (4, 8, and 12) and same age non-infected plants (3, 7 and 11). Bar = 25 μm.

possibly, their protein products, would be even higher in the infected plants.

Because PMEs remove the methyl esters from the pectin backbone, we decided to test this hypothesis by analyzing the level of methyl esterification present in the cell walls of infected cacao seedlings during the asymptomatic and necrotic stages and their respective non-infected same age controls. If there is a higher level of PME enzyme due to the combined effect of the upregulation of *Mp-pme* during the necrotrophic stage and the greater amount of fungal material present in this stage, there should be an inverse correlation with the degree of pectin methyl esterification in the cell wall of infected plants at this stage.

As mentioned in Section 2.6, the monoclonal antibodies JIM5 and JIM7 were used in this work to characterize the level of methyl esterification in the pectic domain homogalacturonan. JIM5 recognizes pectin with low DE (0–40%) and JIM7 labels pectin with high esterification content (15–80%) (Willats et al., 2000).

As shown in Fig. 5C, strong JIM7 labeling was observed in the infected and non-infected asymptomatic samples analyzed (Fig. 4C: 1 and 2); however, comparing the negative control samples (Fig. 5C: 9 and 10), which were labeled only with the secondary antibody, with the samples treated with JIM5 (Fig. 5C: 5 and 6), no JIM5 labeling is observed in the treated samples. JIM5 and JIM7 are able to unspecifically label pectic domains with 15–40% DE. Therefore, the fact that the samples were labeled only by JIM7 indicates that both infected asymptomatic and its same age non-infected control present a high degree of methyl esterification (>40%).

On the other hand, infected plants showing necrosis and their non-infected same age controls show an inverse pattern of methyl esterification: the comparison between the negative control (Fig. 5C: 11) and the samples treated with the primary antibodies leads to the conclusion that non-infected plants were strongly labeled by JIM7 and were not labeled by JIM5 (Fig. 5C: 3 and 7). These data indicate a high level of methyl esterification, while infected plants were strongly marked by JIM5 and weakly by JIM7 (Fig. 5C: 4 and 8), in comparison to their negative control (Fig. 5: 12), thus indicating a low level of methyl esterification. The apparent labeling of some samples from the negative control is related with their auto fluorescence.

These results, in conjunction with the *M. perniciosa Mp-pme* expression *in planta* (Fig. 5A) and the higher amount of fungal material present in the infected tissues (Fig. 5B), strongly suggest a participation of the fungal PME enzyme in the demethylation process of cacao pectin during the necrotrophic stage of WBD.

4. Discussion

Methanol oxidase has been primarily studied in the methylotrophic yeasts *Pichia, Candida, Hansenula,* and *Torulopsis* (Veenhuis et al., 1983). The capability of these organisms to grow on

methanol as the sole carbon source is due to the production of large amounts of MOX; in the presence of methanol, MOX can reach 30% of the total soluble protein produced by these organisms (Giuseppin et al., 1988). However, very little is known about this enzyme among the basidiomycete and/or phytopathogenic fungal species.

In this study, we describe an extracellular methanol oxidase from the phytopathogenic basidiomycete species *M. perniciosa*. The deduced 650 amino acid sequence shows 51–53% identity and 65–67% similarity to MOX from methylotrophic yeasts and ascomycete species and 85–89% identity and 93–95% similarity to other basidiomycete species, indicating that this enzyme is highly conserved among the basidiomycetes.

MOX is a classical peroxisomal enzyme. In methylotrophic yeasts and ascomycetes, the enzyme is produced in the cytosol as 70–75 kDa monomers and post-transcriptionally imported into the peroxisomes, where they are assembled into active octamers (Gunkel et al., 2004). The importation of the protein into the peroxisomes is generally triggered by the recognition of peroxisome targeting signals (PTS) present in the sequence of imported proteins by peroxin (PEX) receptors. PTS1, a C-terminal tripeptide motif, that generally complies with the consensus sequence (S/A/C)(K/R/H)(L/M), is recognized by the protein Pex5p (Gould et al., 1987; Waterham et al., 1997). PTS2, recognized by the peroxin Pex7p, is a N-terminal sequence formed by a very general sequence, (R/K)(L/V/I)X5(H/Q)(L/A), that is present in just a few peroxisomal enzymes (Lazarow, 2006). *M. perniciosa* methanol oxidase, as shown in Fig. 2, lacks both PTS1 and PTS2 sites, which is in agreement with the enzyme activity assays and mass spectrometry analysis showing that this enzyme is secreted to the extracellular space.

To date, the only described extracellular MOX belongs to the lignin-degrading basidiomycete *G. trabeum* (Daniel et al., 2007). However, the protein sequence lacks any clear secretion signal. The authors suggested that the differences in MOX targeting compared with the known yeast peroxisomal localization were traced to a unique C-terminal sequence of the *G. trabeum* enzyme, which is apparently responsible for the protein's extracellular translocation. *M. perniciosa* MOX contains an identical C-terminal sequence when compared to *G. trabeum* and predicted MOX from other basidiomycetes that are completely different from the known peroxisomal MOX. Because *M. perniciosa* MOX also lacks a predicted secretion signal, we suggest that its unique C-terminal sequence is the signal component that leads to the translocation of this enzyme to a secretory pathway, given that this is the only significant sequence difference between *M. perniciosa* and other basidiomycete MOX compared with the yeast and ascomycete peroxisomal *mox* genes.

In methylotrophic yeasts, methanol oxidase is regulated at the transcriptional level by a repression and/or derepression mechanism (Ozimek et al., 2005). In *P. angusta*, the expression of *mox* is subject to a strong carbon catabolite repression, as the *mox* gene is completely repressed by growth on glucose; this substrate inhibits peroxisome proliferation, even in the presence of methanol. On the other hand, *mox* transcripts are detected when cells are grown on methanol or glycerol as the sole carbon sources (Roggenkamp et al., 1984; Veenhuis et al., 1983). In *M. perniciosa*, the *Mp-mox* gene is detected when the mycelia is grown *in vitro* in glycerol and strongly induced in the presence of methanol, in both biotrophic-like and necrotrophic mycelia (Fig. 4). However, gene repression is not observed because the mycelia grown in glucose express *Mp-mox* at a similar magnitude as when grown in glycerol.

The analysis of the *M. perniciosa* genome revealed that the fungus possesses all the genes classically related to the methanol degradation pathway (GenBank ID: JX024739–JX024749). RNA-seq-based transcriptome analysis showed that all of these genes are upregulated in the necrotrophic mycelia supplemented with methanol when compared to those supplemented with glycerol.

Table 4
Prediction of the subcellular localization of the enzymes involved in the methanol metabolism pathway in *M. perniciosa*. Catalase and pectin methylesterase are predicted as secreted; the other enzymes are predicted as cytoplasmatic, except for OAH, predicted as mitochondrial. However, MOX is as secreted protein, as show in Section 3.2, despite the absence of a predicted signal peptide. *Abbreviations*: cyto, cytoplasmatic; nucl, nuclear; pero, peroxisomal; extr, extracellular, mito, mitochondrial.

Gene	SignalP	Psort prediction	PeroxiP
MOX	N	cyto: 19.0, cyto_nucl: 11.5, pero: 4.0, nucl: 2.0	N
CAT	Y	extr: 23.0, mito: 3.0	N
FMDH	N	cyto: 26.0	N
FDH	N	cyto: 15.5, cyto_nucl: 8.5, E.R.: 4.0, mito: 3.0, extr: 3.0	N
DHAS	N	cyto: 16.0, cyto_nucl: 9.5, pero: 5.0, extr: 3.0	N
DHAK	N	cyto: 21.5, cyto_nucl: 11.5, extr: 5.0	N
FBPA	N	cyto: 24.5, cyto_nucl: 13.5	N
FBPT	N	cyto: 17.0, cyto_nucl: 15.3, cyto_mito: 10.5, extr: 2.0	N
OAH	N	mito: 11.0, cyto: 5.0, plas: 3.0, extr: 3.0, pero: 3.0	N
PME	Y	extr: 26.0	N

Based on the values of gene expression and the prediction of the subcellular location of the methanol pathway enzymes, performed by the software SignalP, PsortII and PeroxiP (Table 4), we propose a peroxisome-independent model for methanol degradation in *M. perniciosa* (Fig. 6). According to our model, the methanol released by pectin demethylation is oxidized by an extracellular methanol oxidase, generating formaldehyde and hydrogen peroxide. The peroxide generated may be degraded by an extracellular catalase (CAT) or by a secreted DyP peroxidase (PER), that uses iron ions as co factors. The resulting formaldehyde may be subjected to oxidation by the enzymes formaldehyde dehydrogenase (FMDH) and a NAD-dependent formate dehydrogenase (FDH), generating carbon dioxide. It may also be incorporated in the dihydroxyacetone (DHA) pathway, comprising the enzymes dihydroxyacetone synthase (DHAS), dihydroxyacetone kinase (DHAK), fructose-bisphosphate aldolase (FBPA) and fructose-bisphosphatase (FBPT), whose products are incorporated into the pentose phosphate pathway. One of the subproducts of the DHA pathway is glyceraldehyde 3-phosphate, which can be a precursor for the synthesis of oxaloacetate. Oxaloacetate, an intermediate of the tricarboxylic acid cycle, is also a substrate for the enzyme oxaloacetate acetyl hydrolase (OAH), which generates oxalate. Oxalate is known for its capability to remove calcium ions from the structure of pectin, forming calcium oxalate crystals and allowing further enzymatic pectin degradation by PCWD (Guimaraes and Stotz, 2004). The production of calcium oxalate crystals by *M. perniciosa* necrotrophic mycelia has been previously reported (Rio et al., 2008).

As higher levels of gene expression may correlate to higher amounts of protein produced, we suggest that in *M. perniciosa*, the formaldehyde generated from methanol metabolism is preferentially metabolized by the enzymes FMDH and FDH due to the higher values of RPKM and the fold change when compared with the values of the DHA pathway. Formaldehyde metabolized by the DHA pathway is used in the production of cell constituents used for cell growth and proliferation; on the other hand, its use by FDH and FMDH generates NADH, used as an electron donor for the generation of energy. However, at least a part of the formaldehyde generated is metabolized by the DHA pathway, allowing *M. perniciosa* to grow in methanol as sole carbon source; if all the formaldehyde was metabolized by the FDH and FMDH pathway, no growth on methanol as the sole carbon source would be observed.

In methylotrophic yeasts, the peroxide generated in the peroxisomes by methanol oxidation is decomposed by a catalase (Yurimoto et al., 2011). Because *M. pernciosa* methanol metabolism occurs in the extracellular space, we investigated the presence of an

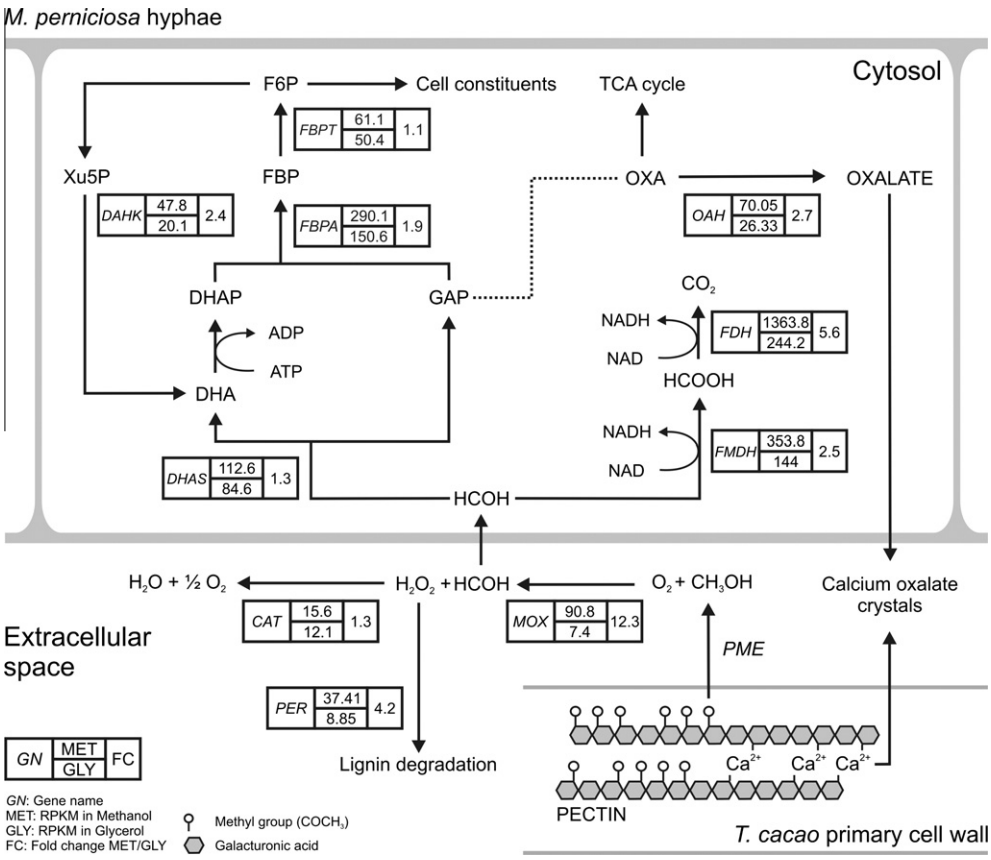

Fig. 6. A peroxisome-independent model for the methanol metabolism pathway in *M. perniciosa*, which is induced at a transcriptional level in cultures supplemented with methanol when compared with cultures supplemented with glycerol. Enzyme abbreviations: MOX, methanol oxidase; CAT, catalase; PER, peroxidase; FMDH, formaldehyde dehydrogenase; FDH, NAD-dependent formate dehydrogenase; DHAS, dihydroxyacetone synthase; DHAK, dihydroxyacetone kinase; FBPA, fructose-bisphosphate aldolase; FBPT, fructose-bisphosphatase; OAH, oxaloacetate acetyl hydrolase, PME: pectin methyl esterase. GenBank ID: JX024739–JX024749. Substrate abbreviations: DHA, dihydroxyacetone; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde 3-phosphate; FBP: fructose-1,6-bisphosphate; F6P: fructose 6-phosphate; Xu5P: xylulose 5-phosphate; OXA, oxaloacetate.

extracellular catalase. As shown in Fig. 6, a catalase showing a clear predicted secretory signal (Table 4) is slightly induced in the presence of methanol and its activity is detected in the fungal culture supernatants (data not shown), indicating a possible role in the degradation of the peroxide generated. Moreover, *M. perniciosa* possesses several peroxidases that are predicted to be secreted, which may also participate in peroxide degradation. One of these peroxidases is induced in the methanol RNA-seq libraries, as shown in Fig. 6. A Dyp peroxidase, which uses iron ions as cofactors, shows a 4-fold increase in its expression when *M. perniciosa* is supplemented with methanol compared with supplemented with glycerol. In known plant–pathogen models involving wood-degrading fungal species, such as *Postia placenta, Phanerochaete chrysosporium* and *G. trabeum*, secreted peroxidases are related to lignin degradation (Daniel et al., 2007; Wymelenberg et al., 2010). Moreover, extracellular sources of hydrogen peroxide, such as that generated by methanol oxidation are related to lignin degradation via the Fenton reaction: $H_2O_2 + Fe^{2+} + H^{1+} = OH + Fe^{3+} + H_2O$. The hydroxyl radicals generated by this reaction (·OH) are able to penetrate the plant cells of infected hosts and act as depolymerizing agents, thus causing extensive cell damage (Schlosser et al., 2000). The necrosis observed in the dry broom phase of WBD as a consequence of plant cell death may be caused by, among other factors, lignin degradation via peroxidases and/or the Fenton reaction. Although *M. perniciosa* is not a classical wood-degrading phytopathogen, studies have revealed that this species is probably derived from a saprotrophic ancestral (Aime and Phillips-Mora, 2005; Tiburcio et al., 2010); thus, *M. perniciosa* may have retained its ability to degrade wood.

As shown in Fig. 4, in addition to methanol, MOX expression is also induced in the presence of esterified pectin. Our results show a correlation between the expression of *Mp-mox* and *Mp-pme* in *M. perniciosa* mycelia grown in esterified pectin, suggesting methanol utilization provided by pectin degradation. Interestingly, both genes also exhibit a transcriptional correlation in infected cacao seedlings at the different stages of the progression of WBD that were analyzed. In plants, one of the main sources of methanol is pectin, which is the main constituent of primary cell walls and the middle lamellae of higher plant cells (Nakagawa et al., 2000; Pelloux et al., 2007). The differential expression of *Mp-mox* and *Mp-pme* during the progression of WBD suggests an important role for these genes in the development of the disease, especially during the beginning of necrosis when the mycelia turn necrotrophic and invade the plant cells. The higher relative expression of MOX and PME, in conjunction with the lower level of methyl esterification in the beginning of necrosis, as shown in Fig. 5 by a weak JIM7 labeling and strong JIM5 labeling (Fig. 5C: 3 and 7), may be key steps in the beginning of cell invasion by the mycelia, given that the activity of PME allows further action of other PCWD, such as polygalacturonases and cellulases.

Although PMEs are constantly reported to be pathogenicity factors, because disruption of the *pme* gene in fungi like *B. cinerea* reduces virulence in their respective plant hosts (Valette-Collet et al., 2003), very little is known about the importance of MOX for pathogenicity. The only well-described case refers to the ascomycete fungus *C. fulvum*, in which the deletion of the methanol oxidase gene reduced its virulence in tomato leaves; however, the mechanism of MOX in the pathogenicity of this fungus remained

uncertain (Segers et al., 2001). In phytopathogenicity, MOX has been related to lignin degradation instead of the pectin degradation as proposed in this work. In the wood-degrading basidiomycetes, such as *P. placenta* and *G. trabeum*, MOX is suggested to generate the hydrogen peroxide used for Fentoń reaction (Daniel et al., 2007; Martinez et al., 2009). Although we cannot discard the possibility that MOX participates in Fentoń reaction, providing the extracellular hydrogen peroxide used for Fentoń reaction, our data strongly suggest that *M. perniciosa* methanol oxidase is related to the pectin degradation. Moreover, as *M. perniciosa* is able to grow on methanol as the sole carbon source and possesses the complete pathway for methanol degradation, we also suggest that this fungus utilizes methanol as an energy source, similar to the methylotrophic yeast species. This is in contrast to wood-degrading fungal species, in which methanol degradation is only described as an provider of extracellular generator of hydrogen peroxide (Daniel et al., 2007). Lastly, MOX also uses ethanol as a substrate; in plants, ethanol is derived from fermentative growth on sugars and used as a carbon source via acetaldehyde and the TCA cycle; however, we carefully inspected over 80 RNA-seq libraries and have found no metabolism compatible with ethanol production by the plant (data not shown); on the other hand, our results show evidences for the presence of methanol and for that reason we consider that this is the substrate utilized by the proposed pathway. The disruption of the *Mp-mox* and *Mp-pme* genes from *M. pernciosa* would be a key next step in confirming the role of these two enzymes in the establishment of WBD and will be performed as soon as gene disruption techniques are developed for *M. perniciosa*.

5. Conclusions

In this work, we demonstrate that *M. perniciosa* produces a methanol oxidase that is secreted into the extracellular space. The relative expression of this methanol oxidase *in vitro* and *in planta* is correlated with the *Mp-pme* expression levels and with the reduction in the level of methyl esterification observed in infected cacao plants, suggesting that this fungus metabolizes the methanol generated by pectin degradation. Moreover, we propose a peroxisomal-independent methanol metabolism pathway for *M. perniciosa*, which begins in the extracellular space and ends in the cytosol.

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