African Journal of Biotechnology Vol. 12(12), pp. 1311-1317, 20 March, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.476
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Protective effect and expression of defense-related ESTs induced by acibenzolar-S-methyl and a phosphorylated mannan oligosaccharide-based product against *Moniliophthora perniciosa* in *Theobroma cacao*

Lívia de Matos Pereira[1], Mário Lúcio Vilela de Resende[1]*, Sandra Marisa Mathioni[1], Magnólia Araújo Campos[2], Pedro Martins Ribeiro Júnior[1], Fabrício Rabelo Camilo[1], Anderson Tadeu Silva[3] and Luciano Vilela Paiva[3]

[1]Departamento de Fitopatologia, Universidade Federal de Lavras, CP 3037, CEP 37.200-000, Lavras, MG, Brazil.
[2]Unidade Acadêmica de Educação-Biologia, Universidade Federal de Campina Grande, Centro de Educação e Saúde, CEP 58175-000, Cuité, PB, Brazil.
[3]Laboratório Central de Biologia Molecular, Universidade Federal de Lavras, MG, Brazil.

Accepted 25 February, 2013

Witches' broom disease (WBD), caused by the fungus *Moniliophthora perniciosa*, is one of the main diseases in cocoa (*Theobroma cacao*) and has caused severe economic losses. Integrated disease management has been the focus for its control and therefore, the identification of new inducers of plant resistance is desirable. Thus, the goal of this work was to evaluate two potential inducers of resistance against WBD. A phosphorylated mannan oligosaccharide-based product (PMO) and acibenzolar S-methyl (ASM) were tested on *M. perniciosa* inoculated seedlings and in field experiments and showed a reduction on the incidence of WBD. The expression of two defense-related expressed sequence tags (ESTs) in cocoa, coding for peroxidase (*Pox*) and chitinase (*Chi*), were accessed by qPCR. Both products induced the expression of the *Pox* defense-related EST. In general, ASM induced the expression of chitinase (*Chi*) and peroxidase (*Pox*) in earlier time-points than PMO. However, PMO provided long-lasting and higher levels of expression. *Chi* expression was triggered in the time-points succeeding the spraying but was very low. On the other hand, peaks of *Pox* transcripts were detected in later time-points for both inducers. ASM and PMO modes of action might be explained, at least partially, by the overexpression of defense-related ESTs.

Key words: Cocoa, witches' broom disease, disease control, peroxidase, chitinase, induced resistance, elicitors, quantitative reverse transcriptase PCR (qRT-PCR).

INTRODUCTION

Witches' broom disease (WBD), caused by the fungus *Moniliophthora* (ex *Crinipellis*) *perniciosa* (Aime and Phillips-Mora, 2005) is one of the three major fungal diseases of cocoa, *Theobroma cacao* L. Severe economic losses in this crop, which ranged from 50 to 90%, were caused by this disease in all growing regions to which it has spread. Even with the introduction of resistant varieties, production of cocoa in South America is still impaired as a result of WBD (Meinhardt et al., 2008). The fungus infects meristematic tissues causing hyperplasia and hypertrophy and breakage of apical dominance, resulting in an abnormality of the meristem called "green broom", a characteristic symptom of the disease (Evans, 1980). Genetic, cultural, chemical and biological control methods are integrated for the

*Corresponding author. E-mail: mlucio@dfp.ufla.br. Tel: +553538291793. Fax: +553538291281.

management of WBD (Luz et al., 1997). Proposed alternative methods include those based on plant extracts (Bastos and Albuquerque, 2004) with toxicity to the pathogen, and those based on the induction of systemic resistance in plants (Resende et al., 2000, 2002; Costa et al., 2010). Plant resistance can be divided into two categories, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Loon et al., 1998). In SAR, the resistance develops systemically either in response to pathogens that cause a hypersensitive reaction or necrosis.

This can be reproduced by the exogenous application of salicylic acid (SA) and synthetic compounds, such as acibenzolar-S-methyl (ASM). In SAR, the expressed resistance, is generally effective against a broad spectrum of pathogens and is associated with the production of pathogenesis-related (PR) proteins (Van Loon et al., 2006). ISR is commonly induced by rhizobacteria, and in contrast to SAR, the signaling is mediated by jasmonate and ethylene, without immediate expression of PR proteins (Van Loon et al., 2006). A phosphorylated mannan oligosaccharide-based product (PMO), in which the main active ingredient is extracted from *Saccharomyces cerevisiae* cell walls, can also induce resistance in plants (Costa et al., 2010).

Certain peroxidases are also involved in the plant defense response by strengthening the cell wall through catalyzing the polymerization of phenolic compounds during lignin synthesis, a process that can delay or prevent pathogen colonization (Van Loon and Van Strien, 1999). Furthermore, peroxidases have been associated with a variety of other plant defense-related processes including the hypersensitive reaction, during which cell wall polymer cross-linking and tissue suberization occur (Baysal et al., 2003).

Plants can recognize many microbial, surface-derived compounds, which induce defense responses in both host and non-host plants (Nürnberger and Brunner, 2002). These structurally conserved molecules including peptides, carbohydrates, glycoproteins and lipids, are generally named microbial associated molecular patterns (MAMPs) (Aslam et al., 2009). Based on the knowledge of defense regulators and MAMPs, synthetic chemical elicitors of plant defense have been developed (Resende et al., 2007). Acibenzolar S-methyl (ASM) was the first commercially available SAR inducer (Lyon and Newton, 2003). Although chemically related to SA, ASM seems to act independently on SA or any other signal molecule, activating a signaling process that result in elevated defense gene expression (Benhamou and Bélanger, 2002). ASM was used with success to prevent the occurrence of WBD in cocoa seedlings (Resende et al., 2002) and is currently registered in the Ministry of Agriculture of Brazil for the control of this disease in nurseries(http://extranet.agricultura.gov.br/agrofit_cons/principal_agrofit_cons).

Oligosaccharide elicitors also have received attention and are important in the signaling response in plant defense. However, little is known either about their perception or the pathways involved in the downstream signaling triggered by those elicitors (Ramonell et al., 2005).

Quantitative reverse transcriptase PCR (qRT-PCR) has been deployed as a sensitive, rapid and reproducible method for measuring defense-related gene expression (Wong and Medrano, 2005). Cools and Ishii (2002) evaluated gene expression in cucumber pre-treated with ASM by qRT-PCR, revealing the induction of acidic peroxidase and pathogenesis-related protein 1 (PR-1) encoding genes. In wheat, Desmond et al. (2006) examined the expression of 26 genes after pre-treatment with methyl jasmonate and ASM followed by inoculation with *Fusarium graminearum* and observed the reproducible induction of eight genes, including a chitinase (*Chi*) and peroxidase (*Pox*) genes, whose proteins act against fungal cell walls that contain chitin and on strengthening of plant cell walls, respectively.

In order to associate cocoa protection against WBD to the possible induction of defense-related genes, the aim of this work was to test two inducers of resistance (ASM and PMO) against WB and assess the temporal expression of two defense-related expressed sequence tags (ESTs), *Chi* and *Pox,* before and after inoculation of cocoa seedlings with *M. perniciosa*.

MATERIALS AND METHODS

Cocoa seedlings (*Theobroma cacao*, cv. Catongo, susceptible to WBD), originating from the Cocoa Research Centre (CEPEC-CEPLAC, Itabuna, BA, Brazil) were sown in polystyrene trays with 72 cells, containing commercial potting mix (Plantmax®, Eucatex, São Paulo, SP) and fertilized weekly with a 10x dilution of Hoagland's solution (Hoagland and Arnon, 1950). Trays were kept in a greenhouse with 25±3°C and 80% relative humidity maintained by automated mist sprinklers.

Inoculum production

M. perniciosa basidiocarps were obtained from infected cocoa branches maintained in a humid chamber with intermittent fog sprays. Basidiospores were harvested in a 16% (v/v) glycerol solution according to Frias et al. (1995) and stored in liquid nitrogen until inoculation on cocoa plants.

Protective effect of ASM and PMO on the cocoa witches' broom incidence in greenhouse and field conditions

Greenhouse trial

To evaluate disease prevention, 30-day old cocoa seedlings were sprayed to run-off with PMO (Agro-Mos®, Improcrop Ltd., Curitiba, PR) at the rate of 10 mL· L^{-1} of water, or with ASM (Bion® 50WG, Syngenta Ltda, São Paulo, SP), at the rate of 0.2 g a.i · L^{-1} using a hand-pump sprayer that delivered 0.3 L· m^{-2}. The mock inoculated control was sprayed with distilled water. The experiment was set as a randomized block design and was repeated twice with eight replicates and six seedlings per replicate.

Seven days after spraying, seedlings were inoculated with *M. perniciosa* basidiospores using agar-droplet inoculation. Each seedling was inoculated with 10 µl of basidiospore suspension (100,000 viable basidiospores per ml in 0.3% agar) on the apical meristem. Plants were maintained in a chamber at approximately 100% relative humidity for 48 h after inoculation to support fungal penetration (Resende et al., 2000).

Witches' broom incidence was evaluated 60 days after inoculation, which is the time required for the formation of brooms (hypertrophy and hyperplasia) (Resende et al., 2002). Data were tested for normality, submitted to analysis of variance (ANOVA) and means were compared according to Tukey test ($P < 0.05$) using SAS v.8. (SAS, Cary, NC).

Field trial

After confirming the control of witches' broom on seedlings by PMO, an experiment was carried out in a 15 year-old cocoa clonal plantation (*Theobroma cacao*, cv. Catongo, susceptible to WBD) with high severity level of witches' broom disease, in Linhares, State of Espírito Santo, Brazil.

A randomized block design was used with eight replicates of ten plants each. The treatments applied in the field experiment were the same with that of the greenhouse experiment and the disease occurred naturally. Before initiating the experiment, the trees were carefully cleaned, removing symptomatic pods and branches. Other conventional crop management practices were adopted, such as pruning and individualization of the canopies. Quarterly applications of the products (February, May, August and November) were conducted during two consecutive years, using a motorized backpack sprayer (400 ml of each suspension was applied per plant at each time).

The cumulative number of vegetative brooms per plot from two consecutive years of assessment was submitted to ANOVA and means were compared as described for the greenhouse experiment. Data were tested for normality and analyzed by a one-way ANOVA followed by a Tukey test ($P < 0.05$) for multiple comparisons using SAS v.8. (SAS, Cary, NC).

Defense-related EST expression bioassay

The experiment for expression analysis was performed under conditions similar to the greenhouse experiment to access the expression of putative defense-related ESTs. 30-day old plants were sampled at 0.5, 2, 4, 7.5, 9 and 11 days after PMO or ASM application (dpa) and *M. perniciosa* inoculation was carried out seven days after the chemical application (chemical application performed as the greenhouse experiment). The epicotyl region was sampled from four plants, each being a technical replicate, and from three biological replicates for each time-point. The four technical replicates were pooled for RNA extraction. Samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction

Total RNA was extracted from 4 g of the pooled technical replicates based on the protocol described by Chang et al. (1993) with modifications, as described. Samples were ground in a mortar with liquid nitrogen and transferred to a 50 mL Falcon tube, into which the extraction buffer was added [2% CTAB ($m \cdot v^{-1}$; Sigma), 100 mM Tris; 25 mM EDTA (Sigma); 2 M NaCl (Sigma); 0.05% spermidine ($m \cdot v^{-1}$; Sigma) and 2% β-mercaptoethanol ($v \cdot v^{-1}$; Sigma)]. Then, they were homogenized by vortex for 20 s and the mixture was incubated at 65°C for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added and the samples were homogenized and centrifuged at 12,000 *g* at 4°C for 30 min. The supernatant was transferred to new 50 mL Falcon tubes. RNA precipitation was performed by adding 2 mL of a 10 M lithium chloride solution and samples were incubated for 16 h in ice and then centrifuged at 12,000 *g* at 4°C for 45 min.

After centrifugation, the supernatant was disposed and the pellet washed with 10 mL 75% cold ethanol and centrifuged at 12,000 *g* and 4°C for 10 min. This step was repeated to eliminate any salt excess and the resulting pellet was purified following the QIAGEN RNeasy Cleanup Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. RNA integrity was visualized in agarose gel (1.2% $m\ v^{-1}$).

DNase treatment and cDNA preparation

Total RNA was treated with DNase I (New England BioLabs, Ipswich, MA, USA), following the manufacturer's instructions, to eliminate the presence of residual genomic DNA. A reaction containing 10 µg of total RNA, 2U of DNase I, 10 µL of 10X reaction buffer and nuclease-free water, to make up a 100 µL final volume was incubated at 37°C for 30 min. Samples were quantified using NanoDrop™ 1000 (Thermo Scientific, Wilmington, DE, USA). For each cDNA synthesis reaction, 1 µg of total RNA was reversely transcribed using the SuperScript™ II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The random primers (Invitrogen Cat No 48190-011) and the dNTP mixture were utilized at 3 and 10 µM per reaction, respectively. The mixture was heated up to 65°C for 5 min and immediately cooled for 5 min, by using the thermocycler (Eppendorf Mastercycler®, Westbury, NY, USA). Then, samples were spun down and 4 µL of buffer (First-Strand 5X and 1 µM of DTT) was added into the same tube, which was gently homogenized by inversion and incubated at 25°C for 2 min. The enzyme SuperScript II™ 200U μL^{-1} was added at 1 µL per reaction to make a 20 µL total reaction by adding DEPC-treated water. Tubes were incubated in a thermocycler at 25°C for 2 min and then at 42°C for 50 min. The enzyme was inactivated by heating the samples at 70°C for 15 min.

qRT-PCR of defense-related ESTS

Peroxidase and chitinase primer design

The primers used in this experiment were designed by using the primer express software (Applied Biosystems) with parameters set to generate 50 to 150 pb amplicons. Specific primers for the putative ESTs for the pathogenesis-related (PR) proteins, *peroxidase* (F-5'ACTGCCAGTGGGATCCAAAC3', R-5'GACCCCGGCCCAAAATATTG3') and class I *chitinase* (F-5'CCACCCTGAGGGCATATTTG3', R-5'CCGATCCGAACCTGAACACT3'), were designed based on GenBank deposited cocoa sequences (http://www.ncbi.nlm.nih.gov, accession numbers: CK144296.1 and U30324.1), that amplified 58 and 60 bp fragments, respectively. Actin was chosen as constitutive endogenous (housekeeping) control based on previous experiments (Alemanno et al., 2008). Conventional RT-PCR followed by agar gel electrophoresis was performed to verify the cDNA amplification. The specificity of each primer was verified by alignment of their sequences using the BLASTn function in the GenBank (Altschul et al., 1997) and by checking the presence of single bands by gel electrophoresis.

qRT-PCR

The qRT-PCR expression analysis was performed using SYBR green in the ABI PRISM 7500 Fast Real-Time PCR system (Applied

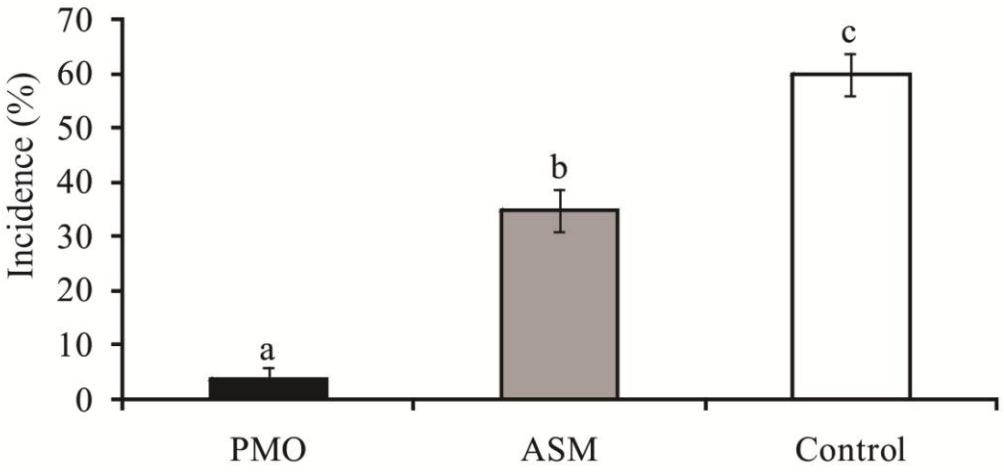

Figure 1. Effect of ASM and PMO on the witches' broom incidence evaluated at 60 days after *M. perniciosa* inoculation of *T. cacao* (cocoa) seedlings. Bars headed with different letters on the top are statistically different (Tukey test, P ≤ 0.05). Error bars = standard error.

Biosystems, Foster City, CA, USA). The reaction conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and ending with 95°C for 15 s. For each reaction, 80 ng of cDNA, 1.5 uM of each primer and 6.25 uL of the Master Mix SYBR green UDG with ROX (Invitrogen, Carlsbad, CA, USA) were added to make a final volume of 12.5 µL per sample. Three technical replicates for the qRT-PCR were performed for each biological replicate. Data were collected and stored in the Sequence Detection Software (1.4 version) provided by the equipment's manufacturer.

Results were normalized using C_t values obtained for the endogenous control present in the same reaction. C_t is defined as the first amplification cycle in which fluorescence indicates that the PCR product is detected above the threshold. The normalization was performed using the equation: $\Delta C_t = C_t$ (target gene) - C_t (endogenous control; actin). The calibration was determined by the formula $\Delta\Delta C_t = \Delta C_t$ (sample) - ΔC_t (calibrator). The relative quantification was obtained by the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The expression of the ESTs was tested for normality and log transformed. For each gene, differences in expression levels for each evaluated time-point were analyzed by ANOVA followed by Tukey test ($P < 0.05$) for multiple comparisons. Analyses were performed using PROC ANOVA, SAS v.8. (SAS, Cary, NC).

RESULTS

Protective effect of ASM and PMO on the cocoa witches' broom incidence in greenhouse and field conditions

In the greenhouse experiment, PMO treatment showed significantly lower incidence of witches' broom symptoms (4.2%) than ASM (35.2%), when compared with water control treatment (60.4%) (Figure 1).

Field trial evaluation of infected branches (vegetative brooms), showed that PMO reduced the incidence of vegetative brooms by ca. 3-fold as compared to the control (mock treatment). ASM, the product registered in Brazil as an inducer of resistance in cocoa, had an intermediate performance, differing from both PMO and the control treatment (Figure 2).

Expression of *Pox* and *Chi* in cocoa seedlings after elicitation with ASM and PMO

The *Pox* EST was slightly induced at 0.5 day after spraying (das), with PMO-treated seedlings being more induced than with ASM-treated ones. However, at 2 and 4 DAS, the trend was reversed. Highest expression levels of about 6 fold-changes occurred at 7.5 and 9 das for PMO-treated and 9 das for ASM-treated seedlings. By 11 das, expression of the *Pox* EST had declined (Figure 3A). Considering *M. perniciosa* inoculated plants sprayed with ASM or PMO, they showed induced expression at 9 DAS when compared with 7.5 das for the *Pox* EST (Figure 3A). By analyzing the contribution of the pathogen inoculation on the expression of the *Pox* EST, at 7.5 das, that is, 12 h after inoculation (hai), there was a significant reduction in the transcript levels induced by the pathogen in PMO-treated and inoculated plants, but not in ASM-treated and inoculated seedlings. Although, *Pox* transcripts decreased at 11 das for all treatments, in ASM-treated and inoculated plants, their levels were significantly higher (Figure 3A).

The *Chi* EST showed a slight induction at the three time-points (0.5, 2 and 4) anteceding the pathogen inoculation, which occurred at 7 das (Figure 3B). The ASM-treated seedlings showed higher expression levels than PMO-treated ones at 0.5 das, however, the expression was very low. At 2 and 4 das, the *Chi* expression level was higher in PMO-treated than in ASM-treated seedlings. After the pathogen inoculation, the levels of *Chi* expression were low and considered not significant (Figure 3B).

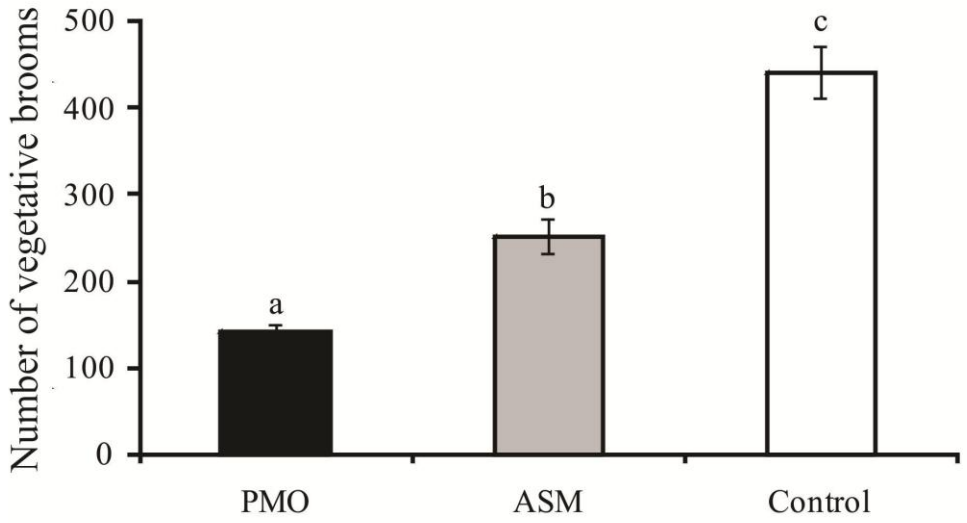

Figure 2. Effect of ASM and PMO on the total number of infected *T. cacao* (cocoa) branches (vegetative brooms). Bars headed with different letters are statistically different (Tukey test, P ≤ 0.05). Error bars = standard error.

DISCUSSION

Studies on the effect of inducers on plant resistance to diseases are needed and very important for their applied aspects; however, very little is known about their molecular and physiological effects on the plant protection and how plants respond to the application of such inducers. Thus, in the present study, we have shown the effect of inducers on resistance to protect cocoa plants against WBD. The treatment with PMO, applied on seedlings or on adult plants in the field, significantly reduced the incidence of witches' broom, when compared with the treatment with ASM.

On the expression of ESTs, we found that peroxidase *(Pox)* and chitinase (*Chi*) related ESTs were induced in cocoa by treating seedlings with either of the resistance inducers, ASM or PMO. However, the inducers showed a more significant effect on the *Pox* EST expression.

Inducers of plant resistance can differ on the genes they affect, which might explain the more significant effect observed in this study for ASM and PMO on the *Pox* EST than on the *Chi* EST in cocoa plants. Studies have shown that peroxidase like-proteins directly respond to resistance inducers (Katz et al., 1998; Conrath et al., 2002; Thulke and Conrath, 2002).

There are some reports of a not yet well characterized signaling cascade in plants induced by oligosaccharides, in which the expression of defense-related genes was detected (Potin et al., 1999). This may be the case of PMO, a mannan oligosaccharide-based inducer of resistance. On the other hand, the salicylic acid pathway followed by ASM, a SA analogue, has already being the focus of several studies. Peroxidase and chitinase genes have been reported as directly responsive to resistance inducers such as ASM (Katz et al., 1998; Conrath et al., 2002; Thulke and Conrath, 2002).

Induced resistance phenomena may be associated with an increased capacity for a rapid and effective activation of cellular defense responses, which are induced after challenged by a pathogen, in a so-called priming effect (Conrath et al., 2002). The activation of signaling components is suggested to contribute to an accelerated and enhanced response when the cells are challenged by a secondary and likely stressful stimulus (Conrath et al., 2002). However, the results of the current study show no priming effect since there was no significant second increase in *Pox* and *Chi* expression in cocoa seedlings upon inoculation with *M. perniciosa.*

Responses elicited by pathogens in susceptible genotypes occur in smaller magnitude and more slowly than those elicited in resistant genotypes, and this trend has also been shown in cocoa (Leal et al., 2007). Comparing the expression profile of ESTs in ASM/PMO-treated and inoculated susceptible cv. Catongo seedlings, *Pox* transcripts remained high (up to 9 das). However, *Chi* transcripts were gradually reduced to almost undetectable levels by 11 das. Thus, *Chi* gene expression may have been silenced in the presence of the pathogen. Suppression of defense by pathogens in susceptible genotypes of host plants has been shown previously as an important component in the pathogenesis process (Nomura et al., 2005). On this way, Leal and colleagues (2007) have already demonstrated that expression of *Chi* was not induced in cocoa plants inoculated with *M. perniciosa*.

In an ASM-mediated study of induced resistance on cocoa seedlings against *Verticillium* wilt, peroxidase activity increased from 1.5 to 2.0-fold as compared to the

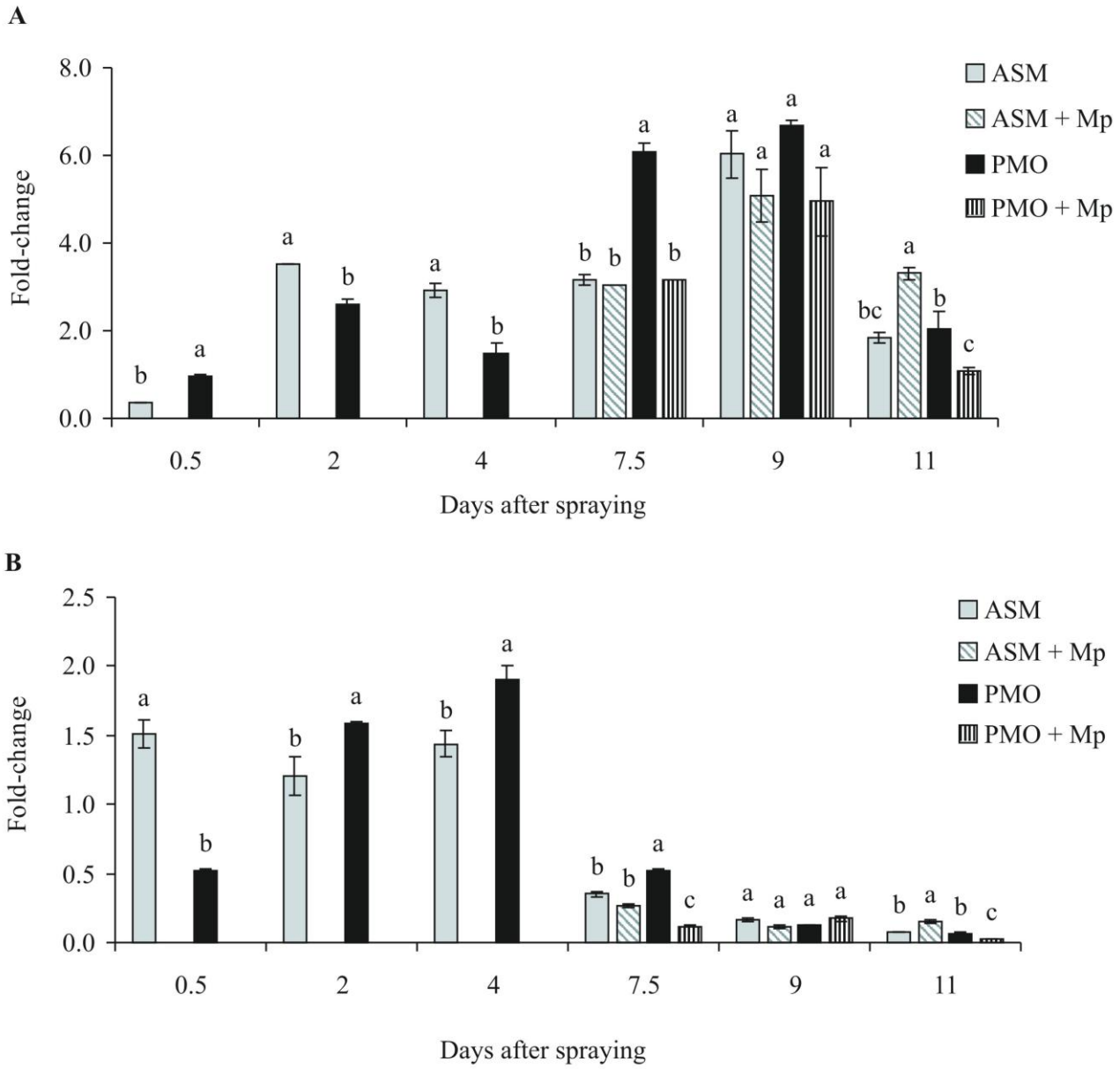

Figure 3. Expression profile of peroxidase (A) and chitinase (B) ESTs at 0.5, 2, 4, 7.5, 9 and 11 days after spraying with ASM (acibenzolar-S-methyl; Bion®) or PMO (phosphorylated mannan oligosaccharide; Agro-Mos®). Inoculation with *M. perniciosa* (Mp) was performed at 7 days after spraying. Bars represent the fold-change of ESTs in elicited plants relative to the control (mock treated - sprayed with water). Bars headed with different letters are statistically different (Tukey test, $P \leq 0.05$). Error bars = standard error. Actin was used as housekeeping (endogenous) control.

control, either before or after inoculation with *Verticillium dahliae* (Cavalcanti et al., 2008). By contrast, chitinase activity (*Chi*) increased only before Verticillium-inoculation. These authors suggest that peroxidase acts as a general stress response enzyme, and chitinase is a more specific response of plants against intruders, therefore may be suppressed by some fungal pathogens.

ASM and PMO modes of action might be explained, at least partially, by the over-expression of the *Pox* EST. Costa and colleagues (2010) reported that PMO containing copper sulphate in its formulation (Agro-Mos® commercial) has a direct effect on the pathogen as copper sulphate has antifungal effect. Treatments with commercial and experimental PMO, performed by Costa and colleagues (2010), showed similar *Chi* activity at 7 das, however commercial PMO presented the first peak of *Chi* activity at 1 DAS and experimental PMO showed an early peak at six hours after spraying. ASM also has higher *Chi* activity than control at 6 h after spraying. Moreover, commercial and experimental PMO treatments

showed different *Pox* activity profiles. In non-inoculated plants, the induced *Pox* activity was observed at 1 das and experimental PMO showed no difference as compared to the control. After inoculation, both presented an increase in the enzymatic activity at 10 das. However, commercial PMO peaked at 6 h after inoculation also on seedlings treated with ASM.

As the priming effect generated by different inducers might vary, that is, the induction of different genes, it is reasonable to speculate that other defense-related genes (or ESTs) are also likely to contribute to the reduction of witches' broom disease incidence in cocoa and thus deserving future studies.

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