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Research Article

Protein extraction for proteome analysis from cacao leaves and meristems, organs infected by *Moniliophthora perniciosa*, the causal agent of the witches' broom disease

Preparation of high-quality proteins from cacao vegetative organs is difficult due to very high endogenous levels of polysaccharides and polyphenols. In order to establish a routine procedure for the application of proteomic and biochemical analysis to cacao tissues, three new protocols were developed; one for apoplastic washing fluid (AWF) extraction, and two for protein extraction – under denaturing and nondenaturing conditions. The first described method allows a quick and easy collection of AWF – using infiltration–centrifugation procedure – that is representative of its composition in intact leaves according to the smaller symplastic contamination detected by the use of the hexose phosphate isomerase marker. Protein extraction under denaturing conditions for 2-DE was remarkably improved by the combination of chemically and physically modified processes including phenol, SDS dense buffer and sonication steps. With this protocol, high-quality proteins from cacao leaves and meristems were isolated, and for the first time well-resolved 1-DE and 2-DE protein patterns of cacao vegetative organs are shown. It also appears that sonication associated with polysaccharide precipitation using *tert*-butanol was a crucial step for the nondenaturing protein extraction and subsequent enzymatic activity detection. It is expected that the protocols described here could help to develop high-level proteomic and biochemical studies in cacao also being applicable to other recalcitrant plant tissues.

Keywords:
Apoplastic washing fluid / Cacao vegetative organs / Enzymatic activity / Plant protein extraction / Two-dimensional gel electrophoresis
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1 Introduction

Cacao (*Theobroma cacao* L.) is a tropical subcanopy tree originally from the rain forest of the Amazon basin. It is cultivated primarily to provide cacao liquor, butter and powder for the chocolate industry, not only due to flavor properties, but also due to emerging health benefits [1]. Cacao is an important commodity: more than 20 million people depend directly on cocoa for their livelihood, and approximately 90% of the production is exported in the form of beans or semimanufactured cocoa products to Europe and USA (Food and Agriculture Organization, http://www.fao.org). Pathogenic diseases are a major problem for cocoa production, causing annual crop losses of 30–40% [2]. Cacao is susceptible to a number of coevolved pathogens, such as *Moniliophthora* (=*Crinipellis*) *perniciosa*, the causal agent of witches' broom disease [3], which has spread throughout Brazil, destroying cacao tree plantations and leading to important economical and social changes in affected areas [4, 5]. Due to its environmental and economical importance, recent studies to understand the genomic program of cacao during its infection by witches' broom disease were developed [6–9]. However, cacao has received little attention with respect to proteomic research and until now, no study has been carried out to analyse the protein pattern of cacao during its infection by pests or diseases. Some studies to understand and improve the chocolate quality and flavor by characterization of protein

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Abbreviations: ADP, acetone dry powder; **AWF,** apoplastic washing fluid; **HPI,** hexose phosphate isomerase; **PCD,** programmed cell death; **POD,** peroxidase; **TE,** total extract

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changes during ripening of the cacao fruit, postharvest fermentation of cacao beans and key processing steps in the chocolate factory, namely, roasting and conching, were developed [10, 11]. However, these studies carried out for cacao beans, were not related to the high level of polysaccharides present in the vegetative organs such as leaves and meristems [12, 13], which are specifically infected by the hemibiotrophic fungus *M. perniciosa*. One of the challenges associated with the present research is the isolation of intact proteins (under native or non-native conformation) from cacao leaves and meristems, avoiding the high content of polyphenols and polysaccharides which disturb protein separation and proteome analysis in 2-DE [14–16].

On the other hand, the plant cell apoplast, which includes the cell wall matrix, is a dynamic compartment involved in a variety of functions during normal growth and under stress conditions, including maintenance of tissue shape, development, nutrition, signalling, detoxification and defence [17]. The plant cell apoplast plays a major role in defence mechanisms and has been found to respond to biotic and abiotic stresses such as air pollutants, heavy metal toxicity, drought, salinity, temperature extremes, pathogen invasion and oxidative conditions. Stress conditions have been shown to alter protein composition of the apoplast both qualitatively and quantitatively. Most of these stress-induced changes observed in the apoplast appear to be related to an increased expression of putative defence proteins [17–20]. Therefore, protein analysis of apoplastic fluid seemed to be a crucial step in order to understand the cacao–*M. perniciosa* interaction. It was previously shown that, in the apoplastic space, the biotrophic phase of *M. perniciosa* progressed and also changed from the monokaryotic (parasitic/biotrophic) to the dikaryotic (saprophytic/necrotrophic) mycelium [7, 21]. Presumably, plant defence compounds and substances responsible for the phase transition of the fungus could be located in the apoplast [21]. For this reason, we report here a new method of apoplastic washing fluid (AWF) extraction based on vacuum application and centrifugation, very useful for cacao–*M. perniciosa* interaction proteome analysis.

On the other hand, although many protocols are reported to define good conditions for sample preparation from recalcitrant plant tissues prior to IEF or 2-DE gel [22, 23], in the case of cacao, the polysaccharides are still contaminating the protein extract and interferes with such protein analysis. To overcome this problem, we developed two new protocols of cacao protein extraction (under denaturing or non-denaturing conditions) that attempt to minimize the presence of such compounds. The first protocol allowed the extraction of cacao proteins under denaturing conditions using phenol/dense SDS extraction buffer in combination with a sonication step. This method could be used from plant total extract (TE; with a previous step of acetone dry powder (ADP) preparation) or from AWF, and was necessary to produce high-quality proteins suitable for 1-DE and 2-DE. The second protocol allowed the extraction of native proteins using *tert*-butanol and sonication and may be used previously to enzymatic activity detection. As an example of the efficiency of these two protocols for further applications, many results such as Western blot of cacao protein extracts and enzymatic activities (peroxidase (POD), chitinases and proteases) are demonstrated. These results allow new perspectives regarding the understanding in proteome studies of the infection of the cacao tree by different devastating pathogens such as *Moniliophthora perniciosa*.

2 Materials and methods

2.1 Plant material

The plant material (leaves and meristems) was obtained from cacao trees (*T. cacao* L.) infected or uninfected by *M. perniciosa*, growing in the field of the Universidade Estadual de Santa Cruz, Bahia, Brazil. The development stages and symptoms of the disease were evaluated as described by [24] and [7].

2.2 Infiltration of cacao leaves and extraction of the AWF

Collected leaves were washed with distilled water and the central nervure was removed. The leaf pieces were immerged in K_2HPO_4 equilibrated with ascorbate (except for POD analyses in which ascorbate was not used) and introduced in a desiccating chamber. Vacuum (1 bar) was applied for 3 min, followed by the application of atmospheric pressure for 2 min. The process was repeated until the end of the bubble emission (corresponding to approximately 27 min of vacuum applied) (Fig. 1A).

Pieces of leaves were rolled with an adhesive plastic sheet around a rubber tube of 0.5 cm diameter × 8 cm length used as support (Fig. 1B). The "leaf cartridge" was inserted in the body of a syringe, and the syringe was inserted in a 15 mL Falcon tube. The "apparatus" was centrifuged for 25 min. After centrifugation, the AWF extracted from the tissue leaf was collected and concentrated by lyophilization for subsequent utilization in biochemical and proteomic experiments. AWF may be used pure, for enzymatic analyses (*e.g.* PODs), or may be submitted to a protein extraction step (Fig. 1A).

The influence of the centrifugal force on AWF extraction was evaluated by testing our protocol at 1000, 2000, 3000, 4000 and 4500 × *g*. In each condition, AWF volume was measured and AWF proteins were quantified using the 2-D Quanti Kit according to the recommendations of the manufacturer (GE Healthcare). Symplastic contamination in AWF was also analysed by detection of cytosolic hexose phosphate isomerase (HPI; EC 5.3.1.9) activity as described by [25]. Activity of HPI was determined using fructose-6-phosphate as substrate, which is converted into glucose-6-phosphate. The resulting glucose-6-phosphate is oxidized by added glucose-6-phosphate dehygrogenase (G6P-DH) and the correspond-

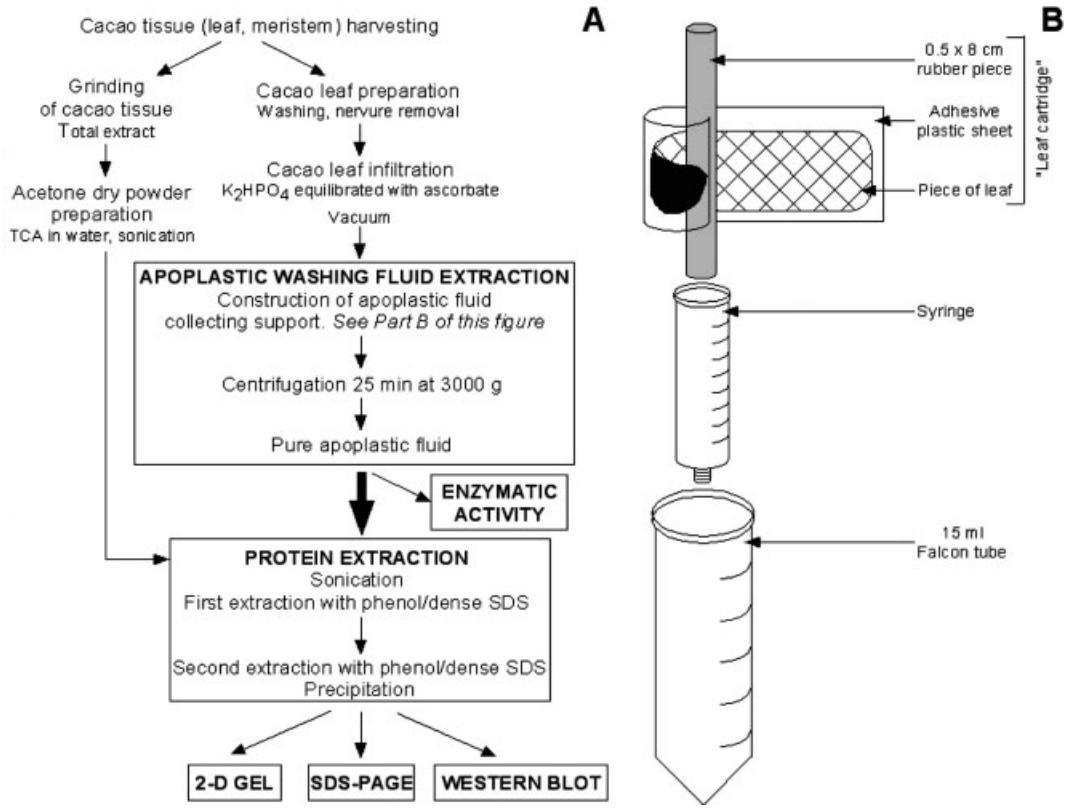

Figure 1. Extraction of AWF and proteins from cacao tissues. (A) Scheme showing the steps of AWF extraction, and of protein extraction from AWF and ADP. (B) Construction for collecting AWF from cacao leaves by centrifugation.

ing reduction of $NADP^+$ is measured by the increase in OD at 340 nm. The final reaction mixture (pH 8) contained 50 mM/L Tris, 5 mM/L MgCl2, 1 mM/L NaCl, 0.39 mM/L $NADP^+$, 0.46 U/mL G6P-DH and 1.4 mM/L fructose-6-phosphate. In this study, HPI activity is shown as the oxidation of $NADP^+$/min/g of protein.

2.3 New protein extraction methods developed for cacao vegetative organs

2.3.1 Preparation of ADP from cacao TE

The preparation of the ADP from plant TE was based on the protocol described by [22] with modifications (Fig. 1A). The plant material (leaf or meristem) was ground in liquid nitrogen using mortar and pestle. About 150–200 mg of ground tissue were resuspended in 1.5 mL of acetone, and centrifuged at 10 000 × *g* for 3 min at 4°C. The pellet was washed one more time with the same volume of cold acetone. After centrifugation, the pellet was dried at room temperature for 20 min. Then, the plant powder was washed three or four times with TCA 10% in acetone until the loss of coloration. At every step of the protocol described above, the pellet was completely resuspended by sonication (3 pulses of 5 s each, 40% output, with 10 s intervals) on a Ultrasonic processor (Gex 130, 130 W), and then centrifuged at 10 000 × *g* for 3 min at 4°C. Finally, the powder was washed once in 10% TCA in water and sonicated (3 pulses of 5 s each, 70% output, with 10 s intervals), and then washed once in acetone 80%. At this step the presence/absence of polysaccharides became more evident (see results and Figs. 4B and C). The final pellet was dried at room temperature, used for protein extraction, or stored at −80°C for future use. As other modification to the protocol described by [22], 2-mercaptoethanol 0.07% which prevents oxidation was added in all the solutions used in this protocol.

2.3.2 Protein extraction under denaturing conditions

Extraction of proteins from ADP or AWF was based on the protocol described by [22] with modifications (Fig. 1A). About 10–50 mg of ADP of cacao tissue or 15 mL of leaf AWF concentrated by lyophilization were resuspended in 0.8 mL of dense SDS extracting buffer (sucrose 30%, SDS 2%, 0.1 M Tris-HCl pH 8.0, 2-mercaptoethanol 5%). Samples were submitted to sonication (3 pulses of 8 s each, with 10 s intervals) with the setting at 50% output on a Ultrasonic processor (Gex 130, 130 W), incubated 10 min in ice, and then one volume of saturated phenol pH 8.0 was added. After 30 min of incubation at room temperature under agi-

tation, samples were centrifuged at 5000 × *g* for 10 min at 4°C. The proteins from the phenol phase were extracted again with one volume of dense SDS buffer. Phenol phases from the two extractions were grouped and incubated 14 h at −20°C with five volumes of $AcNH_4$ 0.1 M in methanol. Samples were centrifuged at 20 000 × *g* for 5 min at 4°C. Proteins were washed three times with $AcNH_4$ 0.1 M in methanol, twice with acetone and once with ethanol 70%, and then the pellets were finally dried at room temperature. Depending upon the following use, the pellets were resuspended in the electrophoresis buffer or in distilled water. Proteins (three repetitions for each sample) were quantified using the 2-D Quanti Kit according to the recommendations of the manufacturer (GE Healthcare).

2.3.3 Total native protein extraction method

Extraction of native proteins from plant TE (meristems and leaves) was based on the protocols described by [26] and [12] with modifications. About 1 g of plant tissue powder was resuspended in 5 mL of extraction buffer (Tris-HCl 10 mM pH 7.5, Triton X-100 1%), vortexed for 10 min and kept on ice. Samples were submitted to sonication (8 pulses of 3 s each with 10 s intervals) with the setting at 70% output on a Ultrasonic processor (Gex 130, 130 W), and then centrifuged at 15 000 × *g* for 20 min at 4°C. The pellet was submitted to a second extraction (Fig. 7), whereas the supernatants were mixed with 0.4 volume of *tert*-butanol and 1/10 of 3 M NaAc pH 4.5. Samples were kept on ice for 30 min with a vortex step at each 10 min, and then centrifuged at 15 000 × *g* for 10 min at 4°C. The supernatant containing total proteins under native conditions were stocked at 4°C for subsequent protein activity detection.

2.4 Electrophoresis and electroblotting

For 1-DE (SDS-PAGE), the Laemmli buffer system [27] was used to cast 5% stacking and 12.5% resolving gel. After denaturation at 95°C for 3 min, proteins were resolved under 150 V, 280 mA/60 W in a BioRad mini-Protean II apparatus until bromophenol blue reached the bottom of the gel. After electrophoresis, proteins were visualized with 0.1% w/v colloidal Coomassie G 250 [28], or electroblotted onto hybond™-C extra NC support (Amersham Biosciences).

For 2-DE, first-dimensional electrophoresis was performed on an Ettan IPGphor system (GE Healthcare). Protein samples were applied in 250 μL of 2-DE rehydratation solution by reswelling 13 cm Immobiline DryStrip (pH 4–7, GE Healthcare) for 12 h. Afterwards, focusing was performed on the same apparatus under the following conditions: step and hold at 500 V for 1 h, gradient 1000 V for 1 h, gradient 8000 V for 2:30 h, and step and hold 8000 V for 55 min. After IEF, the strips were stored at −80°C until second-dimensional analysis. Before the SDS-PAGE electrophoresis, the strips were incubated for 15 min in equilibration buffer (6 M urea, 7.5 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue) with DTT 1% w/v, and for another 15 min in equilibration buffer with iodoacetamide 2.5% w/v. The strips were transferred onto vertical 12.5% SDS-PAGE gel. The second dimension (SDS-PAGE) was performed on a Ruby SE600 system (GE Healthcare): 80 V for 45 min and 50 mA *per* gel for 3 h 30 min for every strip at a constant temperature of 5°C. After electrophoresis, proteins were visualized with 0.1% w/v colloidal Coomassie G 250 [28]. 2-DE images were processed using the Kodak EDAS 290 imaging system. The High-Range Rainbow Molecular Weight Marker was used (GE Healthcare).

2.5 Immunoblotting

The protein blot was blocked with 5% casein in TBST buffer (20 mM Tris-HCl, pH 7.6, 0.8% NaCl, 0.1% Tween 20) and incubated with polyclonal anti-AWF antibody (dilution 1:1000) for 1 h at room temperature. An alkaline phosphatase-labeled antirabbit antibody (Sigma, dilution 1:5000) was used as a secondary antibody. The detection system was NBT/BCIP (Promega).

2.6 Chitinase assay

Chitinase activity was determined by colorimetric assays using the purple dye-labeled biopolymeric substrate, CM-chitin-RBV (Loewe Biochemical, Germany). Two hundred microliters of CM-chitin-RBV (2 mg/mL) was mixed to 300 μL of protein extract (under nondenaturing conditions) and 300 μL of Tris-HCl 10 mM, pH 7.5; Triton 1%. The mixture was incubated at 37°C for 3 h. The reaction was stopped by the addition of 200 μL of 2 M HCl. Samples were cooled on ice for 15 min, then centrifuged at 20 000 × *g* for 10 min to remove the nondegraded substrate. The supernatant was collected and the assay was performed spectrophotometrically at 550 nm. The chitinase activity was described by unit/ng protein/h. One unit of chitinase activity corresponded to an increase of absorbance of 0.1 [29, 30]. For each sample, nine independent repetitions were used.

2.7 POD activity

AWF extract was fractionated by IEF on 5% polyacrylamide stacking and 7.5% resolving 0.5 mm thick gel containing 9% v/v Ampholines (pH range from 3.5 to 10; Pharmacia). Fifty microliters of AWF was resuspended in 50 μL of distilled water in which was added 1 μL of Ampholines pH range from 3.5 to 10, and resolved in a BioRad mini-Protean II apparatus using NaOH 0.08% w/v as cathode running buffer and phosphoric acid 0.06% v/v as anode running buffer in the following conditions: prerun at 200 V for 15 min, and then 300 V for 15 min; run at 200 V for 10 min, and then 400 V for 3 h. After electrophoresis, POD activity was detected by incubating the gel in the buffer A (phosphate buffer 10 mM pH 6.0, guaicol 20 mM) for 20 min, and revealed by incubating the gel in buffer A containing H_2O_2 0.3% v/v. The

pH was checked on 0.5 cm pieces cut from the gel. This new method of POD activity visualization was compared with the classic method on seminative gel using the detection reaction as described by [31].

For the POD assay, 1.5 μL of proteins extracted under native conditions were mixed with 148.5 μL of Tris-HCl pH 7.5 and then with 150 μL of 2 × activity buffer (40 mM guaiacol, 20 mM NaH_2PO_4 pH 6.0, 0.6% H_2O_2). Activity was detected on microplate reader VERSAmax (Tunable Molecular Devices) at 470 nm for 1 min 30.

2.8 Electrophoretic detection of proteases on a caseine-polyacrylamide gel

Protease activity stain followed the protocol reported by [32] and [33]. Protein extracts of cacao leaves and meristems were mixed with loading buffer 6 × (glycerol 60%, bromophenol blue 0.4% and Tris-HCl 0.5 M pH 6.8) and loaded in a 12% SDS polyacrylamide gel copolymerized with 1% w/v casein. Gel running was made on the BioRad mini-Protean II apparatus at 160 v/250 mA/60 W. The gels were washed in Triton X-100 at room temperature for 30 min for SDS removal and protease renaturation, transferred to 0.1 M sodium acetate pH 4.0 and kept at 30°C overnight for proteolysis. After incubation, the gels were stained with 0.1% w/v colloidal Coomassie G 250 [28] then finally destained with distilled water. Protease activity was visible as clear bands against a dark-blue background.

3 Results

3.1 Preparation of AWF extracts

AWF from infected and uninfected cacao leaves for proteomic analysis were prepared using the infiltration–centrifugation procedure (Fig. 1). Several centrifugal forces were used (from 1000 to 4500 × *g*) and the collected volume of AWF increased linearly with the increase of the centrifugal force (Fig. 2A). At 3000 × *g*, the collected volume of AWF was about 200 μL/g of fresh weight (Fig. 2A). The AWF protein amount also increased linearly with the increase of the centrifugal force and about 35 μg/mL of protein was obtained at 3000 × *g* (Fig. 2B). This method was based on preliminary work from cacao for AWF extraction limiting intracellular contamination [34]. The level of intracellular contamination in the AWF was here evaluated by detection of the activity of the cytosolic HPI. The HPI activity in the AWF extracts was always weak regardless of the centrifugal force used, from 1.1 to 3.1 mmol of $NADP^+$/min/g of protein at 1000–4500 × *g*, respectively (Fig. 2C). At 3000 × *g*, 1.6 mmol of $NADP^+$/min/g of protein was detected, which could be considered as negligible compared with the activity detected in the total protein extract (50.1 mmol of $NADP^+$/min/g of protein).

The AWF may be directly used for protein activity detection by assay or in gel. As example, POD activity from mature and young uninfected leaves and from young leaves infected by *M. perniciosa* was detected on gel using our protocol (Fig. 3). Several isoforms were observed in all the studied samples: two bands in the young uninfected leaves, eight bands in the young infected leaves and nine bands in the mature uninfected leaves (Fig. 3A). These isoforms were clearly observed in AWF samples while only one band is observed in the TE protein sample (Fig. 3B, line 2). Considering that both AWF and TE samples contain apoplastic proteins such as PODs, this result showed that the AWF extraction method greatly enriched the AWF fraction with apoplastic proteins. The method of POD activity detection developed in this paper was compared with the classic detection method on seminative gel as described by [31] (Fig. 3C). The resolution and the amount of detected bands were greatly increased when the protocol we developed was used (Fig. 3C). With the classic protocol, only two bands of low intensity were detected in young infected and uninfected leaves, without pattern difference between these two samples. Our method provided better band separation and the detection of different POD activity pattern depending of the physiological conditions studied.

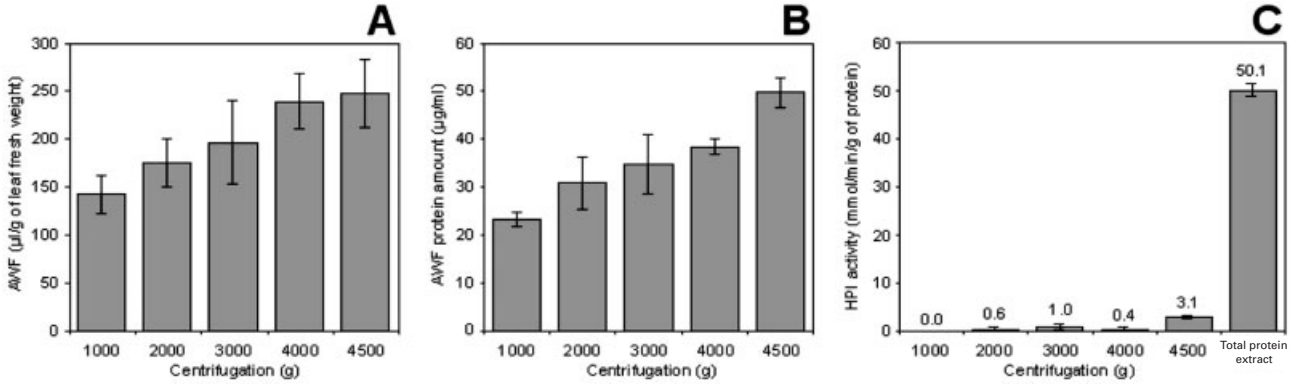

Figure 2. Characterization of AWF by centrifugation with increasing forces. (A) Amount of AWF obtained X centrifuge force. (B) Protein amount in the AWF extract X centrifuge force. (C) HPI activity in AWF extracts obtained under different centrifuge forces and in the total protein extract.

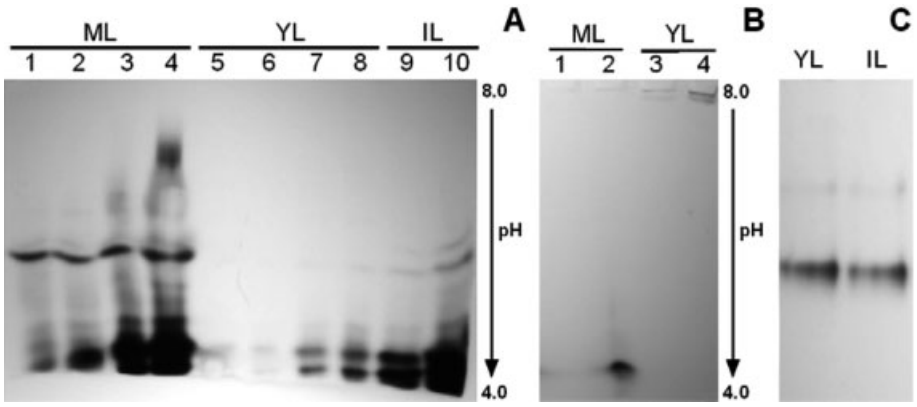

Figure 3. POD activity of AWF and TE proteins from cacao leaves uninfected or infected by *M. perniciosa*. (A) POD activity of pure AWF using the protocol described in this paper (see Section 2). Lanes 1, 5 and 9: 10 μL; lanes 2 and 6: 20 μL; lanes 3 and 7: 40 μL; lanes 4 and 8: 80 μL; and lane 10: 30 μL of AWF. (B) POD activity of TE proteins using the protocol described in this paper (see Section 2). Lanes 1 and 3: 10 μL; lanes 2 and 4: 30 μL of extract. (C) POD activity of AWF using the classic detection method on seminative gel as described by Alfenas *et al.* [31]. In each line of the gel, 18 μL of AWF were applied. IL, young leaf infected by *M. perniciosa*; YL, uninfected young leave; ML, uninfected mature leave.

3.2 Protein extraction under denaturing conditions and proteomic analyses

As determined by 1-DE, proteins isolated from cacao ADP by the phenol/dense SDS protocol (Fig. 4A, lines 1 and 2) showed very high quality and very little contamination by polysaccharides. Sharp polypeptide bands ranging from 100 to 14.3 kDa are excellently resolved with a low background (Fig. 4A, line 2). During the ADP preparation, the last sonication step, associated with TCA 10% in water washing step, was essential as shown in Figs. 4B and C. When the sample was sonicated, the polysaccharides were completely solubilized (Fig. 4C) whereas without sonication the polysaccharides still remained in the supernatant hindering the following steps of protein extraction (Fig. 4B, red arrow). This difference was also visible on the SDS-PAGE gel: more bands were observed in the sonicated sample (Fig. 4A, line 2) than in the sample without sonication (Fig. 4A, line 1). The quantification of the sample with or without sonication is presented Fig. 4D. The protein concentration was about 12 times higher in the sample treated by sonication (Fig. 4D, line 2) than in sample without sonication (Fig. 3D, line 1), showing that our protocol improved the quality as well the quantity of proteins extracted from cacao vegetative tissues.

The AWF and TE proteins from infected and uninfected cacao leaves were analyzed and compared on SDS-PAGE and immunoblotting (Fig. 5). All extracts present an excellent resolution with bands of polypeptides ranged from 100 to 14.3 kDa (Fig. 5A). TE protein (Fig. 5A, lines 3 and 4) present more bands of polypeptides than AWF protein extracts (Fig. 5A, lines 1 and 2). Howeve, the polypeptides detected on AWF extracts present a higher size than those detected on TEs (from 100 to 60 kDa, approximately). Interestingly, a prominent band of approximately 55 kDa characteristic of leaf protein fractions of many plant species and representing

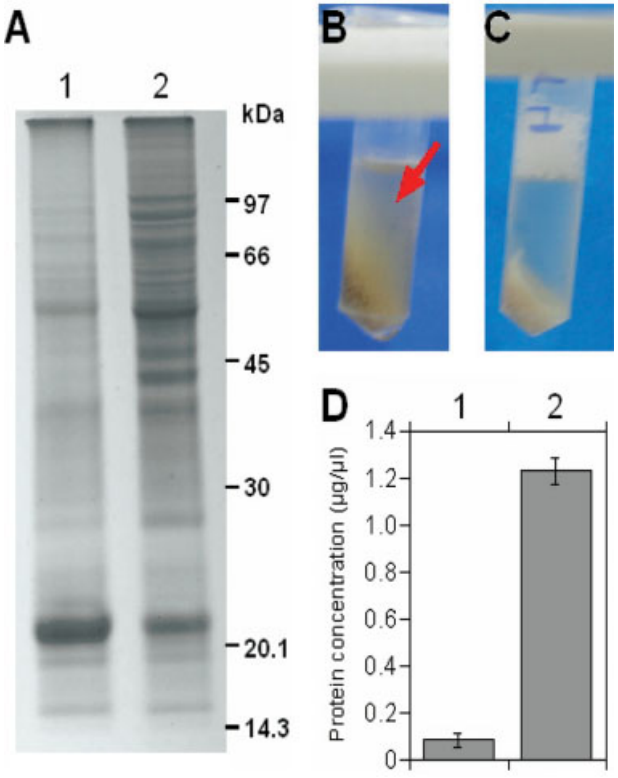

Figure 4. Protein extraction from ADP of cacao meristem. (A) SDS-PAGE of TE proteins: 20 μg of proteins were run on gel. 1, phenol method extraction without the last sonication step; 2, phenol method extraction with the addition of the last sonication step (see Section 2). (B), (C) Visualization of the extraction steps corresponding to the phenol method without sonication (B) and with sonication (C). The red arrow indicates the presence of non-dissolved polysaccharides. (D) Protein quantification after phenol method extraction without (line 1) or with (line 2) the last sonication step.

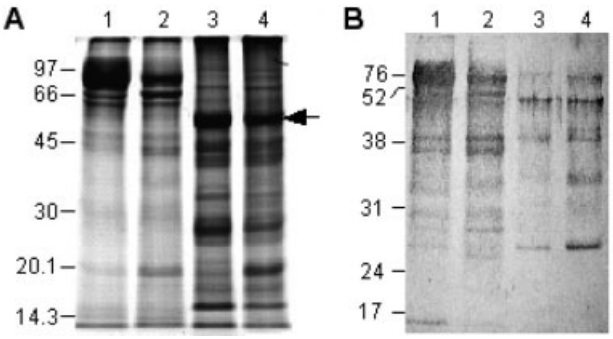

Figure 5. SDS-PAGE and immunoblotting of AWF and ADP proteins. (A) SDS-PAGE. Twenty micrograms of proteins was loaded on acrylamide 12.5% gel. (B) Immunoblotting obtained using an anti-AWF antibody. Each line contains 10 μg of proteins. 1, AWF from healthy leaf; 2, AWF from leaf of green broom; 3, TE (ADP extraction) from healthy leaf; 4, TE (ADP extraction) from leaf of green broom. LMW-SDS Marker Kit and High-Range Rainbow Markers (GE Healthcare) were used for SDS-PAGE and immunoblotting, respectively.

the large subunit of the ribulose-1,5-biphosphate carboxylase (Rubisco) was detected only on TEs (Fig. 5A, lines 3 and 4, black arrow). Differences between protein pattern from infected and uninfected plants were also observed. A protein of approximately 90 kDa was more abundant in the healthy AWF extract than in the infected one (Fig. 5A, lines 1 and 2). Two proteins of approximately 35 and 15 kDa were more abundant in healthy TE than infected one (Fig. 5A, lines 3 and 4). On the other hand, a 20.1 kDa protein was more abundant in infected samples (AWF and TE) than in healthy ones (Fig. 5A, lines 2 and 4). This SDS-PAGE gel was transferred on an NC membrane and blotted with an anti-AWF antibody (Fig. 5B). As expected, the anti-AWF antibody recognized the pure AWF extracts much better (Fig. 5B, lines 1 and 2) than the TEs (containing both cytosolic and apoplasmic proteins).

The AWF and TE proteins from uninfected cacao plants were analyzed by 2-DE. All polypeptides maps showed a broad distribution of spots in pI range from 4.0 to 7.0 and a mass range from 220 to 14.3 kDa (Fig. 6). The results displayed satisfied 2-DE pattern with the AWF leaf extract (Fig. 6A) as well as the TE of the meristem (Fig. 6B). These results were in accordance with the results obtained on SDS-PAGE (Fig. 5A): more proteins are observed on TE than on AWF and the AWF proteins have a molecular mass higher than the TE proteins (Fig. 6). These results showed that our extraction protocols of AWF and TE proteins allowed further 2-DE analysis of high resolution quality.

3.3 Native protein extraction and protein activity analysis

Since studies regarding enzymatic analysis detection require nondenaturing extraction and electrophoresis conditions, we developed a new protocol of cacao protein extraction without use of denaturing compounds such as SDS, β-mercaptoethanol, *etc*. This protocol was based on sonication and use of *tert*-butanol which allowed the breaking of polysaccharides and their precipitation, respectively (Fig. 7). As examples, we tested different protocols of enzymatic activity detection (Fig. 8). Figure 8A shows chitinase activity on leaves and brooms of cacao infected or not by *M. perniciosa*. It was

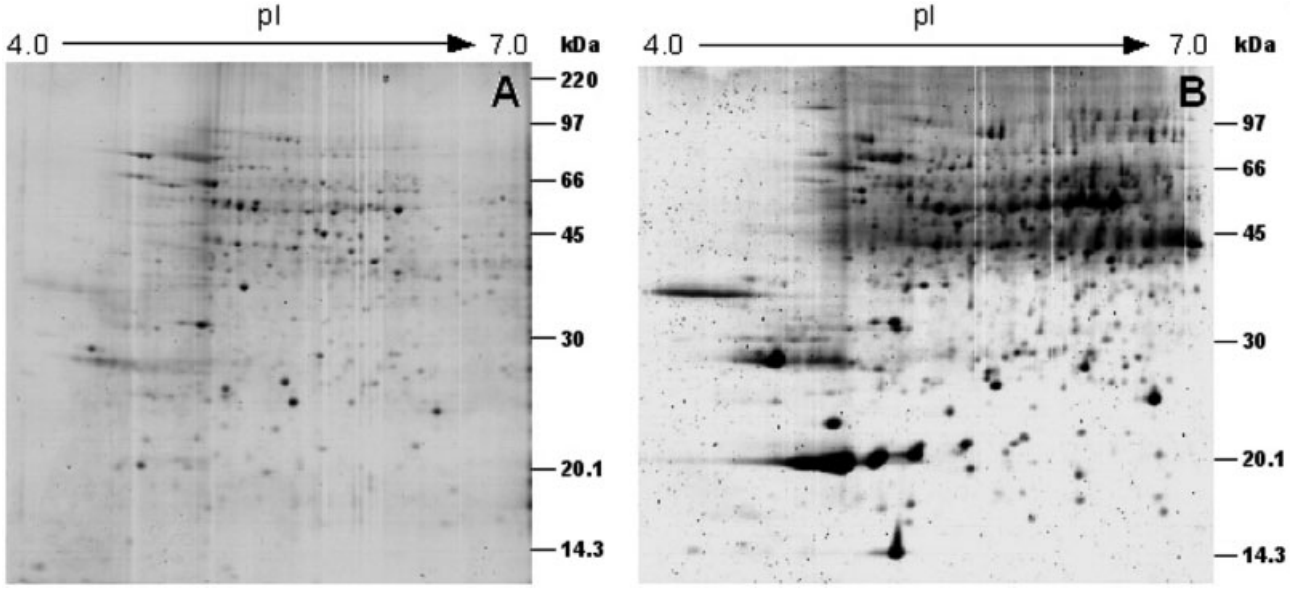

Figure 6. 2-DE proteins from cacao. (A) AWF from uninfected leaves. (B) Total proteins from noninfected meristems. AWF and TE proteins were obtained as described in Fig. 1.

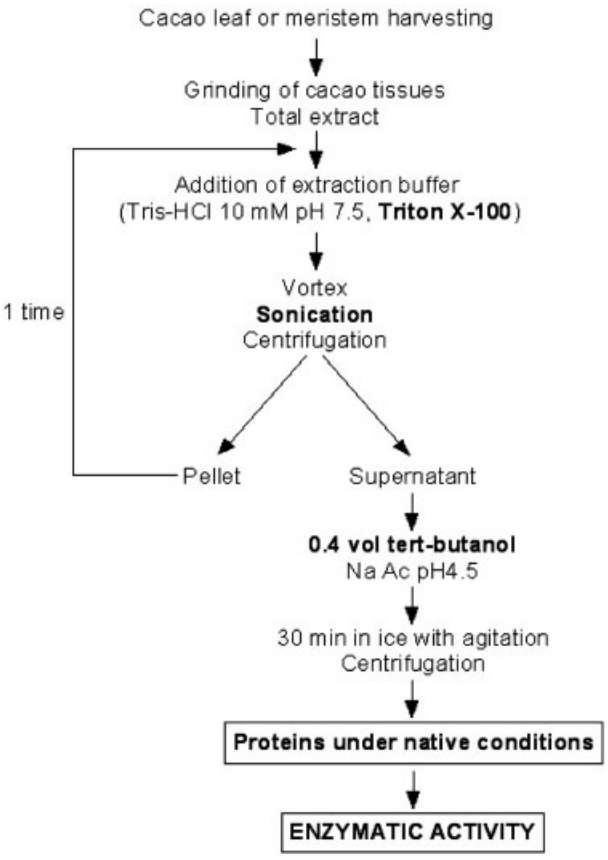

Figure 7. Scheme of extraction of cacao proteins under native conditions.

observed a higher chitinase activity in infected leaves than in uninfected ones. However, no chitinase activity difference was observed between infected and uninfected meristems. As observed in Fig. 8B, infected organs (leaves as well as meristems) present a higher POD activity than uninfected ones. The developed protocol also allowed the detection of enzymatic activity on gel as observed for the detection of proteases in infected and uninfected meristems (Fig. 8C). Four isoforms of proteases were observed (Fig. 8C, a–d), three of them were encountered in all the studied samples (a–c) whereas the last one (d) was found only in dry broom.

4 Discussion

4.1 New protocol of extraction of AWF from cacao leaves

One of the aims of our work was to collect AWF representative of the intact composition of the organ, and in sufficient amounts in order to develop subsequent proteomic experiments. Several methods of isolation of apoplastic fluid have been developed such as application of pressure using the Scholander bomb [35], the vacuum perfusion method [36], the elution method [37] and the infiltration–centrifugation method. The latter has been used in different variations: centrifugation technique without infiltration process [25], infiltration technique without a centrifugation process [38] or a combination of both, the infiltration–centrifugation technique [39–41]. The infiltration–centrifugation technique was evaluated for different plant species and it was shown that this method allowed quick and easy collection of apoplastic fluid which is representative of its composition in intact leaves [42]. For cacao, we developed a protocol based on leaf infiltration–centrifugation using 1 bar infiltration pressure and testing different centrifugation forces (Fig. 1). The infiltration pressure used for cacao leaves was closer to the one used on tobacco leaves (0.8 bar; [17]) and higher than the one used for other plant species [18, 42]. In our conditions, the higher infiltration pressure and the application time used were necessary because of the structure of the cacao leaf, its low potential of transpiration, its high cuticular diffusive resistance [43] and because of the high amount of polysaccharides present in the cacao tissues [12, 13]. Lower infiltration pressures were tested but because of the low amount of AWF collected in these conditions (data not shown) we evaluated that the 1 bar pressure applied until the end of bubble emission (applying approximately nine times 3 min of pressure) allowed the extraction of a sufficient amount of AWF. To avoid alterations of the AWF composition by contamination by intracellular solutes, several increasing centrifugal forces were tested. We observed that increasing centrifugal forces increased the volume of AWF extracted as well as the corresponding amount of proteins. Moreover, we assessed cellular damages by the use of HPI as marker enzyme characteristic of the symplasm [25, 40]. This marker

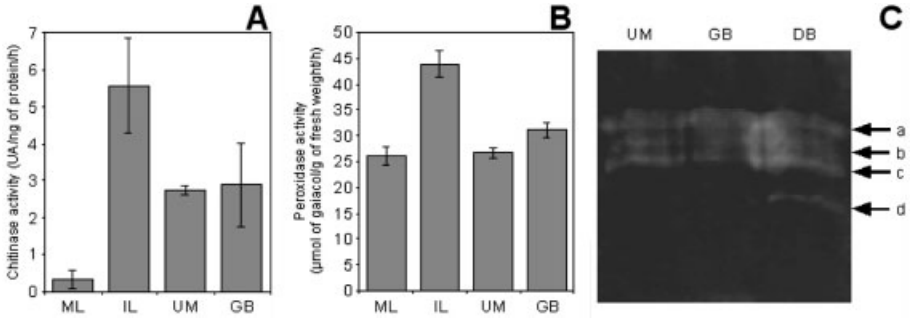

Figure 8. Detection and analysis of enzymatic activity from proteins extracted under native conditions. (A) Chitinase activity assay. (B) POD activity assay. (C) Protease activity detected in polyacrylamide-casein gel. ML, mature leaf; IL, infected mature leaf; UM, uninfected meristem; GB, green broom; DB, dry broom; a–d, protease isoforms.

was preferred to the malate dehydrogenase (MDH) also used in other similar investigations [39, 44]. Li *et al.* [45] demonstrated that MDH was reported to be present in soluble apoplasm in leaves of barley and oats, and, under these conditions, the use of MDH as marker contamination might lead to an overestimation of cytosolic contamination in AWF. In our experiments with the HPI marker, we observed only little contamination of the AWF with cytosolic compounds. No contamination was observed at $1000 \times g$; the lowest centrifugal force tested. At 2000, 3000 and $4000 \times g$, the contamination is quite similar and comprised between 0.4 and 1 mmol of $NADP^+$/min/g of protein. At $4500 \times g$, the highest centrifugal force tested, the contamination increased and reached 3.1 mmol of $NADP^+$/min/g of protein. The HPI activity at 2000, 3000, 4000 and $4500 \times g$ corresponded to 1.2, 2, 0.8 and 6.2% of the total HPI activity detected in the total protein extract. As reported by [46], symplastic contamination lower than to 2% could be considered as negligible, and, consequently, cacao leaf AWF extracted with a centrifugation force lower than $4000 \times g$ is representative of the intact leaf apoplasm. Not only for this reason but also because of the sufficient amount of proteins obtained, $3000 \times g$ as centrifugation force for the following experiments using AWF was chosen. The AWF obtained could be directly used for detection of some enzymatic activities such as POD in gel (Fig. 3). In conclusion, the described method allows a quick and easy isolation of apoplastic fluid in sufficient amount in order to develop subsequent analyses. It therefore provides a suitable tool for studying the apoplasmic compartment of cacao leaves.

4.2 Optimized protein extraction protocols for cacao vegetative organs

Cacao tissues, in particular leaves and meristems, have a very high level of compounds such as polysaccharides and phenolic compounds which greatly interfere with protein stability and separation in 2-DE [12, 13]. Mostly because of these disadvantages, to date no successful 2-DE proteome studies on cacao vegetative tissues have been reported. Of the published protocols for analysing recalcitrant plant material containing high levels of interfering compounds tested in our laboratory [22, 23], none was highly effective on cacao extracts (data not shown). For this reason, a new protocol for protein extraction from cacao vegetative tissues based on TCA-ADP preparation, phenol extraction combined with the use of dense SDS and sonication steps (Fig. 1) was developed. In the entire protocol – from ADP preparation to TE or AWF protein extraction – the sonication steps were very important for breaking down polysaccharides, and more generally macromolecules, through the effects of the shock waves generated from ultrasound [47]. Without the sonication steps the extraction of cacao proteins from vegetative organs was not successful and a lot of polysaccharides remained in the extract avoiding correct subsequent manipulation in proteomic experiments (data not shown). In the ADP preparation, the last washing of the powder using TCA 10% in water appeared also to be a crucial step for diluting the polysaccharides (Figs. 4B and C) and facilitating the subsequent protein extraction. The combination of sonication and washing with TCA 10% in water was a key process in order to obtain an ADP of high quality.

The proteins were extracted from ADP and from AWF with a mixture of phenol and dense SDS buffer. SDS is an excellent solubilizing agent, which allows the recovery of membrane-bound proteins. With 30% of sucrose, the SDS extraction buffer was heavier than the Tris-buffered phenol, and a phase inversion was created. The buffer forms the aqueous lower phase containing carbohydrates, nucleic acids and insoluble cell debris; the upper phenol phase, easily collectable, contains cytosolic and membrane proteins, lipids and pigments. The proteins in the phenol phase were purified and concentrated together with the subsequent AcNH4/methanol precipitation. Another advantage of phenol extraction is that it minimizes the protein degradation often encountered during sample preparation due to endogenous proteolytic activity [48]. As shown in Figs. 5A and 6, under these protein extraction conditions, nondegraded products were detected, indicating that our protocol is efficient to obtain proteins of high quality and in high quantity. Because 2-DE is quantitatively a strong technique, cost-effective and the most suitable approach for species of which the genome is not (fully) sequenced, the sample preparation is a critical step essential for obtaining good results. The fact that the proteins extracted could be used for immunoblotting (Fig. 5B), is also shown.

However, enzymatic activities detection such as protease activities in gel, demands a previous nondenaturing protein extraction. For this reason, we also developed a protocol of protein extraction under native conditions based on sonication and use of tertiary-butanol (Fig. 7). The *tert*-butanol was successfully used for cacao RNA extraction in previous molecular biology experiments [12] and allowed the precipitation of polysaccharides. The sonication step first allowed the breaking down of the polysaccharides which were in a second time easily precipitated in presence of the *tert*-butanol and the ammonium acetate pH 4.5. The proteins extracted under native conditions using this protocol were subsequently used in the detection of chitinase (Fig. 8A), POD (Fig. 8B) and protease activities (Fig. 8C). It was observed that chitinase and POD activities was higher in infected organs than in healthy ones (Figs. 8A and B excluding chitinase activity in meristem), and because chitinases and PODs are members of the pathogenesis-related protein family (PR-proteins), the detected activity could be related to their role in plant defence. It has been shown that plant chitinases are involved in defence by degrading the chitin of fungal cell walls [49] and that several physiological functions for PODs in plants have been reported, including defence against pathogen or insect attack [50]. PODs are heme-containing

monomeric glycoproteins that utilize either H_2O_2 or O_2 to oxidize a wide variety of molecules and are involved in the oxidative burst processes during plant resistance (hypersensitivity) or susceptible plant–pathogen interactions (programmed cell death, PCD). Recently, it was observed in cacao that PCD is triggered by *M. perniciosa* infection, likely involving calcium oxalate crystal accumulation and subsequent degradation through activation of an oxalate oxidase gene expression and H_2O_2 production [7]. Here, the increase of the POD activity in infected organs may be related to the use of the oxidative burst occurring during PCD in susceptible plants. Moreover, differential pattern of protease isoforms was observed between healthy or non-necrotic organs and necrotic ones (Fig. 8C). Three protease isoforms were observed in noninfected meristems and green broom, whereas a fourth band was observed in dry necrotic broom. This supplementary band was specific of plant organ as shown by comparing it to *M. perniciosa* protease isoform pattern under same experimental conditions (data not shown). Because proteases are well known to be involved in PCD, in particular cysteine proteases or caspases-like proteases [51], it could be hypothesized that some specific protease isoforms may be involved in the PCD process, helping the degradation of the cell content occurring in cacao organs as described by [7]. Moreover, according to the acid buffer pH (4.0) used in our experiments, the detected proteases isoforms may be aspartic or cystein proteases, but not serine protease which have a higher enzymatic activity at alkaline pH [52]. More experiments, using protease inhibitors [52], may be carried out in order to confirm these results and refine the identification of the protease group involved in the necrotic process of the susceptible interaction between cacao and *M. perniciosa*.

In conclusion, an important area of proteomics is to study global changes in protein expression in tissues or organs using 2-DE gel and image analysis. To establish a routine procedure for the application of proteomic analysis to cacao vegetative organs, an efficient protocol of protein extraction was developed using TCA-ADP preparation, phenol extraction combined with the use of dense SDS and crucial steps of sonication. With this protocol, the isolation of high-quality proteins from cacao leaves and meristems was achieved; the resultant 1-DE and 2-DE gels and immunoblots are high in quality and are free of smearing and streaking. In further experiments, this extraction method and 2-DE analysis will be coupled with protein identification by MS in order to establish a proteome map for infected and noninfected cacao plants. The development of protocols for native protein extraction, using *tert*-butanol and sonication steps, allowing biochemical and enzymatic analysis of infected and noninfected cacao organs, was also successful. Identification of markers for cacao resistance or susceptibility remains an important goal, and proteomic and biochemical approaches remain a promising way to obtain answers to these questions.

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