Cacao Phylloplane: The First Battlefield against *Moniliophthora perniciosa*, Which Causes Witches' Broom Disease

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Accepted for publication 13 April 2017.

ABSTRACT

The phylloplane is the first contact surface between *Theobroma cacao* and the fungus *Moniliophthora perniciosa*, which causes witches' broom disease (WBD). We evaluated the index of short glandular trichomes (SGT) in the cacao phylloplane and the effect of irrigation on the disease index of cacao genotypes with or without resistance to WBD, and identified proteins present in the phylloplane. The resistant genotype CCN51 and susceptible Catongo presented a mean index of 1,600 and 700 SGT cm⁻², respectively. The disease index in plants under drip irrigation was reduced by approximately 30% compared with plants under sprinkler irrigation prior to inoculation. Leaf water wash (LWW) of the cacao inhibited the germination of spores by up to 98%. Proteins from the LWW

of CCN51 were analyzed by two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by tandem mass spectrometry. The gel showed 71 spots and identified a total of 42 proteins (28 from the plant and 14 from bacteria). Proteins related to defense and synthesis of defense metabolites and involved in nucleic acid metabolism were identified. The results support the hypothesis that the proteins and water-soluble compounds secreted to the cacao phylloplane participate in the defense against pathogens. They also suggest that SGT can contribute to the resistance of cacao.

Additional keywords: innate defense, plant–pathogen interaction.

The cacao tree (*Theobroma cacao* L.), which originated in the Amazon basin, belongs to the Malvaceae family, and is one of the world's most important tropical crops. Nibs are the raw material for the chocolate, beverage, and butter industries. In addition to their organoleptic properties, cacao products are beneficial to health (Kris-Etherton and Keen 2002). However, the production of cacao is threatened by pathogens such as *Phytophthora* spp., *Ceratocystis cacaofunesta*, *Moniliophthora roreri*, and *M. perniciosa*. The fungal pathogen *M. perniciosa* is the causal agent of witches' broom disease. This fungus has two phases of development. The first phase is a biotrophic one that begins with the germination of basidiospores on the surface of young tissues and the penetration of hyphae into the intercellular space (Sena et al. 2014). In this phase, which lasts from 6 to 15 weeks, hypertrophy and hyperplasia of infected tissues occur, apical dominance is lost, and axillary buds proliferate, resulting in abnormal branches called “green brooms” (Griffith et al. 2003). The second phase is a necrotrophic one in which the fungus develops intracellularly, leading the tissues to necrosis and subsequently their death, causing “dry broom” (Calle et al. 1982; Evans 1980). After this phase and a further dry period, basidiocarps start to form in the necrotic tissues, producing spores that are eventually released to infect other plants, thus restarting the cycle (Evans 1981; Lawrence et al. 1991). This destructive disease can reduce cacao production by 50 to 90% and has been one of the factors responsible for the industrial collapse of this product in countries such as Suriname, Trinidad, Ecuador, and Brazil. Therefore, witches' broom is among the most important diseases

that affect the culture of cacao in the Western Hemisphere (Evans 1980; Purdy and Schmidt 1996).

Similar to most fungi, *M. perniciosa* uses the leaf surface as the first point of contact with its host for germination of basidiospores, which are spread mainly by air (Aylor 1990). Plants use numerous mechanisms to try to prevent the growth of pathogens, including the induction (Chisholm et al. 2006) or production (Kliebenstein et al. 2005) of secondary metabolites and proteins or antimicrobial peptides (De Lucca et al. 2005). Some of these biochemical compounds are probably filtered within the leaf (e.g., sugars). Others, such as the secondary metabolites, are selectively produced by trichomes, which are specialized epidermal cells distributed throughout the phylloplane (Wang et al. 2008). Trichomes may be simple or glandular, and are found in many plants of different families (Wagner et al. 2004). The trichome density (i.e., trichome number per unit leaf area) may imply the susceptibility or resistance of a plant to diseases, and may represent a natural resistance (Shepherd and Wagner 2007). Secondary metabolites related to plant resistance to herbivores, pathogens, and insects are already well documented (Shepherd et al. 2005; Tian et al. 2012). However, the occurrence and role of proteins and peptides in the innate defense of plants are little known. The first-known plant defense protein secreted to the phylloplane was named phylloplanin (Shepherd et al. 2005). High levels of this protein are present in leaf water wash (LWW) of *Nicotiana tabacum*, a species that presents a large number of trichomes. This protein showed inhibitory activity against the fungus *Peronospora tabacina*, the causal agent of blue mold disease in tobacco (Kroumova et al. 2013; Shepherd et al. 2005).

Thus, the function of phylloplane proteins must be investigated in other plants in order to seek defense mechanisms in the phylloplane to prevent the germination of spores before they adhere to the host. This study was based on the hypothesis that plants of *T. cacao* can secrete antipathogenic proteins to the phylloplane, where they may have an important role in the initial defense against plant pathogens, including *M. perniciosa*. Thus, we evaluated the index of short glandular secreting trichomes (SGT) in the meristem and on the surface of young leaves of cacao genotypes with or without resistance

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and two supplementary tables are published online.

to *M. pernicioso*, to assess whether a relationship between the number of trichomes and the susceptibility or resistance of the plant exists. We analyzed the effect of leaf washing (by irrigation) on the infection rate of the two cacao genotypes. We tested the inhibitory activity of the LWW on spores of the fungus and identified proteins present in the leaf surface of *T. cacao* through two-dimensional electrophoresis (2DE) and tandem mass spectrometry (MS/MS) of the LWW.

MATERIALS AND METHODS

Macroscopic analysis of cacao phylloplane. The density of SGT (number per square centimeter) was determined in young leaves and meristems of the Catongo and CCN51 genotypes from germplasm collection of the Cocoa Research Center (CEPEC/CEPLAC) (Supplementary Fig. S1). The young leaves were collected within 5 to 10 days after leaf primordium formation, measuring from 5 to 15 cm. The experimental design was completely randomized with six replicates (three plants $\times$ two leaves). On each leaf, a 1-cm² area in the midrib region was analyzed under a Leica EZ4D magnifier, with magnification of $\times 8$ to $\times 38$, and photographed using the program LAS EZ (Leica Application Suite, version 1.4.0). The trichome index was determined by direct counting and is expressed as the mean of the values obtained from the leaves analyzed. For the meristems, the trichomes were quantified in the four-leaf primordial with approximately 2 cm, where three young plants (10 to 20 days old) of each genotype were used for the analysis.

Inoculation of plants with *M. pernicioso*. In total, 300 plants were inoculated with the fungus *M. pernicioso*: 150 plants of the resistant genotype CCN51 and 150 of the susceptible genotype Catongo. Before inoculation, half of the plants of each cacao genotype was irrigated by sprinkling and the other half of each genotype was irrigated by dripping (no sprinkling on leaves and meristems) 30 days after germination. The plants were placed in a humid incubation chamber and their meristems were inoculated with a 20- μ l drop of 2×10^5 basidiospores ml⁻¹, diluted in 3% glycerol (Frias et al. 1991). After the inoculation, the plants were kept for 48 h in the incubation chamber at a temperature of 25°C and relative humidity of 100%. After this period, all plants were transferred to the greenhouse, where they remained under natural light and 30 $\pm$ 2°C conditions for 60 days.

Plants were evaluated 15, 20, 25, 30, and 45 days after inoculation (DAI) for presence or absence of witches' broom symptoms. Symptoms were recorded 60 DAI, and each plant was evaluated for broom type, amount of axillary brooms, broom diameter, and terminal broom length; in addition, the number of axillary brooms >1 cm was analyzed. In the analysis of data, we considered the percentage of plants showing any symptom of the disease, whether terminal or axillary brooms or any other disease symptom (disease incidence [DI]), and total number of brooms, whether terminal, axillary, or cotyledonary (disease severity [DS]), with variables according to Jucá et al. (2014). The experiments were arranged in randomized design and each plant was considered an experimental unit.

Test of *M. pernicioso* germination in the presence of LWW. The LWW was collected from 60 young leaves of each cacao genotype (5 to 10 days old, kept under soil irrigation and not fungus inoculated) in 40 ml of distilled water. The LWW was freeze dried and resuspended in 2 ml of autoclaved distilled water. From this crude LWW, a 1,200- μ l aliquot was fractionated in a Centricon Amicon Ultra-4 centrifugal filter unit with a membrane nominal molecular weight limit of 10 kDa (Ultracel YM regenerated cellulose membranes; Millipore). The flowthrough was collected as the fraction containing compounds of <10 kDa. The fraction retained in the membrane was collected in 1,200 μ l of distilled water as the fraction containing >10 kDa compounds. All fractions of LWW were used for the in vitro test of *M. pernicioso* germination. An inoculum of fungal spores was diluted to a concentration of 1×10^6 basidiospores ml⁻¹ and added to the different compound

fractions at an LWW/spores ratio of 4:1. Tests were carried out with the LWW in dilutions of 1:1, 1:10, 1:50, and 1:100 in 3% glycerol. Control germination was conducted in 3% glycerol and the spore viability was evaluated. The solutions with the spores were incubated at 24 $\pm$ 2°C for 30 min, placed in four microscope slides containing 0.3% agar for each treatment, and incubated for 4 h at the same temperature in Petri dishes. Then, the germinated and nongerminated spores were counted at 32 random points of the dishes under a microscope and the germination rate was determined (Supplementary Table S2). All experiments were performed at least two times, using four replicate per experiment, in a completely randomized design.

Statistical analysis. For all statistical analyses, including analysis of variance (ANOVA) ($P < 0.05$), SAS software (version 8.02; SAS Institute Inc.) was used. Statistical differences among treatment means of categorical variables were detected by Tukey's test at 5% significance level.

Collection of LWW for proteomic analysis. For the protein analysis by one-dimensional (1D) electrophoresis of the CCN51 LWW, 600 young leaves were collected from the germplasm collection of the Cocoa Research Center (CEPEC/CEPLAC) and grown under field conditions.

For 2DE, 300 seeds of CCN51 were germinated and grown in pots with 20 kg of composted soil. The pots were placed in a greenhouse under natural light at a temperature of 30 $\pm$ 2°C and were watered by drip irrigation onto the soil for 30 days, to avoid leaf washing.

The LWW was collected according to the method of Shepherd et al. (2005). In total, 1,250 young leaves were collected and each leaf was washed by immersion for 30 s in a beaker containing 1,000 ml of distilled water. The LWW was filter sterilized, first through a 0.45- μ m cellulose membrane and then through a 0.22- μ m membrane, to remove fungi and bacteria. Then, the LWW was distributed in 50-ml falcon tubes, frozen at -80°C, and freeze dried until the complete elimination of water.

Extraction of proteins from the phylloplane and electrophoresis. Proteins of the freeze-dried LWW of CCN51 were precipitated with 10% trichloroacetic acid in acetone with 0.07% β -mercaptoethanol, using 1 ml of the precipitating solution for each freeze-dried mass of 300 leaves. The mixture was kept in ice for 30 min, then centrifuged at 12,000 rpm for 10 min in the A-4-44 rotor of the centrifuge (5810 R; Eppendorf), and the supernatant was discarded. The samples were washed with 100% acetone containing 0.07% β -mercaptoethanol and, after removing excess wash solution, they were left to air dry for 20 min. One part of the sample was resuspended in 1 $\times$ sodium dodecyl sulfate (SDS) loading buffer and the other part was resuspended in rehydration buffer (urea at 7 mol liter⁻¹, thiourea at 2 mol liter⁻¹, 1% (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS), and traces of bromophenol blue). The samples were sonicated with three 5-s pulses at 40% amplitude in an ultrasonic processor (PGEX 30) to achieve complete resuspension. The proteins of the samples were determined with the 2D-Quant Kit (GE Healthcare), following the manufacturer's instructions, using bovine serum albumin as the standard.

A sample of proteins (30 μ g) was separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970) using the Mini-Protean II System (Bio-Rad), according to the manufacturer's instructions. The gels were stained with 0.08% colloidal Coomassie Brilliant Blue G-250 (Neuhoff et al. 1988).

A mass of 350 μ g of protein, diluted in 250 μ l of rehydration buffer containing dithiothreitol (DTT) at 50 mmol liter⁻¹ and 0.5% nonlinear (NL) ampholytes (pH 3 to 10) (Amersham Biosciences), was loaded onto 13-cm gel strips which contained NL immobilized pH 3 to 10 gradient (IPG, Immobiline Dry-Strip; Amersham Biosciences). The sample was subjected to rehydration, followed by focusing in the Ettan IPGphor 3 Isoelectric Focusing System (GE Healthcare), according to (Silva et al. 2013). In this step, the proteins were separated on the strip according to their isoelectric points. After focusing, the strips were incubated with 7 ml of equilibrium buffer

containing 1% DTT for 15 min with gentle agitation. Then, they were incubated with equilibrium buffer containing 2.5% iodoacetamide for 15 min with gentle agitation. Finally, the strips were washed with stacking buffer (Tris at 0.025 mol liter⁻¹ [pH 8.3], 0.1% SDS, and glycine at 0.19 mol liter⁻¹) for 15 min and used for separation of the proteins in the second dimension according to their molecular weight.

The 2DE was conducted on a 12.5% gel in a Hoeffer SE 600 Ruby Vertical Electrophoresis System (Amersham Bioscience). The running conditions were similar to those used by (Silva et al. 2013). The gel was stained in 0.08% colloidal Coomassie (Lin et al. 2008; Neuhoff et al. 1988) for 7 days, followed by destaining in distilled water for 5 days, with daily water replacements to remove the excess of colloidal Coomassie.

Protein digestion and MS/MS identification. Of a total of 82 spots detected in the 2DE gel, 71 were excised and treated with Gold trypsin (Promega Corp.) at 25 ng µl⁻¹, following the protocol described by Silva et al. (2013). Subsequently, 25 to 50 µl of 50% acetonitrile containing 5% formic acid was added, and the mixture was shaken gently for 30 min. After transferring again to new tubes, the residual organic solvent was reduced to 10 µl in a Speed Vac

RVC2-25CD apparatus (Martin Christ Gefriertrocknungsanlagen GmbH). Aliquots of the digested samples were applied to the UPLC nanoAcquity Liquid Chromatography unit (Waters), coupled to a Micromass ESI-Q-TOF Spectrometer (MS/MS). The spectra of the trypsinized peptides were detected and analyzed by the ProteinLynx Global Server 4.2 program (Waters). The proteins were identified by comparison with proteins in the Cacao Genome (<http://www.cacaogenomedb.org/>), SWISSPROT, MASCOT, and National Center for Biotechnology Information databases.

RESULTS

Index of trichomes in cacao leaves and meristems. We analyzed the SGT index of cacao genotypes CCN51 and Catongo, which are resistant and susceptible, respectively, to the fungus *M. perniciosa* (Fig. 1). In the meristems and leaf primordium of the two cacao varieties (regions of the plant where disease symptoms were observed), we identified SGT and tall glandular trichomes (Fig. 1A and B). Plants of Catongo presented a mean of 63 SGT per leaf primordia, whereas CCN51 presented a mean of 176 trichomes

Fig. 1. Distribution of short glandular trichomes (SGT) in meristems and young leaves of cacao varieties with or without resistance to witches' broom. **A**, Leaf primordia of susceptible Catongo and of resistant CCN51. **B**, Identification of SGT and tall glandular trichomes (TGT) on the leaf edge. **C**, Incidence of trichomes on the abaxial surface of young leaves. Arrows point to SGT. **D**, Quantity of trichomes present in the leaf primordia and on the surface of young leaves (in square centimeters) of the two genotypes.

per leaf primordia (Fig. 1D; Supplementary Table S1). The surface of young leaves (5 to 10 days old) presented 700 ± 39 SGT cm^{-2} for the susceptible genotype and $1,600 \pm 41$ SGT/ cm^2 for the resistant genotype (Fig. 1C and D). The numbers 39 and 41 refer to the standard deviation.

Disease index in plants under drip irrigation and sprinkler irrigation. In plants inoculated with *M. perniciosa*, we observed a DI of 61.33% for Catongo and 9.33% for CCN51 under soil (drip) irrigation, and 73.91 and 36.48%, respectively, for the same genotypes under sprinkler irrigation (Fig. 2A). The DS showed that the frequency of plants from CCN51 with typical witches' broom disease ranged from 0.16 for plants irrigated only in the soil to 0.58 for plants irrigated by sprinkling (Fig. 2B). For Catongo, frequency ranged from 0.98 for drip irrigation to 1.3 to sprinkler irrigation. ANOVA detected significant *F* values ($P < 0.05$) for the effects of treatment (irrigation system) and genotypes but the interaction between genotype and irrigation was not significant. The effect of the irrigation method on DI and DS was independent of the genotype. At 45 DAI, we observed stability in the rate of infection by the fungus, noting an increase in incidence of the disease of almost two times for Catongo plants and almost four times for CCN51 plants, which had leaves washed by sprinkling (Table 1).

A delay of symptom onset was also observed when both plant varieties were subjected to irrigation into the soil; the first symptoms appeared in only a few CCN51 plants at 25 DAI, whereas the susceptible variety showed symptoms by 20 DAI. For the sprinkler-irrigated plants, the number with symptoms for this same period of time increased to six times more for CCN51 and to more than double for Catongo, presenting symptomatic plants even before this period (Table 1).

Germination of spores in cacao LWW. The LWW of the two varieties under study were tested for their inhibitory effect on the germination of *M. perniciosa* spores (Fig. 3). Concentrated LWW samples of the two varieties analyzed showed high levels of inhibition: 99.7% for CCN51 (resistant) and 99.1% for Catongo (susceptible). The inhibition activity of the total LWW from CCN51 and Catongo was 82 and 86%, respectively, for a 10 $\times$ dilution and 66 and 64%, respectively, for a 50 $\times$ dilution. For the fractions with >10 kDa compounds, the inhibition was 74% for CCN51 and 96% for Catongo. The fractions with <10 kDa compounds had the opposite pattern of behavior, 85% for Catongo and 94% for CCN51. For the dilutions of 1:50 and 1:100 of the aforementioned fractions, the inhibitory activity was 60 to 70% (Fig. 3). The test control, with no presence of LWW, presented a germination rate above 82%, proving the viability of spores germinated in the in vitro test.

Protein profile of the LWW. Using SDS-PAGE, we evaluated the protein profiles of the LWW of young leaves of resistant CCN51 plants grown under rain and wind conditions in the field or under soil drip irrigation in the greenhouse (Fig. 4). A clear band of protein was shown for the LWW of plants from the field, whereas several

bands were shown for the wash of plants kept in the greenhouse (Fig. 4). The band of approximately 35 kDa present in the LWW of field plants showed identity with a new cacao basic protein related to tolerance to desiccation.

Eight bands were excised from the plants kept in the greenhouse, from which 10 proteins were identified. These eight bands showed identity with plant proteins. Of these, seven proteins were related to nucleic acid metabolism and regulation. Two bands represented ribosomal proteins of bacteria (Table 2).

Considering the analyses of data obtained by 1D SDS-PAGE, the LWW from CCN51 was analyzed by 2DE to identify proteins present in the phylloplane (Fig. 5). Most of the spots corresponded to neutral and acidic proteins (Fig. 5A). In total, 71 spots were excised from the gel, processed, and analyzed by MS. Of these, 23 different plant proteins and 11 bacteria proteins were identified (Table 2). Together, the 1D and 2D SDS-PAGE analyses resulted in the identification of 28 plant proteins (42.4%) and 13 bacteria proteins (19.7%), totaling 62% of proteins identified in relation to the total analyzed (Fig. 5B).

DISCUSSION

This study shows that the water-soluble components of the cacao phylloplane correspond to the first line of chemical defense against the fungus *M. perniciosa*, the causal agent of witches' broom disease. It represents pioneering work in the characterization of the phylloplane proteomic profile.

The higher number of trichomes in *M. perniciosa*-resistant cacao genotype CCN51 than in the susceptible genotype Catongo suggests and encourages investigation of whether the defense mechanisms in the phylloplane have an important role in this pathosystem (Fig. 1). The glandular trichomes produce secondary compounds and proteins involved in the plant's defense mechanism in relation to stresses, being a chemical barrier (Aziz et al. 2005; Glas et al. 2012; Shepherd et al. 2005; Tian et al. 2012). This is

TABLE 1. Percentage of cacao plants of the Catongo (susceptible) and CCN51 (resistant) genotypes submitted to sprinkling or drip irrigation that presented symptoms of the disease after inoculation with the fungus *Moniliophthora perniciosa*

Irrigation, genotype	Symptomatic (%) at time of infection				
	15 days	20 days	25 days	30 days	45 days
Aspersión					
Catongo	14.7	34.7	64	66.7	66.7
CCN51	...	12	29.3	30.7	32
Soil					
Catongo	...	12	30.7	33.3	36
CCN51	...	...	5.3	6.7	9.3

Fig. 2. A, Mean percentage for the incidence of witches' broom and B, mean values for disease aggressiveness presented by seedlings of Catongo and CCN51 (susceptible and resistant, respectively) subjected to sprinkler irrigation and soil-only irrigation.

perhaps one of the strategies employed in *T. cacao* for resistance to pathogens, because the sprinkler-irrigated plants have a higher incidence of witches' broom disease. In addition, the trichomes are a physical defense, because they prevent insects from circulating freely, affecting their development and oviposition, which perhaps reduces herbivory and the adhesion of pathogenic microorganisms (often carried on the legs of these insects) to the leaf surface of plants (Tian et al. 2012). However, the same studies showed that nonglandular trichomes are correlated with attacks of certain pests and pathogens, because they favor a leaf surface roughness facilitating spore capture and germination and, consequently, infection (Calo et al. 2006). Considering that CCN51 may have a greater abundance of defense compounds in the phylloplane due to its greater amount of glandular trichomes, the increased amount of SGT may be a physiological advantage for the plant by providing a greater ability to secrete metabolites and proteins to the phylloplane, which may be involved in the plant's defenses.

The incidence and severity of witches' broom disease were lower in cacao plants irrigated only by drip into the soil than in the plants irrigated by sprinkling. There were highly significant differences

($P < 0.001$) in the DI and DS between both treatments and genotypes (Fig. 2; Table 1). These results support the hypothesis that the cacao plants should have compounds and proteins in the phylloplane that participate in the initial combat against the pathogen. Thus, sprinkler irrigation or rain on plants may result in loss of these water-soluble compounds from the surface and, consequently, in reduction of the initial defense against pathogens. The differences found in the levels of incidence and severity of the disease between genotypes were already expected, because CCN51 is known to be resistant to witches' broom disease (Boza et al. 2014). The increase by approximately 1.8× in incidence of the disease in Catongo under sprinkler irrigation compared with soil irrigation contrasts with the increase by approximately 3.5× observed in CCN51. These data suggest that water-soluble compounds or proteins present on the leaf surface—possibly secreted from the interior of the plant by different epidermal openings, glandular trichomes, even endophytic and epiphytic bacteria—may represent an important defense strategy of different cacao genotypes.

Proteins and water-soluble compounds of both the plant and microorganisms present in the LWW (Fig. 5; Table 2) likely

Fig. 3. Inhibition rate of germination of *Moniliophthora perniciosa* basidiospores in the presence of leaf water wash (LWW) at different dilutions. The LWW were fractionated into low molecular weight components (<10 kDa) and high molecular weight components (>10 kDa) and used undiluted and at dilutions of 1:10, 1:50, and 1:100.

Fig. 4. Protein profile of leaf water wash from *Theobroma cacao* CCN51 (resistant variety) after one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. **A**, Molecular weight markers. **B** and **C**, Proteins washed from young leaves of CCN51 grown in the field (**B**) and greenhouse (**C**). Each number indicates the band excised, with its respective protein identified.

TABLE 2. List of tandem mass spectrometry-generated proteins from the cacao leaf water wash from CCN51, the cacao genotype resistant to *Moniliophthora pernicioso*, submitted to drip irrigation method^a

Bands or spots	Protein (organism)	MW (kDa)	pI	Function	Accession number
Bands					
1	Desiccation-related protein (<i>Medicago truncatula</i>)	35	...	Desiccation-related protein	XP_003594696.1
2	Peptide transporter PTR3 (<i>Arabidopsis thaliana</i>)	118	...	Peptide transporter	AED95331.1
3	RNAse_H (<i>Ricinus communis</i>)	116	...	Degrades RNA hybrid RNA:DNA	EEF42034.1
4.1	Protein p14 (<i>R. communis</i>)	67	...	Pre-mRNA splicing	EEF31553.1
4.2	Retrotransposon protein Ty1 (<i>Oryza sativa</i>)	66	...	Binds to DNA; related to disease resistance in plants	AAO66536
4.3	NAC domain-containing protein (<i>R. communis</i>)	64	...	Transcription factor	EEF51544.1
5	CRM family member 2 (<i>A. thaliana</i>)	45	...	RNA splicing and ribosome maturation	AEE73657.1
6	50S Ribosomal protein L4 (<i>Acinetobacter</i> sp.)	35	...	Repressor of transcription or translation	KGT46844.1
7.1	Pentatricopeptide repeat containing protein (<i>A. thaliana</i>)	26	...	Unknown function; may be involved in RNA processing and stabilization	AED91763.1
7.2	DNA-directed RNA polymerase 3 (<i>A. thaliana</i>)	25	...	Synthesizes small rRNA and tRNA complex enzymes that are responsible for transcription of DNA into RNA	AEC07531.1
8	50S ribosomal protein L7/L12 (<i>Acinetobacter</i> sp.)	18.4	...	Ribonucleoprotein involved in protein translation	KGT46581.1
Spots					
2, 7, 15, 34, 41, 42, 45	RNAse_H (ribonuclease) (<i>Populus trichocarpa</i>)	40.56	8.21	Cleaves RNA hybrid RNA:DNA	EEE89879.2
8	β-Caryophyllene synthase (<i>Vitis vinifera</i>)	42.54	6.04	Isoprenoid biosynthesis (plant defense)	NC_012025.3
13	Laccase 11 (<i>A. thaliana</i>)	58.30	9.63	Oxidoreductase, process synthesis of lignin	AED90578.1
27	Transketolase chloroplastic (<i>A. thaliana</i>)	80.60	6.34	Participates in the Calvin cycle in photosynthesis (energy)	NP_567103.1
28	Leucine-rich repeat receptor kinase (<i>A. thaliana</i>)	74.38	5.84	Signal transduction; via signaling (receptor) in plant defense against pathogens	AEE31353.1
29	E3 ubiquitin ligase PUB14 (<i>M. truncatula</i>)	39.70	7.58	Proteins related to plant–pathogen interaction	XP_013469421
30	Retrotransposon/probable polyprotein (<i>O. sativa japonica</i>)	20.76	10.4	Binds to nucleic acids; disease resistance in plants (plant protection)	BAD87808.1
35	Similar to pherophorin-like protein (<i>A. thaliana</i>)	49.22	9.77	Glycoprotein of the extracellular matrix (cell structure)	CAA16736.1
51	α/β-Hydrolase domain-containing protein (<i>A. thaliana</i>)	57.50	6.83	Participates in catalytic functions in lipid metabolism (metabolism)	AEE31698.1
53	Transcription elongation factors IIS (<i>A. thaliana</i>)	50.14	9.21	Efficient formation or stability of the complex with RNA polymerase II (transcription)	AED91455.1
58.1	Serine carboxypeptidase S28 family protein (<i>A. lyrata</i>)	45.6	6.12	Protein degradation—Protease (plant defense)	XM_002869306.1
58.3	Terpene cyclase, C1 domain-containing protein (<i>A. thaliana</i>)	42.54	6.04	Isoprenoid biosynthesis (plant defense)	AAG60132.1
49	Endonuclease/exonuclease/phosphatase (<i>A. thaliana</i>)	12.59	6.06	DNA repair	NP_974691.2
64	RNA methyltransferase (<i>A. thaliana</i>)	63.40	8.76	Processing RNAs (transcription)	AEE85649.1
40	Phosphatidylcholine-sterol O-acyltransferase (<i>A. thaliana</i>)	78.96	6.01	Metabolism of lipids, steroids; important in the process of leaf senescence	EFH68452.1
64.1	B3 domain-containing protein REM16 (<i>A. thaliana</i>)	44.59	8.8	Involved in regulation of transcription (transcription)	Q8RYD1.1
63	Armadillo repeat domain of β-catenin protein (<i>A. thaliana</i>)	72.55	6.76	Signal transduction in hormonal responses	AEE35981.1
37	DNA repair protein Rad4 family (<i>A. thaliana</i>)	78.72	6.35	Participates in repairing damaged DNA nucleotide excision	AED92320.1
58.2	Glicoprotease 1 (<i>A. thaliana</i>)	49.57	9.23	Protease	AEC10532.1
44	Hypothetical protein	12.60	4.62	Unknown	—
55	O-Glycosyl hydrolases (<i>A. thaliana</i>)	46.38	6.45	Carbohydrate metabolism (metabolism)	AEE85258.1
70	Protein 10 type rhomboid (<i>A. thaliana</i>)	38.54	10.47	Serine endoproteinase activity type—Serine protease	AEE30603.1
5	Elongation factor G (<i>Acinetobacter baumannii</i>)	79.13	5.04	Translocation of the ribosome during translation	WP_031974107.1
11	Chaperonin 60 kDa (<i>Acinetobacter</i> sp.)	59.95	4.88	Protein folding (plant defense)	KGT47319
14	ATP synthase subunit α (<i>Acinetobacter baumannii</i>)	55.53	5.29	Regulating the synthesis of ATP (energy)	KIQ72573.1
18, 19, 21, 22, 23	Elongation factor Tu (<i>Acinetobacter</i> sp.)	43.21	5.20	tRNA binds to the ribosome (translation)	ENU30703.1
31, 32	Elongation factor Ts (<i>Acinetobacter</i> sp.)	30.97	5.15	Phosphorylation of GDP to GTP (translation)	KGT48738.1
65	Polyribonucleotide nucleotidyltransferase (<i>Acinetobacter</i> sp.)	75.06	5.07	Processing mRNA	KGT47068.1
38	Outer membrane protein Omp38 (<i>Acinetobacter baumannii</i>)	38.41	5.32	Diffusion of small metabolites (cell structure)	CRX6358.1
61	Phosphate-binding protein PstS (<i>Escherichia coli</i>)	37.02	8.39	Transport of phosphate and response to stress (plant defense)	CAP78186.1
54	ATP-dependent Clp protease proteolytic subunit (<i>Acinetobacter</i> sp.)	22.67	5.29	Degradation of proteins (plant defense)	KGT47985.1
17	ATP synthase subunit β (<i>Pseudomonas putida</i>)	49.38	4.89	Energy	WP_043199911.1

^a Protein listed presented identity with proteins from plants and bacteria. MW = molecular weight.

participated in the high inhibitory activity (up to 98%) of the two varieties against *M. perniciosa* spore germination (Fig. 3). These inhibitors from the LWW were included in the plant's preformed biochemical defense; that is, they are substances and proteins already present in the plant, not induced by attack from pathogens (Garcion et al. 2014). The level of involvement of the structural or biochemical defense mechanisms in the plant's resistance varies according to the pathosystem and even within a pathosystem according to the affected tissue, the age of the plant, environmental conditions, and nutritional status (Johal et al. 1995).

Although Catongo is a susceptible genotype, as shown in the inoculation assay on plants under sprinkler irrigation, its LWW shows inhibition similar to the resistant genotype CCN51. The large amount of glandular trichomes present on the leaf surface of CCN51 suggests that these epidermal appendages actively contribute to the secretion of antimicrobials and their homogeneous distribution throughout the leaf favorable to their initial defense against pathogens. The leaves of susceptible Catongo have a lower number of trichomes and their distribution along the leaf surface is random and spaced. The reduced number of trichomes can result in low distribution of the inhibitory compounds along the phylloplane surface, which may facilitate the deposition of spores in areas that lack inhibitors of germination. We speculate that roughness and composition of the epicuticular wax layer in Catongo is favorable to adhesion and germination of the *M. perniciosa* spores. However, the LWW from Catongo also contains foliar surface defense substances that inhibit the germination of *M. perniciosa* spores as a natural defense mechanism of the plant. Thus, a more detailed study still needs to be performed to identify possible inhibitory substances present in each genotype.

Several proteins that have been identified in the phylloplane of CCN51 are known to perform functions in cytoplasmic metabolic processes (e.g., protein synthesis) or nuclear metabolic processes, such as those involved in nucleic acid metabolism. However, the presence of these proteins in the phylloplane should not be considered a random protein release from within cells; otherwise, proteins commonly more abundant in the cytosol would be identified, such as

those involved in energy metabolism. In addition, some plant and bacterial proteins identified in this study are involved in functions related to the plant's adaptation, growth, and innate immunity, either directly or indirectly, acting as the front line in the fight against microbial invasion. These included the NAC domain-containing protein (Faria et al. 2011), RNase (Luhtala and Parker 2010), O-glycosyl hydrolases (Lammens et al. 2009), serine carboxypeptidase S28 (Shirley et al. 2001), E3 ubiquitin ligase PUB14 (Cho et al. 2008), desiccation-related protein (Wang et al. 1998), leucine-rich repeat receptor kinase (Torii 2004), β -caryophyllene synthase (Köllner et al. 2008), B3 domain-containing protein REM16 (Peng and Weselake 2013), and armadillo repeat domain of β -catenin protein (Funayama et al. 1995).

Some proteins can also be expressed at basal levels in epidermal or peripheral cells, and their secretion to the apoplast can allow for the distribution or leaching of apoplastic fluid to the phylloplane. This deposition of proteins in the phylloplane can also occur through stomata, epidermal pores, hydathodes, trichomes, and other epidermal openings that can release various substances from the tissue interior, whether by secretion or rupture of epidermal cell walls caused by pathogens or wounding (Shepherd and Wagner 2007).

The results presented here provide important data for the understanding of the first defense strategies of the plant against pathogens. The trichomes, their metabolites, and the water-soluble compounds present in the phylloplane represent a preformed biochemical defense of the plant. The compounds and proteins from both the plant and the bacteria present in the leaf are important sources of antimicrobials because, in addition to inhibiting more than 95% of fungal spore germination, they retarded the onset of the disease and reduced its severity when accumulated in the leaf of both genotypes. However, these defense mechanisms were shown to be more important in the resistant genotype, because removal of these compounds from the leaf increased the incidence of the disease by four times, compared with only two times in the susceptible variety. This study encourages further research to understand the plant–pathogen interactions, as well as the symbiotic interactions between a plant and its symbiont hosts.

Fig. 5. Two-dimensional electrophoretic pattern of proteins from leaf water wash of *Theobroma cacao* genotype CCN51, resistant to *Moniliophthora perniciosa*, and their percentage distribution among the organisms of origin. **A**, Proteins extracted were separated by isoelectric focusing using 13-cm strips and pH 3 to 10; then, 350 μ g was applied to a 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and stained with colloidal Coomassie. Numbers correspond to the protein spots identified by electrospray ionization tandem mass spectrometry analysis. **B**, Of the proteins identified, 42.4% were plant, 19.7% were bacterial, and 37.9% were unknown.

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