Physiological and Molecular Plant Pathology 115 (2021) 101656

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

The *Moniliophthora perniciosa* – Cacao pod pathosystem: Structural and activated defense strategies against disease establishment

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ARTICLE INFO

Keywords:
Defense mechanisms
Early response
Infectious process
Resistance
Theobroma cacao
Witches' broom disease

ABSTRACT

The infection process of *Moniliophthora perniciosa* on cacao pods of different resistance sources to witches' broom disease of cacao; i.e., TSH1188 (resistant) and Catongo (susceptible), was evaluated at the histological and enzymatic levels. Cacao pods were inoculated with a basidiospore suspension and were assessed weekly for witches' broom symptoms. Histological evaluations revealed differences between genotypes in the pre and post penetration events of *M. perniciosa*. Compared with catongo, the onset and development of fungal colonization were delayed and less intense in pods of TSH1188. Also, peroxidase activities were higher during the early stages of the infection (0–48 HAI) in the incompatible interaction. These results suggested that TSH1188 cacao pods have structural and biochemical mechanisms which may hinder/delay the infection of *M. perniciosa*.

1. Introduction

Plant infection by pathogenic fungi is a process carried out in several steps. The early stages involve the adhesion of spores to the host cuticle, germination, growth of germ tubes on the plant surface, and penetration [1]. Although similar in most plant-pathogen interactions, these stages can be influenced by environmental conditions, i.e., leaf wetness and the plant phylloplane features, such as stomata and trichrome, that may be a barrier inhibiting the entry and spread of the pathogen [2,3]. Indeed, some pathogens can actively seek out and find stomata to penetrate the host's tissues [4]. Stomatal type, density, size, and opening period can reduce the probability of pathogen colonization, therefore acting as a defense mechanism against plant pathogens [5]. Also, glandular trichomes, their pattern (spatial distribution), and density on the host surface may act as a natural physical or chemical barrier, i.e., secreting defensive proteins that inhibit pathogen germination [6,7].

Additionally, upon pathogen infection, plants attempt to hamper the pathogen invasion by activating various defense mechanisms resulting in an antioxidant system that develops metabolites and enzymes, such as peroxidases (PODs) [8]. These enzymes can be either directly toxic to the pathogen or create conditions that inhibit their development in plants. The PODs family includes ascorbate peroxidase (APX) and guaiacol peroxidase (GPX). They are involved in the detoxification of H_2O_2, leading to lignification, secondary wall formation, and defense against biotic and abiotic stress [9]. The H_2O_2 acts as a signaling molecule and second messenger promoting responses to biotic and abiotic stresses. Its accumulation is directly linked to oxidative burst, which is among the first plant responses to pathogen infection [10].

The chocolate tree, *Theobroma cacao* L. (cacao), is valuable for the food industry for processing and consuming countries. Its seeds, called cocoa beans, are the raw material for chocolate manufacturing and derivatives [11]. The top seven producer countries include Côte d'Ivoire,

Abbreviations: APX, Ascorbate peroxidase; CaOx, Calcium oxalate; GPX, Guaiacol peroxidase; POD, Peroxidase enzyme; H_2O_2, Hydrogen peroxide; WBD, Witches' broom disease.

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https://doi.org/10.1016/j.pmpp.2021.101656
Received 26 March 2020; Received in revised form 25 April 2021; Accepted 26 April 2021
Available online 9 May 2021

Ghana, Ecuador, Cameroon, Nigeria, Indonesia, and Brazil [12]. Pests and diseases are the major constraints of cacao plantations, causing 30–40% losses in global cacao production [12]. Among them, witches' broom disease, caused by the Basidiomycota *Moniliophthora perniciosa*, is the second most important disease of cacao. It can cause losses that can reach up to 100% depending on the environmental conditions and the level of resistance of the host genotype [11]. In Brazil, its introduction in 1989 led to an enormous socioeconomic crisis in the region, causing Brazil to shift from being an exporter to a cacao importer [13,14].

Moniliophthora perniciosa has multiple penetration modes, mainly through stomata and trichrome [15–17]. The fungus can infect the different cacao plant organs, branches, flower cushions, and pods. The typical symptoms, abnormal shoots that display stem swellings and bud proliferation, the vegetative brooms, inspired the name witches' broom disease of cacao (WBD). Diseased pods develop distortion, abnormal ripening, and external and internal necrosis of the tissues and seeds, rendering seed viability. Infection of flower cushion may form hypertrophied flowers, vegetative brooms, and parthenocarpic pods.

The use of plant genetic resistance is the preferred management strategy for WBD control. It provides reasonable disease control, is economically sound, feasible, and ecologically safe. Sources of genetic resistance to WBD have been found in *T. cacao* [11]. Nevertheless, no cacao sources were found completely resistant (immune) to *M. perniciosa*. It appears that the genetic resistance of cacao to WBD is quantitative and polygenic, as different levels of disease severity have been observed among cacao genotypes. Modes of penetration and colonization of *M. perniciosa* on young cacao leaves [15] and apical shoots of resistant and susceptible cacao genotypes have been characterized [16]. Resistant genotypes hindered the growth and/or development of *M. perniciosa* by impeding further colonization, whereas the susceptible genotype showed intense fungal colonization [16,17]. On the other hand, little is known about how the infectious process occurs in cacao pods, the main cacao product. Accordingly, considering the impact of WBD on bean production and the need for a better understanding of how *M. perniciosa* infects cacao pods, the present study examined the infection process of *M. perniciosa*, the antioxidant response, and disease symptom development on cacao pods with known levels of resistance to WBD.

2. Material and methods

2.1. Plant material

Pods of TSH1188 and Catongo were used because of their respective resistance and susceptibility in the field and greenhouse trials for selecting resistant genotypes to WBD [11,18,19] and several molecular and histological *M. perniciosa*/cacao studies [6–8,16,17,20,21]. The cacao trees are maintained at the germplasm of the Centro de Pesquisas de Cacau (CEPEC) at the headquarters of the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC/MAPA), Ilhéus, Bahia. Brazil. The International Cocoa Germplasm Database – ICGD (www.icgd.rdg.ac.uk/) provides further information on those accessions.

To obtain disease-free pods of known age, cacao flowers from each genotype were hand-pollinated. The flowers of the self-compatible Catongo were self-pollinated and used as pollen donors to the self-incompatible TSH1188. During pollination, the flower buttons were protected with a transparent hose tube removed 24 h after pollination and then protected with transparent polyethylene bags to avoid pod infection. Flowers were monitored weekly and, 30 days later, checked for pod set.

2.2. Inoculation procedure

Field inoculations were performed according to Oliveira e Silva [22] with minor modifications; briefly, each cacao pod was inoculated with a 20 μL drop of a basidiospore suspension at a concentration of 2×10^5 basidiospores mL^{-1} in 0.2% water-agar solution, deposited on the center of the pod. After that, cacao pods were exposed to a high-RH treatment (95 ± 5%) in enclosed bags for 48 h. Control pods were mock-inoculated with sterile 0.2% water-agar solution. The experiments were carried out using inoculum from isolate MpCeplac4145 maintained in the *M. perniciosa* collection of CEPEC/CEPLAC/MAPA (CEGEN N° 109/2013/SECEXCGEN). The basidiospores used for inoculation were obtained following Dickstein et al. [23]. Before the inoculation, basidiospores were tested for spore viability, and only those with germination above 80% were used in this study.

First, an experiment was carried out to establish the optimum pod age for artificial inoculation tests (Supplementary Fig. S1). Catongo (susceptibility standard) pods of 45, 115, 128, and 148 days after pollination (DAP) were artificially inoculated; from early stages (~45–60 days) to fully develop, and from unripe (~90–100 days) to ripe (~120–150 days). At least six pods/treatments were used. Sixty-ninety days after inoculation, the pods were assessed individually for WBD symptoms, according to Silva et al. [20]. Briefly: (i) apparent maturation, (ii) the presence of black punctuations, (iii) extension of necrotic lesion and, (iv) and seed hydrolysis.

Infected cacao pods were removed and taken to the Plant Molecular Laboratory (FITOMOL/CEPEC/CEPLAC/MAPA) for *M. perniciosa* isolation. Pods were disinfected, and small segments of the exocarp were transferred to Petri plates containing potato dextrose agar amended with streptomycin (200 μg/mL) and carbendazim (100 μg/mL). The growth of the fungal mycelial mass was accompanied and recorded by photographic captures with the Canon® Power ShotSX530 HS camera. Also, the morphology of all isolates was verified using a microscope (Leica) at 40× to certify the presence of clamp connection and typical hyphae of the species.

2.3. Infection process, sample preparation for scanning electron and light microscopy evaluations

For this study, cacao pods of TSH1188 and Catongo at 45–60 days old (6–8 cm) were inoculated as aforesaid (Supplementary Fig. S1). This pod stage was chosen according to the pod age experiment above. Each treatment consisted of 5 pods (replicates) per collecting time. Cacao pod segments were excised into approximately 1–5 cm segments and immediately fixed in the appropriated buffer. All subsequent microscopic analyses were performed on at least three biological replicates, and the images shown were typical of what was observed.

For observation under scanning electron microscopy (SEM), pod segments of 1 cm^3 from inoculated and control cacao pods were collected at 3, 5–6, 8, and 12 h after inoculation (HAI) and immediately fixed in 2% glutaraldehyde and 1% cacodylate buffer. The post-fixation process was performed using osmium tetroxide vapor impregnation according to Sena et al. [16] and Kitajima and Leite [24]. The material was mounted on aluminum stubs, coated with an approximately 20-nm layer of gold, and analyzed using a Jeol JSM 6390LV scanning electron microscope with a voltage of 200 kV at the Microscopy Platform at the Oswaldo Cruz Foundation, FIOCRUZ, Salvador, Bahia, Brazil.

For examination of the infection under light microscopy, pod segments of susceptible and resistant cacao genotypes at 15, 30, 45, 60, and 120 days after inoculation (DAI) were excised and fixated in a 1:1:18 solution of formaldehyde-propionic acid-ethanol (50%) (FPA). After fixation, the samples were dehydrated in a graded ethanol/*tert* butyl alcohol series (50–100%) for 2 hs each and thermally embedded in paraffin (melting point 56.5 °C Paraplast Plus; Fisher). Embedded tissues were prepared in a longitudinal and transverse direction (15-15 μm thickness) with a rotary Sc 2400 sledge microtome (Leica). Serial sections were thermally mounted on a microscope slide coated with Haupt's gelatin adhesive and 4% formalin solution. Sections were later dewaxed in three changes of 100% xylene and passed through a series of xylene and ethanol (EtOH) 1:1, 100% EtOH, and 70% EtOH. The slides were submitted to a double stain process with naphthol blue-black and

periodic acid Schiff (PAS) and analyzed using a DMRXA microscope (Leica) according to Sena et al. [16]. Three slides with five histological samples were analyzed for each collecting time.

2.4. Data analysis of the histological events

For SEM study, one stub with three independent biological replications was visualized for each genotype, treatment, and collecting time. Five representative sections of 400 μm (250×) were selected randomly and visualized for each biological replication. The frequency mean of each penetration type was estimated on three independent biological replications per genotype in the inoculated samples. Other variables evaluated were: (i) number and type of trichomes, (ii) number and type of stomata, (iii) number of adhered spores; the last considered a qualitative variable (±), and (iv) germination of basidiospores; a spore was considered germinated when the length of the germ tube was equal to or greater than the diameter of the spore.

For light microscopy, at least two paraffin-embedded blocks from independent biological replications were stained, sectioned, and visualized for each genotype, treatment, and collecting time. For each block, 15 representative sections were selected randomly and visualized. The relative frequency of each defense reaction was thus estimated. A reaction detected in less than 10% of samples was considered rare, low in 10–30%, and frequent if present in 30–60% of sections. A reaction detected in more than 60% of the sections was considered highly frequent.

2.5. Antioxidative enzyme assays

The tests were carried out in the Laboratório de Proteômica da Universidade Estadual de Santa Cruz (UESC). The analysis of enzymatic activities was conducted in four replicates, of already-obtained samples of three pods pools, of inoculated and non-inoculated pods, collected at 0 (sample collected right before deposited of inoculum suspension), 3, 6, 12, 24 (HAI), and, 2, 3, 15, 45 and 120 DAI. The zero HAI collecting time acts as a control to identify which subsequent reactions occurred due to fungal infection. The collected samples were immediately submerged in liquid nitrogen and store at −80 °C for 24 h, followed by lyophilization until the extraction was conducted. Then, they were macerated in liquid nitrogen and homogenized in 800 μL of potassium phosphate buffer. K–P buffer was prepared by mixing 50 mmol L^{-1} KH_2PO_4, pH adjusted to 7.0.

Guaiacol Peroxidase (GPX; E.C. 1.11.1.7) activity was investigated according to the methodology described by Rehem et al. [25], with some modifications. The activity buffer used was composed of sodium phosphate at 20 mmol L^{-1} (pH 6.0) and 40 mmol L^{-1} of guaiacol and hydrogen peroxide at 0.06%. A volume of 30 μL of the previously diluted extract was added to that solution. The absorbance readings were performed at a 470 nm wavelength at 25 °C. GPX activity was expressed according to the increased guaiacol consumption in μmol h^{-1} g^{-1} of lyophilized biomass. The conversion of the obtained data was performed according to a standard curve prepared by Rehem et al. [25].

Ascorbate peroxidase (APX; E.C. 1.11.1.11) activity was investigated according to the methodology described by Nakano and Asada [26] with some modifications. The reaction buffer was composed of 50 mmol of potassium phosphate, 0.5 mmol of ascorbate, 0.1 mmol of EDTA, and 0.1 mmol of H_2O_2. From the previously diluted extract, 30 μL were added to that solution. The reaction was started after the addition of the ascorbate. The absorbance readings were performed every 30 s (totalizing 5 min) at 240 nm wavelength at 25 °C in a microplate spectrophotometer (Spectramax Paradigm; Molecular devices, CA USA). APX activity was expressed according to the decreasing absorbance and expressed in nmol g^{-1} h^{-1} of lyophilized mass.

2.6. Statistical analysis

All experiments followed a complete randomized design. The SAS (Statistical Analysis System) software was used to analyze all variables using the ANOVA PROC GLM and Multiple effects analysis (SLICE) to compare genotype x collecting time and determine the interaction between variables. According to Duncan's multiple range test, the differences between means were determined through multiple comparisons ($p < 0.05$).

3. Results

3.1. Influence of pod age on infection of susceptible cacao pods by Moniliophthora perniciosa

Regardless of ages (45, 90–110, and >120 days old), pod infection resulted in disease. However, the intensity of disease severity (≥70%) and fungal colonization were high on younger pods, 45–60 days after pollination (DAP) (Fig. 1A). Therefore, 45 DAP pods were selected for further experiments. It was also observed that the percentage of diseased pods decreased with an increase in pods' age (Fig. 1A–E). Although there was no difference in disease incidence between pods at age 90–110 and > 120 DAP, the severity of the internal symptom and colonization decreased with the pod's age. Thus, the older the infected pod, the fewer the internal symptoms. There were viable seeds observed only in the later stage of pod development (Fig. 1D). Highlighting that, in this study, the disease severity in the interior of the cacao pods reduced with pod age (Fig. 1E).

3.2. Infection process of Moniliophthora perniciosa on cacao pods of contrasting genotypes to witches' broom disease

In general, the infection process of *M. perniciosa* on cacao pods was characterized by (i) the adhesion of the fungal basidiospore to the pod surface and the formation of the germ pore (Fig. 2A), (ii) germ tube emission and elongation (Fig. 2B), and (iii) presence of an amorphous mucilaginous matrix at the site of contact between the fungal structure and the pod surface (Fig. 2C–F). However, the timeline and frequency of these events differed between the cacao genotypes.

Moniliophthora perniciosa showed multiple penetration modes (Fig. 3); through stomata pore (Fig. 3A), directly through the pod cuticle (Fig. 3B), and at the base of glandular trichomes (Fig. 3C). Regardless of the genotype and collecting time, the predominant type of penetration was through stomata, and less expected was the penetration at the base of glandular trichomes (Fig. 3D and E). The differences in the pre-penetration events were significant between genotypes (Table 1). In Catongo, fungal penetration events occurred earlier, starting at 3 HAI and reaching its peak at 8 HAI (Fig. 3D), and regardless of time, spore germination was 50%. On the other hand, the onset of fungal infection on TSH1188 was 6 HAI with a peak at 12 HAI (Fig. 3E), and the events of germ tube adherence, spore differentiation, and germination on the pod surface were significant at 8HAI (Table 1).

3.3. Histological characterization of Moniliophthora perniciosa colonization process in cacao pods of contrasting genotypes to witches' broom disease

The colonization events of *M. perniciosa* in cacao pods tissues were similar between the contrasting genotypes; however, differences in temporal patterns were seen among them (Fig. 4). The kinetics of the histological events showed that colonization of the susceptible cacao pods tissues occurred earlier than in the resistant ones. Catongo tissues were rapidly colonized by *M. perniciosa* hyphae since the first stages of the infection, from 5 to 15 DAI (Fig. 4A–C). At 5 DAI, swollen, flexuous, septated, branched, and unclamped hyphae grew in the apoplast space of the parenchyma cells about 5 μm below the outer layer of epidermal cells of the susceptible genotype (Catongo). From 60 DAI to 150 DAI (Fig. 4E, G), hyphae were already visible in the susceptible genotype. Intensive and intracellular colonization (thin hyphal segments) and lysis

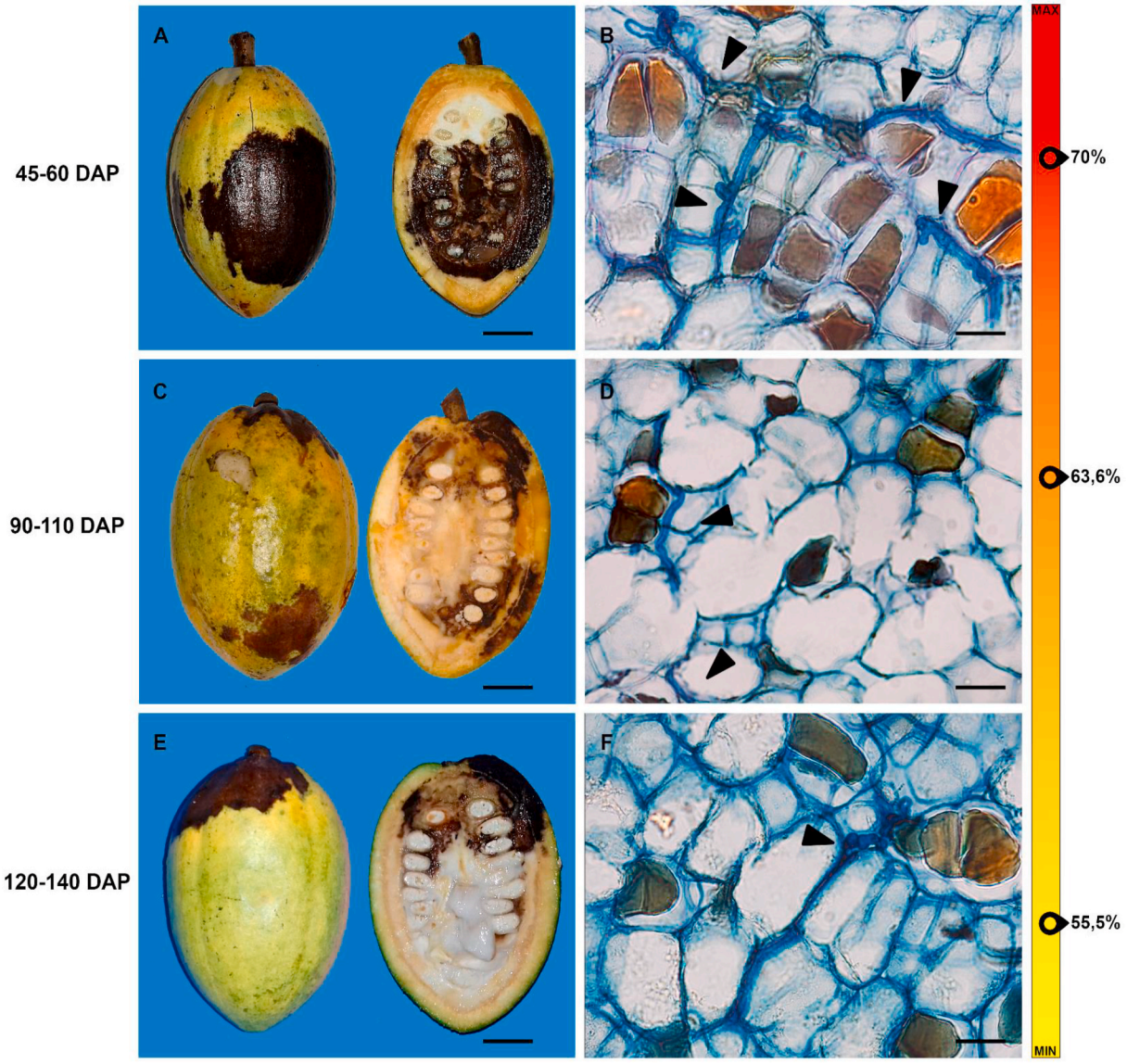

Fig. 1. Influence of pod age on susceptibility of Catongo, a standard susceptible genotype to witches' broom disease of cacao: macroscopic symptoms showing dark brown irregular lesion (A, C, E) and light micrographs of histological characterization of colonization of Catongo pods inoculated with *Moniliophthora perniciosa* (B, D, F):at different age: (AB) 45 days after pollination (DAP); 90–110 DAP (CD), and 120–150 DAP (EF). Black arrowheads: hyphal segments. Scale bars: 1.5 cm (A, C, E) and 20 μm (B, D, F). The color code depicted at the right of the images ranges from red (maximum value) to yellow (minimum value) of disease incidence. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of pod parenchyma cell walls were noted. These characteristics were conspicuous with the necrotrophic phase of *M. perniciosa* (late-stage).

In the resistant genotype, cell colonization was rare to low. Convoluted and small hyphae segments, typical of *M. perniciosa*, were observed at 5 and 15 DAI (Fig. 4B, D) below the epidermal layer. By 60 DAI, the colonization continued until the hyphae segments reached the parenchyma cells (Fig. 4F). At 150 DAI, few short and thin hyphae segments were observed in the pod parenchyma cells (Fig. 4H). Notably, the prevalent hyphal segments of *M. perniciosa* were typical of the biotrophic phase. The defense reactions noted were cell wall strengthening, papilla formation, accumulation of (poly) phenolic, and carbohydrate compounds (Fig. 4B–H). The details of the temporal characterization of *M. perniciosa* colonization in cacao pods of resistant and susceptible genotypes are presented in Supplementary Table 1 and Supplementary Fig. S2. Some of these defense responses were observed in the inoculated susceptible pod tissues, but they were not considered significant (Fig. 4E).

3.4. Characterization of pre-formed defense mechanisms of cacao pods of contrasting genotypes to witches' broom disease

Both cacao genotypes showed glandular and non-glandular trichomes with morphological characteristics consistent with those described in the literature (Fig. 5). The non-glandular trichomes were presented as multicellular, composed of a basal cell and three ramifications (Fig. 5A), and it was sporadic. In contrast, glandular trichomes were composed of a basal cell adhered to the pod epidermis, a pedicle that constitutes the body of the trichomes, and a unicellular secretory head (Fig. 5B). Glandular trichomes were the predominant type in both genotypes (Fig. 5C and D); their density per cm2 was significantly higher ($p < 0.05$) on TSH1188 (569.8 trichomes-cm2) than on pods of (292 trichomes-cm^2) (Fig. 5E).

Regarding the type and density of stomata and cacao genotype, anomocytic stomata were characterized by a pore surrounded by two guard cells with the absence of subsidiary cells characterizing this type of stomata. A variation in the stomata location on the pod surface was

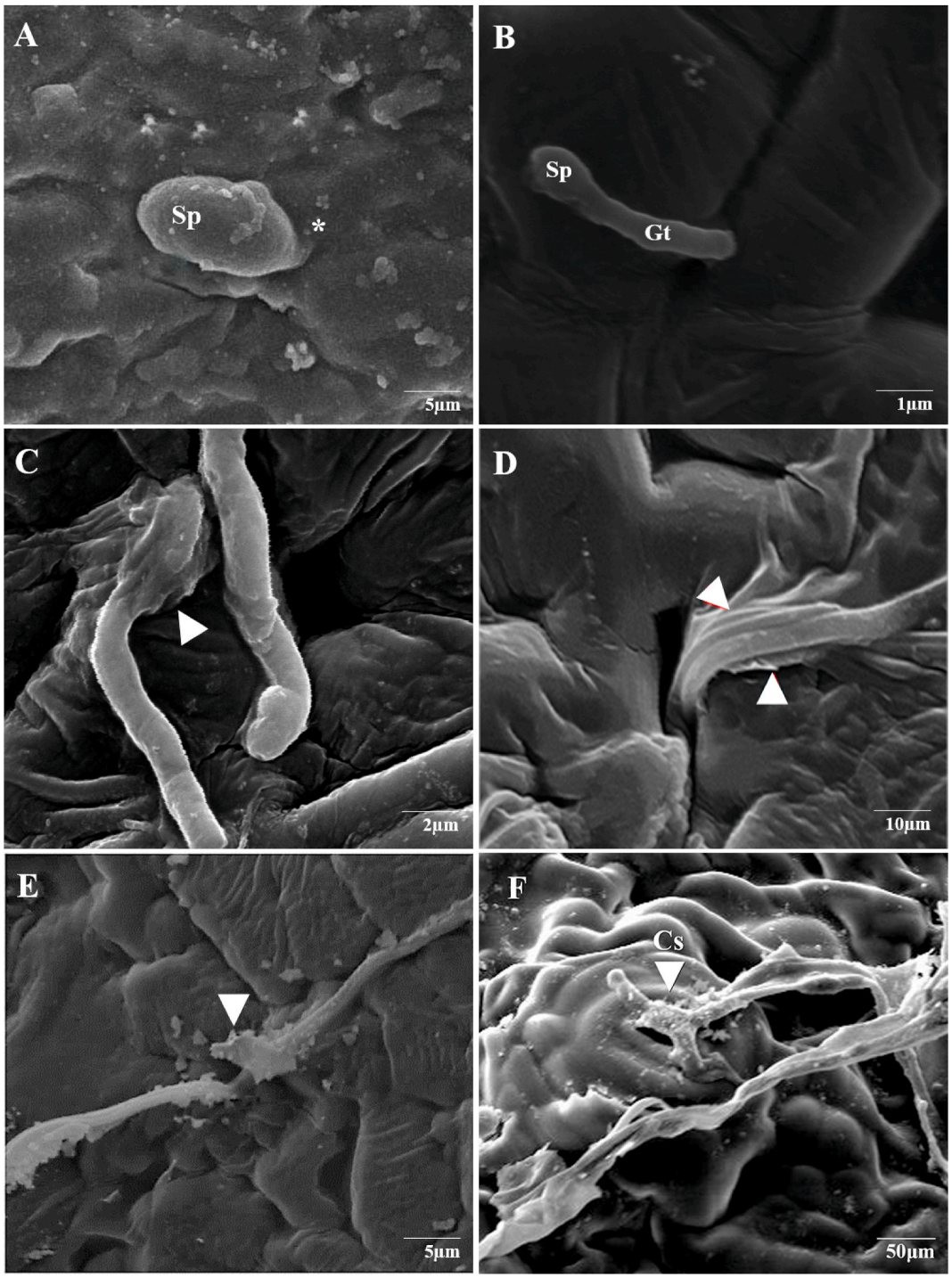

Fig. 2. Scanning electron micrographs showing *Moniliophthora perniciosa* pre-penetration events on cacao pods of contrasting genotypes (resistant, TSH1188 and susceptible, Catongo). A) Basidiospores adhesion and germ pore (*) on TSH1188 at 5 h after inoculation (HAI); B) germ tube development on Catongo pods surface at 3 HAI, basidiospores were considered germinated when the germ tube length exceeded the spore diameter; C) structural modification and apparent dissolution of the TSH1188 pod surface at the site of contact between the pathogen and host (arrowhead) at 12 HAI; D, E) primary hyphae surrounded by mucilage matrix (arrowhead) on Catongo pods at 6 HAI; F) hyphae, with club-shaped tips (Cs), growing around guards cells of TSH88 pods at 12 HAI. Sp: basidiospore; Gt: germ tube.

observed between the genotypes. In Catongo pods, stomata were often observed in large substomatal chambers in the epicarp, sunk within the mesocarp, known as "stomatal crypts" (Fig. 6A and B). In contrast, in the TSH1188 pods, the stomata commonly appeared in the phylloplane of the pods (Fig. 6C and D). Nevertheless, in this work, no significant differences in stomatal density were observed between the studied genotypes (Fig. 6E). However, more detailed studies are needed to investigate stomatal patterning and development on cacao pods.

3.5. Characterization of antioxidative enzymatic activities of cacao pods in response to Moniliophthora perniciosa infection

In general, it was observed that the activity of guaiacol (GPX) was higher than ascorbate (APX) peroxidase in all treatments, collecting time, and genotypes (Fig. 7). For the GPX activity, there were differences between the genotypes (Fig. 7A and B). In the susceptible genotype, Catongo, this activity was the same in both inoculated and non-

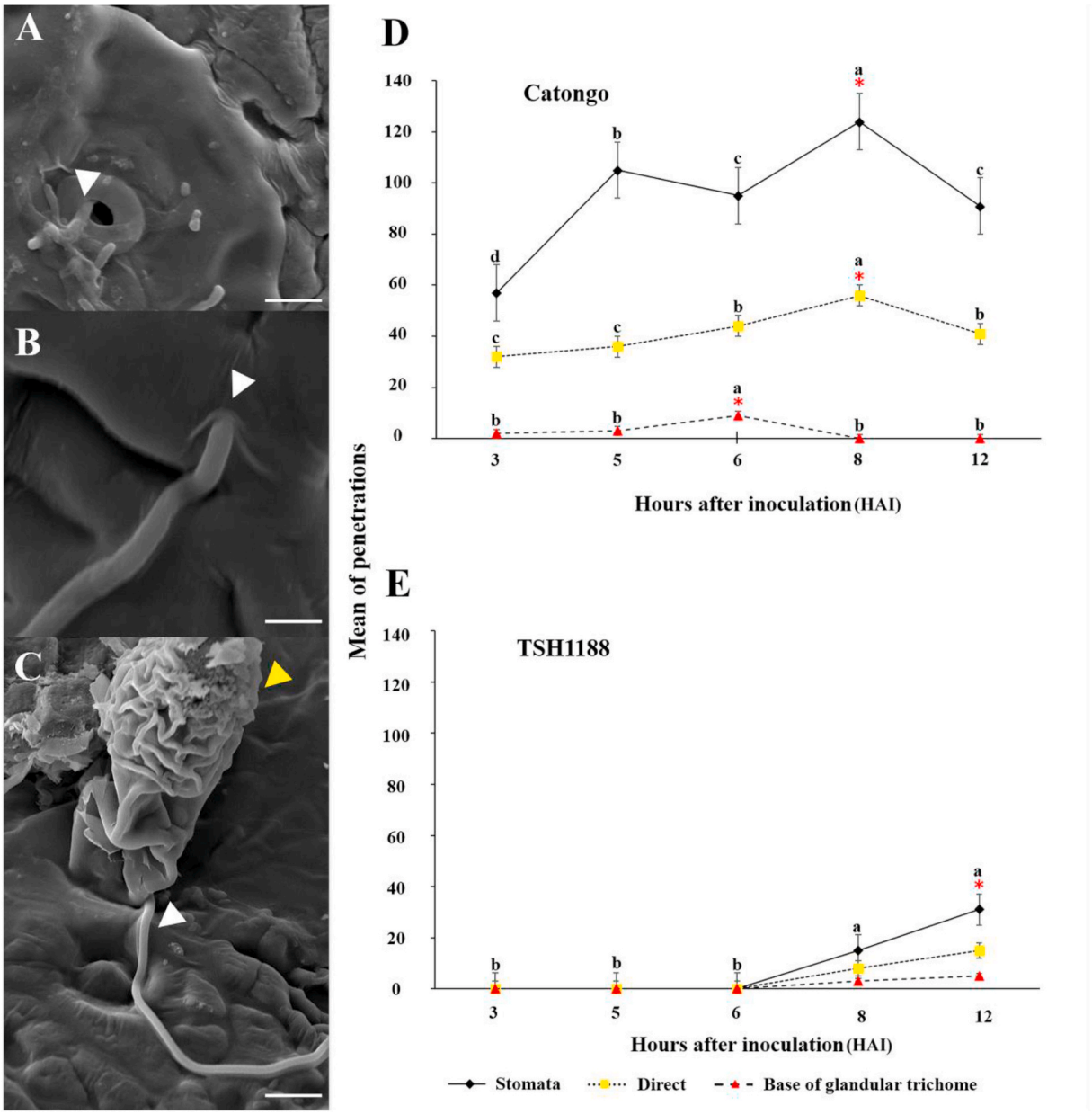

Fig. 3. Scanning electron micrographs showing penetration of cacao pods by *Moniliophthora perniciosa*: A) through the stomatal pore, bar = 5 μm; B) directly by the pod cuticle, bar = 2 μm, both, on Catongo pod at 3 HAI, C) through the base of glandular trichomes on a TSH1188 pod at 12 HAI, white arrowhead points the site of penetration, yellow arrowhead shows the secretory gland of the trichome, bar = 10 μm; D) mean number of *Moniliophthora perniciosa* penetrations by μm2 on a Catongo pod; and E) on a TSH1188 pod. Mean values with different letters show significant differences ($p < 0.05$) by the Duncan test at 5%. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Kinects of pre-penetration events of *Moniliophthora perniciosa* on cacao pods of contrasting clones.

RATED TIME (HAI)	ADHESION INDEX		GERM TUB LENGTH (μm)		GERMINATION (%)	
	CATONGO	TSH1188	CATONGO	TSH1188	CATONGO	TSH1188
3	1.17 Aa	0.00 Bb	6.08 Aa	0.00 Ba	50.00 Ba	0.00 Ba
5	0.90 Ab	0.03 Bb	7.82 Aab	0.00 Ba	50.00 Ba	0.00 Ba
6	0.93 Ab	0.13 Bb	9.55 Abc	0.00 Ba	50.00 Ba	0.00 Ba
8	0.70 Ac	0.23 Ba	11.02 Ac	2.24 Bb	50.00 Aa	50.00 Ab
12	0.83 Ab	0.17 Bb	13.55 Ad	5.02 Bc	50.00 Aa	50.00 Ab
Total Mean	0.91A	0.11B	9.60A	1.45B	50A	20B

According to the Duncan test, averages with the same letter did not differ among them ($p < 0.05$). Mean followed by the same lowercase letter in columns (collecting time) and uppercase in rows (genotype) do not differ by Duncan test (5% probability). HAi: Hours after inoculation.

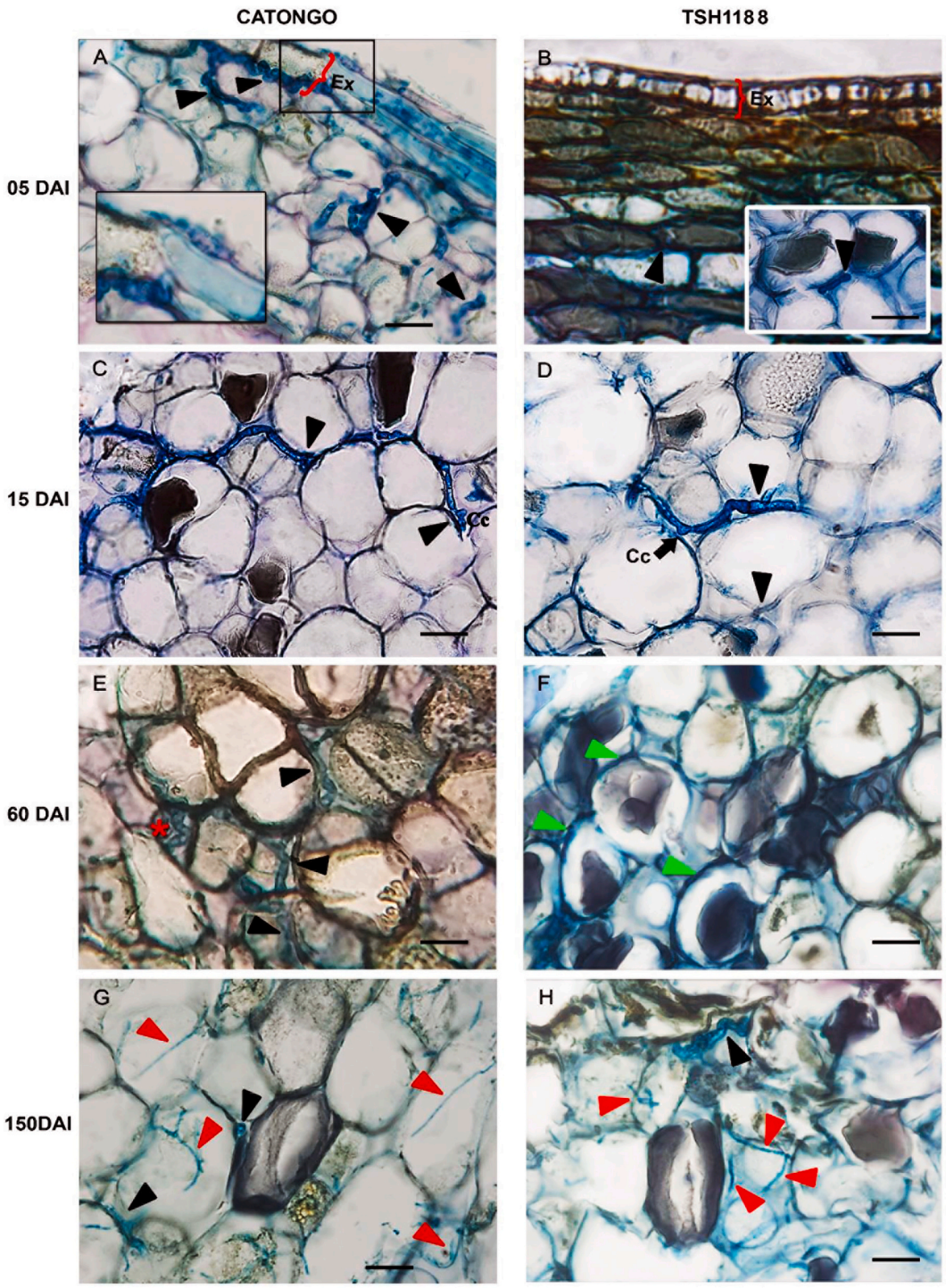

Fig. 4. Light micrographs of cacao pod colonization by *Moniliophthora perniciosa* 5–150 days after inoculation (DAI). A, C, E, G (susceptible genotype, Catongo). B, D, F, H (resistant genotype, TSH1188). Black arrowheads: hyphal segments at biotrophic stage, red arrowheads: hyphal segments at necrotrophic stage, green arrowheads: thickening of the cell wall, Cc = clamp connexion, Ex = exocarp, *intracellular hyphae, bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inoculated pods (Fig. 7A). In TSH 1188 pods, the GPX activity was higher during the first hours in the inoculated when compared to the non-inoculated pods (Fig. 7B). In contrast, in Catongo, this activity peaked only with 15 days of infection (Fig. 7A and B). For APX, compared with Catongo, it was observed an earlier and higher activity in the resistant genotype-TSH1188 (Fig. 7C and D).

3.6. Witches' broom disease evolution in cacao pods of contrasting genotypes

The macroscopic symptoms varied between cacao genotypes. Catongo pods showed a percentage of disease incidence of 70%. The first WBD symptoms were observed at 15 DAI (Fig. 8B). A yellowed zone with tiny, small necrotic spots (Fig. 8B). Internally, the endocarp of the pod and its seeds were mainly asymptomatic, showing just a necrotic spot on

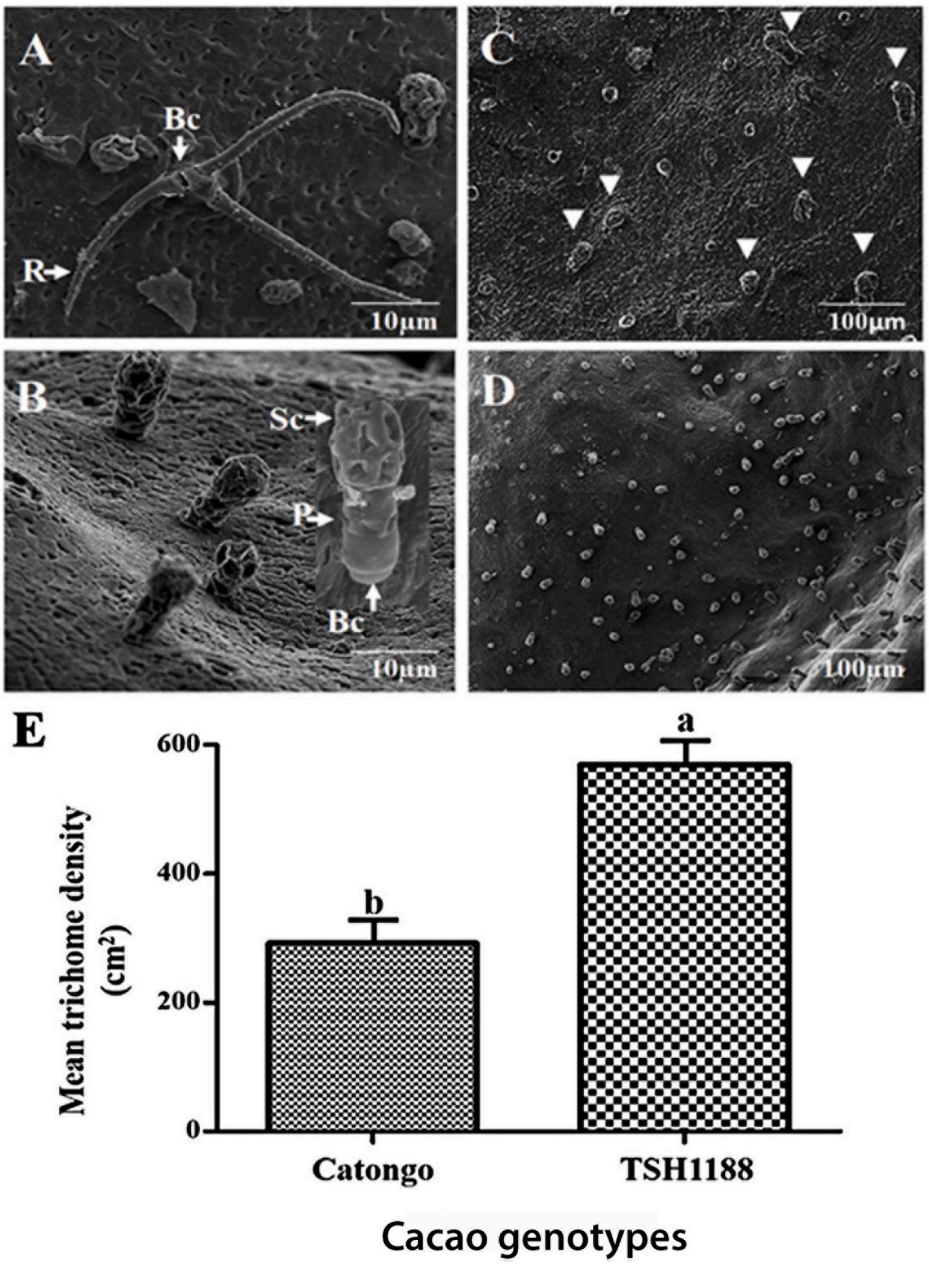

Fig. 5. Scanning electron micrographs showing the trichome characterization and density on cacao pods of resistant, TSH1188, and susceptible, Catongo, genotypes. A) non-glandular trichome characterization, Bc: basal cell, R: ramification; B) glandular trichome characterization, Sc: secretory cell, P: pedicle, Bc: basal cell; C) glandular trichomes in the surface of the susceptible genotype; D) glandular trichomes on pod of the TSH1188; E) glandular trichome density among contrasting genotypes. Mean values with different letters show a significant difference by the Duncan test at 5% ($p < 0.05$).

the mesocarp region, close to the pod peduncle. The symptoms at 30 and 45 DAI were very similar. It was observed that the small necrotic spots at the epicarp of the pod evolved to a stiff and irregular necrotic lesion that covered almost half of the pod epicarp. Pods internal symptoms included intense tissue necrosis of the mesocarp and endocarp region, and about 60% of the seeds were non-viable for planting or industrial use (Fig. 8C). Macroscopically, by 60 DAI, all cacao pods showed an extensive and stiff necrotic lesion. Internally, the tissues were also necrotic and partially dry, with no indication of endocarp hydrolysis (Fig. 8D). About 95% of the seeds were not useable for any purpose.

Contrary to the susceptible genotype, the TSH1188 showed low disease incidence and severity (<20%), and the symptoms have been seen only 60 DAI (Fig. 8E–G). Still, the fungus was restricted to the mesocarp, and seeds were viable (Fig. 8H). The majority of the cacao pods of this genotype were utterly asymptomatic, retaining their characteristic color (reddish-purple), size consistent with their chronological development (5–30 cm), and their internal tissues, mesocarp, and endocarp, intact with viable seeds for consumption/planting.

4. Discussion

This study describes for the first time the factors involved in the *M. perniciosa* infective process on cacao pods with contrasting resistance levels to witches' broom disease. The level of resistance or susceptibility shown by the different cacao genotypes was probably influenced by their pre-formed surface characteristics, the topography of their surface, and the type and density of trichomes stomata.

In this present study, the predominance of glandular trichomes was observed and quantified (570-cm2 in TSH1188 and 292-cm^2 in Catongo). Compared to the susceptible Catongo, TSH1188 had a higher density of glandular trichomes, suggesting a positive relationship between the type and density of trichomes and the resistance level presented by the host. Besides being a morphological characteristic that can act as a pre-formed barrier [27], glandular trichomes are also known to exude water-soluble compounds and proteins, such as α-1,3-glucanase, probably affecting the germination of *Fusarium oxysporum* on cowpeas [28] and perhaps phylloplanin, a protein from the phylloplane of cacao

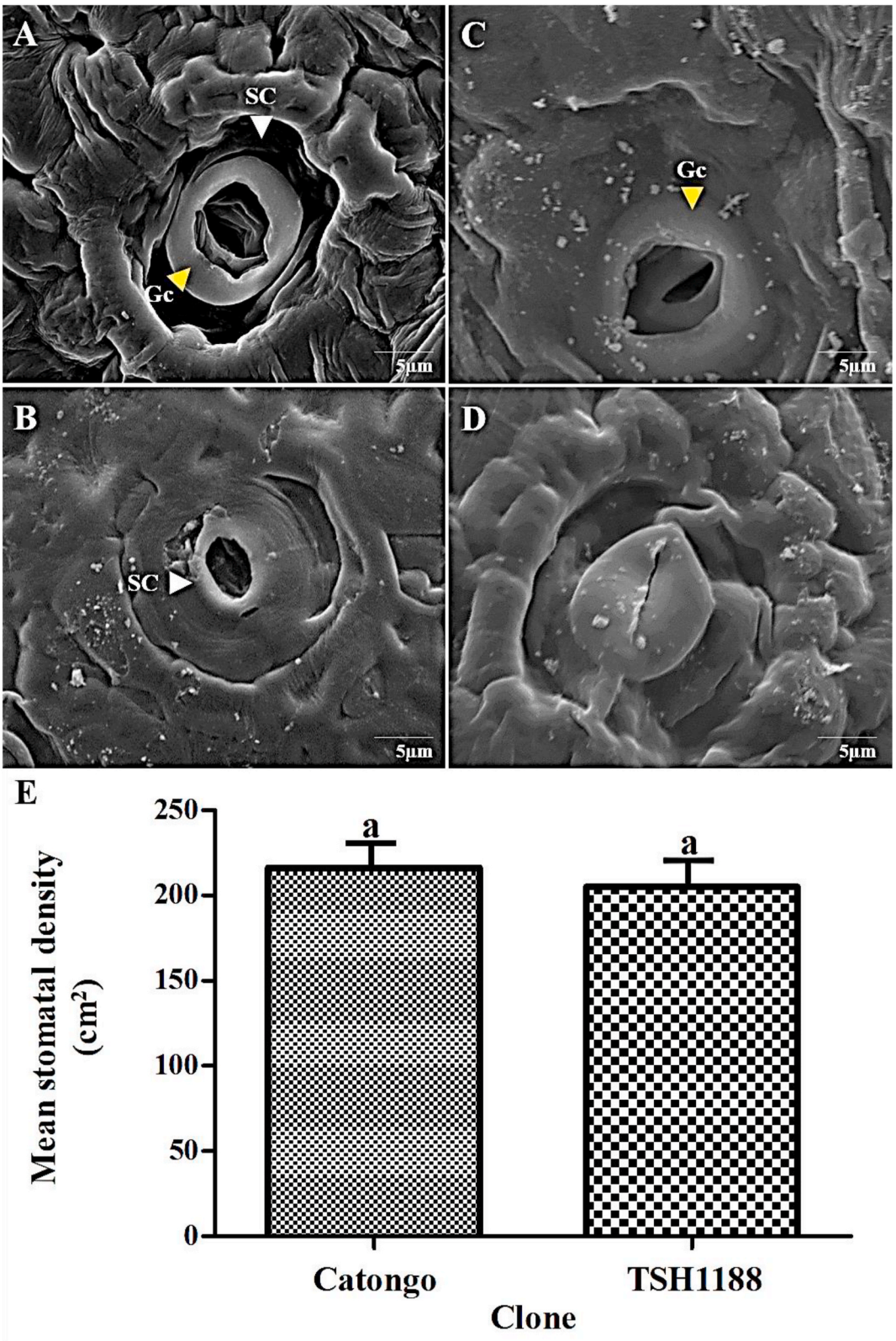

Fig. 6. Scanning electron micrographs showing stomatal characterization and density on cacao pods phylloplane of contrasting genotypes (susceptible, Catongo and resistant, TSH1188) to witches' broom disease of cacao. A, B) anomocytic stomata inside a stomatal crypt (chambers in the epidermis) on the susceptible genotype C, D) anomocytic stomata on the phylloplane of resistant genotype; E) stomatal density of two genotype. Mean values with different letters presented a significant difference ($p < 0.05$) by the Duncan test at 5%. SC: stomatal crypt, Gc: guard cell.

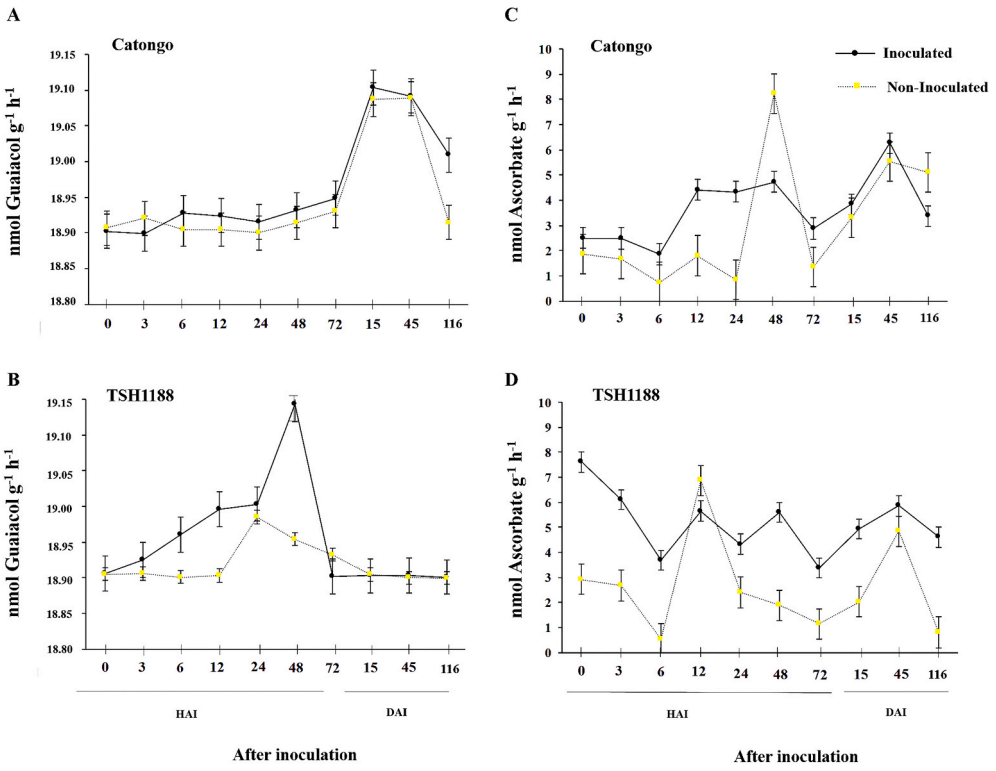

Fig. 7. Kinetics of guaiacol (GPX; A, B) and ascorbate (APX; C, D) peroxidases activity of cacao pods of contrasting genotypes to witches' broom disease of cacao in response to *Moniliophthora perniciosa* infection; Catongo = susceptible genotype; TSH1188 = resistant genotype.

leaves of resistant cacao genotype CCN51, that seems to inhibit *M. perniciosa* germination [7]. Although these cited compounds on the cacao pod's surface are also possible, further studies are needed to investigate the involvement of glandular trichomes as a chemical barrier to *M. perniciosa*.

The influence of stomata and their characteristics on disease resistance have been exposed by several authors in different pathosystems, being *Oidiopsis haplophylli* – pepper [29] and *Puccinia graminis* f. sp. *tritici* – wheat [30] among them. In this latter plant pathosystem, some wheat-resistant varieties showed a late stomatal opening provoking desiccation of the germ tube due to the evaporation of dew on its surface. Besides that, the stomata structure themselves (narrow opening, high guard cells) were are also considered resistance factors [30].

Surprisingly, there was also no significant difference in stomatal density or aperture size between the studied genotypes. However, *M. perniciosa* was more commonly observed penetrating stomata with crypts. This type of stomata was predominantly observed in Catongo pods, the susceptible cacao genotype. Stomatal crypts are invaginations on the pods' surface produced when the epicuticular wax layer coats the epidermal cells and not the outer stomatal cavity, thus, developing a cave-like structure [31]. Little information associates this type of stomata with plant diseases. However, it is mentioned that this type of stomata favors resistance to drought by preventing water loss caused by the opening and closure of the stomata, thus maintaining the humidity inside the crypt [32]. Considering that *M. perniciosa* needs high moisture to germinate and infect the plant organ, anomocytic stomata in stomatal crypts would retain or increase moisture on the pod surface, therefore, favoring the deposition, adhesion, and subsequent germination of basidiospores of *M. perniciosa*.

The histological events of the infection process of *M. perniciosa* in cacao pods were similar in both genotypes. However, the resistance level shown by TSH1188 pods was associated with a slower growth rate of *M. perniciosa* and restriction of germ tube elongation, which was probably influenced by the surface characteristics of the pods discussed above. The germination process started 5 h before in the susceptible cacao pods compared to the resistant cacao pods. This data corroborates the results found by Sena et al. [16] in a study of the shoot apex of cacao plantlets infected by *M. perniciosa*, where the temporal pattern of emission and lengthening (μm) of germs tubes were similar to those observed in the interaction *M. perniciosa* – cacao pods.

In this present study, *M. perniciosa* also presented multiple modes of penetration on cacao pods; through the base of glandular trichomes, directly through the cuticle of the fruit [33], and through stomata [34,35]. This latter mode of penetration appears to be independent of the host resistance level and, in general, is the preferred mode of penetration of the pathogen into host tissues. This result contrasts with those observed in the interaction *M. perniciosa* – cacao shoot apex by Sena et al. [16], where penetrations through stomata were not predominant.

Although structures on the host surface are essential components of host defense against disease, another mechanism that worth attention is the signaling events triggered within the first few minutes of contact between the pathogen and the host. This process is initiated with the perception of an extracellular signal transmitted through the plasma membrane, which results in the accumulation of intracellular molecules and the induction of a signaling cascade that allows the expression of specific genes. Thus, occurring several physiological processes that also determine the post-formed resistance to diseases [36]. High activity of peroxidases and isoenzymes have been associated with resistance in the pathosystems *Bremia lactucae* Regel – lettuce and *Pseudoperonospora cubensis* – melon. They are, in most cases, the first enzymes to have their activity altered when the plant is submitted to any condition of stress (physical, chemical, and biological) [32,37–40].

A significant guaiacol peroxidase activity was observed in TSH1188 during the first stages of the infection; but, the opposite occurred in Catongo. In the susceptible genotype, the activity of this enzyme increased in the late stages of the infection. The same trend was observed for the activity of ascorbate peroxidase. Van Loon et al. [36] suggested that these enzymes have immediately acted in the plant

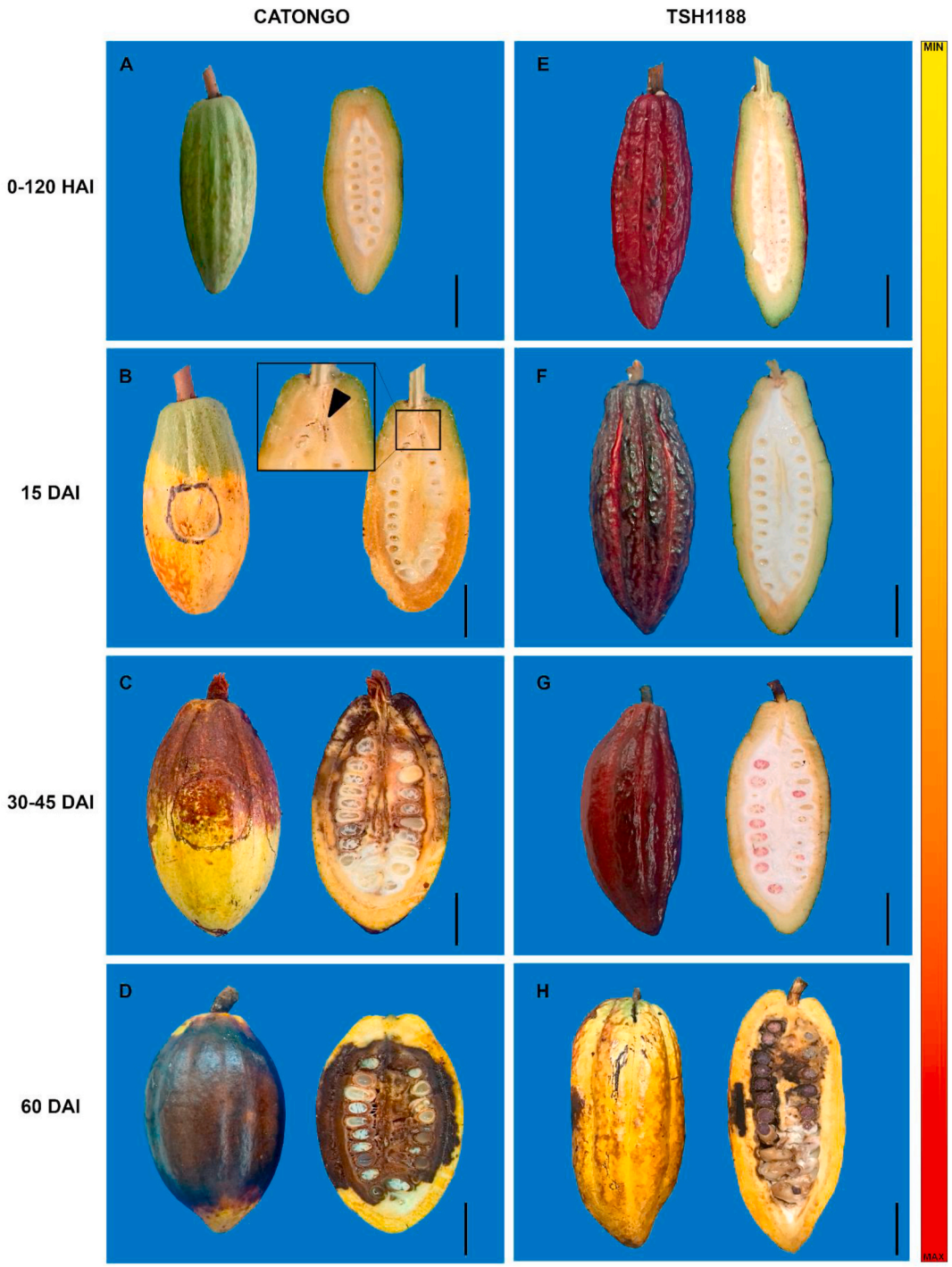

Fig. 8. Macroscopic evolution of witches' broom disease symptoms, external and internal, on susceptible (Catongo; A-D) and resistant (TSH1188; E-H) cacao pods under artificial field inoculation with *Moniliophthora perniciosa*. The color code depicted at the right of the images ranges from yellow (minimum value) to red (maximum value) of disease incidence. Scale bars: 1.3 cm (A), 2.6 cm (B), 3.5 cm (C), 3.8 cm (D), 1.7 cm (E), 2.5 cm (F), 4.1 cm (G), 5.08 cm (H). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

defense, directly acting on the invading structures. Therefore, the temporal pattern of guaiacol and ascorbate peroxidase activities of TSH1188, resistant genotype, suggests an early recognition of the pathogen and subsequent induction of the defense cascade restricting the colonization of *M. per*niciosa in its tissues. The induction of peroxidase activity at the beginning of infection in the resistant genotype, TSH 1188, was also observed by Camillo et al. [41], suggesting the production of H_2O_2 in this genotype as identified by Dias et al. [42]. Altogether, these results led us to associate the TSH1188 pod resistance with the fast ability of the plant to respond to *M. perniciosa* infection, therefore, avoiding further colonization. In Catongo, on the contrary, it suggests a late recognition of the plant to the action of the pathogen, which allows its establishment and subsequent colonization of host tissues. The evidence obtained in this work supports the hypothesis that TSH1188 cacao pods have structural mechanisms that hinder or delay the action of *M. perniciosa*. The interaction of TSH1188 with *M. perniciosa* was characterized by a longer latency period (60 DAI), reduced infectious process, wax layers, rigid cell walls, and the presence of polyphenolic substances.

Also, disease incidence and severity were low in this genotype. Therefore, confirming that the TSH1188 shows resistance to WBD. It is important to highlight that these results were observed under artificial inoculation, wherein the achieved disease severity is usually higher than in field conditions. Under field conditions, infection of TSH188 pods by *M. perniciosa* is minor compared to the results observed in this study.

5. Conclusion

In summary, the results of this study showed that TSH1188 cacao pods have resistance to WBD. The presence of pre-and post-formed defense mechanisms restricted the development and subsequent colonization of *M. perniciosa*. Although pods are most susceptible in the early stages, cacao pods can be infected by *M. perniciosa* at all pod development stages. These findings must be considered to develop control strategies such as chemical applications and the deployment of genotypes with pod resistance to WBD.

Authors contributions

I.M. Meraz-Pérez: Methodology, Original data preparation, Investigation, manuscript writing- Reviewing and Editing; **M. R. Carvalho:** manuscript writing- Reviewing and Editing, Figure reviewing and editing; **Y. J. B. Soares:** Methodology, Investigation; **K. F. Sena:** Conceptualization, Manuscript writing- Reviewing and Editing; **A. S. Estrela Júnior:** Methodology, Investigation; **U. V. Lopes:** Statistical Methodology, Data curation; **L.P. dos Santos Filho:** Statistical analyses, Data curation; **S. A. Araújo:** Figure reviewing, design and editing; **V. L. F. Soares:** Data curation and manuscript reviewing and editing; **C. P. Pirovani:** Data curation and manuscript reviewing and editing; **K. P. Gramacho:** Manuscript conceptualization, Methodology supervision, Original data preparation, Writing- Reviewing and Editing, Validation.

Funding

Funding for the involvement of I.M.P in this study was provided by the Mexican National Council of Science and Technology (CONACYT México), Y. J. B by The Fundação de Amparo à Pesquisa da Bahia (FAPESB/Grant number 6741/2013, Edital 21/2013), K.F. S by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and K.P.G. and C.P.P were supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

I.M. Meraz-Pérez acknowledges her gratitude to the Mexican National Council of Science and Technology (CONACYT México) to sponsor her with a scholarship during her Master's program. The authors would like to thank all personnel of Plant Molecular Laboratory (FITOMOL/CEPEC/CEPLAC/MAPA) and the Universidade Estadual de Santa Cruz (UESC) for the support in the development of this project. The author thanks C. Forte (EMBRAPA), R. Valle (CEPLAC) and Augusto R Sena Gomes for the english review of this article and the anonymous reviewers for their insightful suggestions and careful reading of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2021.101656.

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