Research article

Hydrogen peroxide formation in cacao tissues infected by the hemibiotrophic fungus Moniliophthora perniciosa

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In plant–pathogen interaction, the hydrogen peroxide (H2O2) may play a dual role: its accumulation inhibits the growth of biotrophic pathogens, while it could help the infection/colonization process of plant by necrotrophic pathogens. One of the possible pathways of H2O2 production involves oxalic acid (Oxa) degradation by apoplastic oxalate oxidase. Here, we analyzed the production of H2O2, the presence of calcium oxalate (CaOx) crystals and the content of Oxa and ascorbic acid (Asa) – the main precursor of Oxa in plants – in susceptible and resistant cacao (Theobroma cacao L.) infected by the hemibiotrophic fungus Moniliophthora perniciosa. We also quantified the transcript level of ascorbate peroxidase (Apx), germin-like oxalate oxidase (Glp) and dehydroascorbate reductase (Dhar) by RT-qPCR. We report that the CaOx crystal amount and the H2O2 levels in the two varieties present distinct temporal and genotype-dependent patterns. Susceptible variety accumulated more CaOx crystals than the resistant one, and the dissolution of these crystals occurred in the early infection steps and in the final stage of the disease in the resistant and the susceptible variety, respectively. High expression of the Glp and accumulation of Oxa were observed in the resistant variety. The content of Asa increased in the inoculated susceptible variety, but remained constant in the resistant one. The susceptible variety presented reduced Dhar expression. The role of H2O2 and its formation from Oxa via Apx and Glp in resistant and susceptible variety infected by M. perniciosa were discussed.

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1. Introduction

Reactive oxygen species (ROS) are continuously produced in plants as a result of the aerobic metabolism. ROS are originated from the excitement of the molecular O2, forming simple molecular oxygen (O2'), or from the transfer of one, two or three electrons to the O2, leading to the formation of superoxide radical (O2·−), hydrogen peroxide (H2O2) or hydroxyl radical (OH·), respectively. The fast increase of ROS levels in cells is called oxidative burst and constitutes one of the fastest responses to pathogen attack. These molecules can be harmful to the cell, but on the other hand they can activate defense pathways or metabolic responses to biotic and abiotic stresses [1]. Hydrogen peroxide, the chemically more stable ROS, is involved in a series of cell processes related to plant defense to pathogens via the biosynthesis of other important signaling molecules such as salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), ethylene and Ca2+ [1]. However, depending on the plant–pathogen interaction type, the H2O2 may play a dual role; its accumulation inhibited the growth of biotrophic pathogens, while...
it has been ignored by necrotrophic pathogens or reported as a helper during the infection/colonization process of plant by these ones [2–5]. In the case of the hemibiotrophic fungus Septoria tritici, the accumulation of H$_2$O$_2$ inhibited the infection during the initial stage (biotrophic phase) of the disease in wheat [6]. Hydrogen peroxide is produced in plants via two possible pathways: i) cell wall oxidases catalyze the oxidation of the NADH to NAD$^+$, which in turn enables reduction of O$_2$ to O$_2^-$, and then, the dismutation of O$_2^-$ produces O$_2$ and H$_2$O$_2$ [7]; ii) apoplastic oxalate oxidase and amine oxidase have been proposed to generate H$_2$O$_2$ [8,9].

In 2007, Ceita et al. [10] analyzed the interaction between a susceptible variety of cacao (Theobroma cacao L.; Catongo variety) and the hemibiotrophic fungus Moniliophthora perniciosa. During the time course disease, two distinct phases were observed, i) a biotrophic phase, in which the mycelia grows only in the apoplast and presents monokaryotic hyphae; and ii) a saprotrophic phase, in which the mycelia invades the cell, presenting dikaryotic hyphae and formation of clamp connections. At the end of the saprotrophic phase, plant cell death occurs, followed by basidiocarp production and releasing of spores. The authors also reported an increase of calcium oxalate (CaOx) crystals concomitant with the fungus colonization (biotrophic phase). During the necrotrophic phase, an increase in H$_2$O$_2$ level was observed, associated with the dissolution of the CaOx crystals and the degradation of the generated free oxalic acid (Oxa) by the germin-like oxalate oxidase (Glp). Although a considerable knowledge on the whole fungus cycle and in –omics of T. cacao has been accumulated in the last 10 years [11], little is known about the cellular mechanisms of the resistant cacao–M. perniciosa interaction.

In this study, we analyzed the production of H$_2$O$_2$ and the content of free Oxa and ascorbic acid (Asa) – main precursor of Oxa in plants [12,13]. We also quantified the expression of ascorbate peroxidase (Apx; which converts Asa into dehydroascorbate), dehydroascorbate reductase (Dhar; which uses dehydroascorbate and glutathione to form Asa and glutathione disulfide) and Glp genes in two cacao varieties (susceptible vs resistant) infected and non infected with M. perniciosa. We showed that the CaOx crystal amount and the H$_2$O$_2$ levels in the two varieties present distinct temporal and genotype-dependent patterns. High expression of the Glp and accumulation of Oxa were observed in the resistant variety. The content of Asa increased in the inoculated susceptible variety, but remained constant in the resistant one. The susceptible variety presented reduced Dhar expression. The role of H$_2$O$_2$ and its formation from Oxa via Apx and Glp in resistant and susceptible cacao varieties inoculated by M. perniciosa were discussed.

2. Results

2.1. Detection of H$_2$O$_2$ and CaOx crystals in resistant and susceptible cacao varieties

An increase of H$_2$O$_2$ production was observed at early stages (24 hai, Fig. 1D; 48 and 72 hai, data not shown) in the resistant cacao variety inoculated with M. perniciosa, particularly in the tissues adjacent to the vascular system (Fig. 1D and H). No presence of H$_2$O$_2$ in the susceptible variety was observed (Fig. 1C); at 24 hai the susceptible variety did not differ from the control inoculated by M. perniciosa and infiltrated with water (Fig. 1A and B).

CaOx crystal were visible on sections of both varieties as bright white spots (arrows; Fig. 1E–G). However, at 72 hai, much more CaOx crystals were observed in the susceptible variety (Fig. 1E) in comparison to the resistant one (Fig. 1G). The resistant variety presented less CaOx crystals at 72 hai (Fig. 1G) than before the inoculation (0 hai, Fig. 1F), suggesting a dissolution of the CaOx crystals after the plant infection. This CaOx crystal dissolution was associated with the production of H$_2$O$_2$ in the resistant plants (Fig. 1H). At 24 hai, some remaining CaOx crystals were still observed, surrounded by adjacent cells containing H$_2$O$_2$ (DAB staining; Fig. 1G). In the susceptible variety, no reduction of CaOx crystal number was observed.

2.2. Quantification of Oxa and Asa in resistant and susceptible cacao varieties inoculated by M. perniciosa

The basal level of Oxa was about 2.5 times higher in the resistant variety than in the susceptible one (Fig. 2A and B, 0 hai). Both inoculated varieties showed an increase of Oxa at 3 dai. However, in the inoculated susceptible variety, the Oxa level decreased at 15 dai (returned to basal level), then increased at 30 dai and finally decreased at 45 and 60 dai (with no significant difference with the plant control; Fig. 2A). In the inoculated resistant variety, the Oxa...
level remained high at 15 dai, then decreased at 30 dai and increased at 45 and 60 dai (with no significant difference with the plant control at 45 dai; Fig. 2B).

The basal level of Asa was about 1.5 times higher in the resistant variety than in the susceptible one (Fig. 2C and D, 0 hai). In the inoculated susceptible variety, the Asa level increased from 3 to 30 dai, decreased at 45 dai (without significant difference with the plant control) and then increased at 60 dai (same level than at 30 dai; Fig. 2C). In the inoculated resistant plant, the Asa level at 3 and 15 dai remained constant and equal to the basal level (without significant difference with the plant control), then slightly decreased at 30 dai and finally slightly increased at 45 and 60 dai (without significant difference with the plant control for 45 and 60 dai; Fig. 2D).

2.3. Transcript level of Apx, Glp and Dhar in susceptible and resistant cacao varieties inoculated with M. perniciosa

The expression patterns of Apx, Glp and Dhar were obtained in the early stages of the infection (up to 72 hai). No significant variation of the expression of the three genes studied was observed in the inoculated susceptible variety (Fig. 3B). In the inoculated resistant variety, a significant increase of Apx, Glp and Dhar transcript levels was observed at 48 hai. At 72 hai, Apx transcripts level was still high (and significantly different from the one observed before inoculation; Fig. 3A, 0 hai) while Glp and Dhar transcript levels were as low as the ones observed at 0 hai (Fig. 3A). The expression of the Dhar and Glp in the resistant variety followed the same pattern as the Apx throughout the time course, with a correlation coefficient of 0.96 and 0.95 for Dhar/Apx and Glp/Apx, respectively (data not shown).

3. Discussion

Hydrogen peroxide plays an important role in plants under stress conditions as a signaling molecule which intermediates a series of important cellular responses [1,14]. In the resistant cacao variety infected by M. perniciosa, we observed a significant accumulation of H2O2 throughout the stem apex vascular system in the first 72 hai (Fig. 1D) while no H2O2 accumulation was observed at the early infection stages in the susceptible variety (Fig. 1C). In a previous work, we showed a significant accumulation of H2O2 in the advanced stages of infection (45 dai) in the susceptible cacao infected by M. perniciosa facilitating the transition phase of the fungus from biotrophic to necrotrophic [10] and corroborating other works involving necrotrophic or hemibiotrophic pathogens [2,5,6]. For example, the infection of Arabidopsis thaliana by Botrytis cinerea led to the accumulation of H2O2, which killed the host cells facilitating the pathogen invasion [2].

The successive sequence of production of Oxa (higher after 72 hai), transcript level of Glp (higher at 48 hai), dissolution of CaOx crystals and H2O2 accumulation (visible up to 72 hai) in the resistant infected variety suggested that the Glp participates in Oxa degradation leading to the formation of CO2 and H2O2. These observations corroborate with previous results obtained for infected susceptible cacao plants in which the dissolution of CaOx crystals was followed by a massive accumulation of H2O2 in infected tissues at the late infection times [10]. Together with the increase of the Glp transcript level in the resistant inoculated cacao variety, we observed the increase of the Apx and Dhar transcript levels (Fig. 3A). Apx uses H2O2 to convert Asa into dehydroascorbate, and Dhar uses dehydroascorbate to react with glutathione and form Asa and glutathione disulfide. Therefore, at the same time, Asa was used as substrate of Apx, and was regenerated by Dhar reaction, which may explain the similar Asa contents found in the inoculated and non-inoculated resistant plantlets (Fig. 2D). Despite the existence of several reports describing the function of the CaOx in the free calcium amount regulation process in plants [15,16], a novel and more dynamic role for this molecule was raised by the discovery of the wheat germans [17], opening to other possibilities regarding the functionality on the formation and/or dissolution of CaOx in plants.

The over-expression of an oxalate oxidase (OXO) in transgenic

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Fig. 2. Quantification of free Asa and Oxa in resistant and susceptible cacao varieties. Oxa level on susceptible (A) and resistant (B) varieties. Asa level on susceptible (C) and resistant (D) varieties. Results are the average of three biological replicates ± standard error. Different letters indicate significant statistical difference between samples by the Duncan test. Dark gray bars: plant control; light gray bars: plant inoculated com M. perniciosa.
plants triggered the production of \( H_2O_2 \) and defense responses by the plant to the fungal infection [18–20]. It was also demonstrated the involvement of OXO in the reduction of the progression of some fungal diseases, conferring, in some cases, partial resistance [21]. It was also reported the existence of a crystal matrix protein associated with CaOx precipitation [22]; our group recently identified cacao proteins associated with crystal formation which are involved in the formation/dissolution of CaOx crystals (data not shown). The participation of Asa in the generation of Oxa has been previously proposed [23], and since then, has stimulated the interest of the researchers [13]. By using Asa, Oxa, eritroascorbic acid, galactose and glycolate labeled with \(^{14}C\), it has been demonstrated that Asa acts as precursor of Oxa and CaOx in idioblasts of \( P\). siatodes, and that glycolate is a very poor substrate for Oxa synthesis and CaOx generation. This work confirmed the results of Wheeler et al. [24], which proposed that the \( \delta \)-galactose may be a key intermediate in the conversion of the \( \delta \)-glucose to Asa in plants.

In the susceptible cacao variety, we observed that the CaOx crystals were not dissolved after the infection by \( M.\) perniciosa (Fig. 1E). In addition an increase of Asa in the susceptible plants (Fig. 2C) was observed but without variation of Glp, Apx and Dhar transcripts levels (Fig. 3B) suggesting a relation between Asa and CaOx contents. Ceita et al. [10] already suggested the participation of Asa in the interaction of cacao—\( M.\) perniciosa as a pro-oxidant molecule [25]. In \( M.\) truncatula mutants, a relationship between Asa levels and the presence of CaOx crystals was demonstrated, stating that, in this plant model, Asa is the main source of Oxa [26]. Other studies showed that Oxa itself is involved in programmed cell death (PCD) and is responsible for the increase of ROS in plant. However, when ROS production was inhibited, the apoptotic-like-cell death induced by Oxa does not occur [27]. According to these findings Glp, CaOx and Oxa are involved in CaOx crystal dissolution and subsequent \( H_2O_2 \) formation, and implicated, in one hand, in plant resistance process (acting at the early stages of the interaction) or, on the other hand, contribute to pathogen life cycle in the susceptible variety (at later stages of the disease). It is interesting to note that even \( M.\) perniciosa also produces CaOx crystals [28], these probably are not involved in the earliest stages of disease, but contribute, in the susceptible variety, to the development of the pathogen. The low expression of Dhar in the susceptible variety explains the differences found in the behavior of ascorbate and oxalate between the contrasting varieties. In conclusion, the increased \( H_2O_2 \) levels in the two cacao varieties present distinct temporal and functional patterns. Once produced at the beginning of the infection by the resistant variety, it contributes to the infection control and to plant resistance. In advanced stages of the disease in the susceptible variety, it promotes the pathogen development and the finalization of its life cycle.

4. Material and Methods

4.1. Plant material

Plantlets of \( T.\) cacao L. susceptible (Catongo variety) and resistant (TSH 1188 variety) to \( M.\) perniciosa were cultivated in greenhouse at CEPEC/CEPLAC (Centro de Pesquisas do Cacau da Comissão Executiva do Plano do Lavoura Cacaueira, Ilhéus, Bahia State, Brasil) under natural light and 90% of relative humidity as described by [29]. Apical meristems of 6-week old-plantlets were inoculated by the spraying method [30] using a \( 10^6.ml^{-1} \) basidiospore suspension from the \( M.\) perniciosa C14141 CEPEC/CEPLAC strain. After inoculation, plantlets were kept during 24 h at 25 °C ± 2 °C in a water-saturated atmosphere to allow \( M.\) perniciosa spore germination, penetration and infection [30]. A spore viability test was conducted in the humid chamber (25 °C) 24 h after inoculation and compared to spore viability obtained before inoculation. Apical meristems of three individual inoculated plantlets of each variety (biological replicates) were collected before inoculation (0), at 24 h, 48 h, 72 h after inoculation (hai) and at 15, 30, 45 and 60 days after inoculation (da). The time course disease, 24–72 hai corresponded to the early stages of the infection, 30 dai to wilt and necrosis of the young leaves, and 45 dai to hypertrophy of the stem (“green broom”) and first microscopic necrosis symptoms [10]. At 60 dai, the infected plant presented macroscopic symptoms called “dry broom” and at 90 dai the plant was considered as completely necrotic. Plantlets inoculated with water, and maintained and harvested in the same condition as the inoculated ones, were used as control. Inoculated and control samples were frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C.

4.2. Detection of \( H_2O_2 \) by DAB staining method and visualization of CaOx crystals

Apical meristems of TSH 1188 and Catongo varieties harvested at 24, 48 and 72 hai were immersed in 1 mg/ml of 3′–3′
diaminobenzidene (DAB) — HCl, pH 3.8 (Sigma) and infiltrated under vacuum for 4 h, as described by [31]. Samples were cleared in boiling ethanol (96%) for 20 min, then hand-sectioned with a razor blade, mounted in 50% glycerol and examined using an optical microscope (Olympus CX41). Images were obtained using a digital camera Olympus C-7070. H2O2 was visualized as a reddish-brown coloration. Control samples were immersed and infiltrated with distilled water.

For CaOx crystal visualization, apical meristems were cleared in boiling ethanol (96%) for 20 min, then hand-sectioned with a razor blade, mounted in 50% glycerol and examined using optical microscope with dark field (Olympus CX41). Crystals were visualized as bright spots at low magnification and perfect faceted crystal at high magnification.

4.3. Quantification of ascorbic and oxalic acid by HPLC

Organic acid extraction and quantification was carried out as described by [13] with some modifications. In 2 ml tubes, 6 mg of frozen material were mixed with 1 ml of extraction buffer (4 mM H2SO4, 5 mM DTT, PVPP added to a concentration of 10 mg/ml). The sample was vortexed for 15 min and centrifuged at 26,200 g for 20 min at room temperature. One hundred microliters of filtered supernatant (0.45 μm) was injected into the HPLC (ÄKTAbasisspace, GE-Health Care). Asa and Oxa were separated in a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) at a flow rate of 0.7 ml/min, with detection of Oxa at 210 nm (Rt = 8 min) and Asa at 243 nm (Rt = 10 min). The chromatogram peaks were integrated and analyzed using the UNICORN™ version 5.0 software. Oxa and Asa concentrations in cacao samples were obtained by comparison with a calibration curve obtained by separation in the HPLC of known amounts of pure standards (Sigma—Aldrich). Three biological replicates (3 meristems for each cacao variety at a given point of the time course) and three experimental replicates (3 quantifications of Asa and Oxa for each meristem) were performed. Statistical quantification of Oxa e Asa were made concomitantly, in the same run and from the same sample to minimize bias in the analysis. Statistical analysis was performed with the software SASSM — Agri which tested the experiments as a completely randomised design. Analysis of variance (ANOVA) was applied and for means comparison Duncan’s test was employed, with a critical value of P = 0.05. CaOx quantification was not performed since its formation/dissolution pattern was well established by [10] in both varieties.

4.4. RNA extraction and RT-qPCR analyses

Total RNA from frozen inoculated and control tissues was isolated as described by [32], and cleaned using the Rneasy Plant Mini kit as described by the manufacturer (Qiagen). The RNA was treated with DNase RNase-free according to the manufacturer (Fermentas), and then separated on 1% DEPC-treated agarose gel and stained with ethidium bromide to confirm RNA integrity and the estimated RNA concentration. The first strand cDNA was synthesized in 10 μl from 1 μg of total RNA using the SuperScript II kit (Invitrogen) according to the manufacturer’s instructions. RT-qPCR analysis was carried out for the germ-in-like oxalate oxidase (Glp), ascorbate peroxidase (Apx) and dehydroascorbate reductase (Dhar) cacao genes [29]. The tubulin gene (Tub) from cacao was used as reference (endogenous control) in the qPCR experiments (Supplementary material 1). The qPCR reaction containing 150 ng of cDNA, 0.2 mM of each primer and 10 μl of Applied Mix was performed using the Syber Green Kit PCR master mix on the Applied 7500 Real Time PCR (Applied Biosystems). The expression of each gene was obtained in comparison to the expression level of the Tub gene (relative quantification/2△△CT). Cycling parameters were as follows: 95 ºC for 10 min and 45 cycles of 95 ºC for 15 s and 60 ºC for 45 s. Automated gene expression analysis was performed using the equipment’s software (Applied Biosystems 7500 System SDS Software v1.3.1.21).

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.plaphy.2011.05.004.

References


