

Necrotrophic phase of *Moniliophthora perniciosa* causes salicylic acid accumulation in infected stems of cacao

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Abstract

Moniliophthora perniciosa infects young meristematic tissue of *Theobroma cacao* and induces abnormal shoot development and ultimately necrosis. We analyzed culture filtrates of *M. perniciosa* to identify metabolites with possible effects on witches' broom disease symptom development. All isolates produced salicylic acid (SA), phenyllactic acid and mandelic acid. When SA levels were measured in plant tissues, infected brooms had 5 times more SA compared to healthy shoots. In contrast, leaf inoculation with basidiospores and application of chemical elicitors did not alter leaf SA content. These findings suggest that the necrotrophic phase of *M. perniciosa* causes SA accumulation in diseased tissues.

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1. Introduction

Moniliophthora perniciosa, recently renamed from *Crinipellis perniciosa* [1], is the causal agent of witches' broom disease in cacao (*Theobroma cacao*). This disease is a major constraint for cocoa bean production in South America and the Caribbean, as well as a threat to other cacao growing regions still free of the pathogen. The fungus infects young meristematic tissue and grows intercellularly as a monokaryotic biotroph. Subsequently, the plant develops abnormal growth patterns including clustered shoots with hypertrophied internodes that resemble a broom. Throughout this period, the fungus becomes necrotrophic (dikaryotic) and infected plant parts turn necrotic. Basidiocarps then emerge from dead brooms and release spores that start a new infection cycle. Although most steps of the infection biology are known [2], a few key points remain unclear. The symptoms appear to be hormonally induced, yet application of hormones to

uninfected actively growing leaves fails to induce broom development. Another aspect not well understood that could be linked to symptom development, is when and how the pathogen switches from a biotrophic to a necrotrophic phase. Scarpari et al. [3] conducted a thorough study of the biochemical changes in cacao during the development of witches' broom; however, salicylic acid (SA) levels were not analyzed. SA, an extensively documented plant hormone, triggers systemic acquired resistance [4], thermogenesis [5], and regulates coupled cell enlargement and cell death [6,7]. In cacao, SA and chemical elicitors, including an SA analog, benzothiadiazole, induce chemical changes similar to those observed in infected tissues when applied to young shoots [8]. Moreover, both L. Meinhardt (personal communication) and A. Kilaru (personal communication) suggested that *M. perniciosa* produces SA. We hypothesize that the chemical changes leading to broom development (cell enlargement and death) involve regulation via the SA pathway. In this paper, we identify SA and two related phenolic compounds as products of fungal metabolism by *M. perniciosa* during the dikaryotic growth stage, and we quantified the levels of this hormone in filtrates of fungal

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cultures, in infected and uninfected plant tissues, and in leaves after treatment with chemical elicitors.

2. Material and methods

2.1. Chemical reagents

All solvents used were HPLC grade. The chemical elicitors used were adenine (AD), aminoethoxyvinylglycine (AVG), benzothiadiazole (BTH) (Acti-guard, product containing BTH, from Novartis, USA), glutathione (GL) and jasmonic acid (JA). BSA (N,O-bis(trimethylsilyl)-acetamide) (Pierce, USA) was used as derivatizing reagent prior to GC-MS analysis. SA was used as the standard for HPLC and GC-MS analyses (Chemicals were purchased from Sigma-Aldrich unless indicated otherwise).

2.2. Fungal cultures

M. perniciosa isolates 278, Alf42, 1103, PS12, 16avd1, 1130, Hrvd1, PS9, PS6 and PS10 collected in Bahia, Brazil, from diseased cacao tissues, were grown from mycelium in potato dextrose broth (PDB) for three weeks. The mycelia were then filtered off using glass wool, and the broth was lyophilized and stored at -80°C until analysis.

2.3. Plant material

Trees of two *T. cacao* varieties, Cacao Comum (CCO), disease susceptible, and Scavina 6 (SCA), much less susceptible to disease, grown on a cacao farm in Bahia, Brazil were used in this study. The youngest pair of leaves in a flush was inoculated with basidiospores of *M. perniciosa*. After 48 h, the inoculated leaves (L1) and the two older proximal pairs in the same flush (L2 and L3) were collected. The leaves were freeze-dried and stored at -80°C until analysis.

Infected brooms and healthy shoots were also collected, freeze-dried and stored at -80°C . For the investigation of the effect of chemical elicitors on SA levels, detached cacao leaves from greenhouse-grown trees were used. Young flush leaves of approximately 1 cm long were placed in a 30 mM phosphate buffer solution (pH 5.6) containing 10 mM sucrose and 1 mM of the chemical elicitor, and placed on a shaker for 24 h. After this period leaves were rinsed with distilled water, blotted and frozen at -80°C until extraction.

2.4. SA extraction and HPLC analyses

SA extraction and HPLC analyses followed the method of Verberne et al. [9]. A Shimadzu LC-10AT equipped with a RF-10A spectrofluorometric detector and a C18 column ($140 \times 4.6 \text{ mm} \times 0.5 \mu\text{m}$ particle size) was used. The fluorescence detector was set at an excitation wavelength of 305 nm and emission of 407 nm; the flow rate was

0.8 mL min^{-1} using a mobile phase of 90% 0.2 M sodium acetate buffer pH 5.5 and 10% methanol.

2.5. GC-MS analyses

The samples analyzed by HPLC were dried in nitrogen, resuspended in acetonitrile and derivatized with BSA (reaction vial was heated at 70°C for 15 min). Samples were injected in the GC without further purification. GC-MS analyses were performed using a Hewlett Packard (HP) gas chromatograph 6890 series, equipped with an Agilent column DB-1MS ($60 \text{ m} \times 250 \mu\text{m i.d.} \times 0.25 \mu\text{m}$ film thickness) using helium (chromatographic grade, from Airgas, NJ, USA) as carrier gas with a flow rate of 1 mL min^{-1} . Oven temperature was set at 70°C , held for 2 min and increased to 240°C at $10^{\circ}\text{C min}^{-1}$ and held for 30 min. Spectral data were obtained from an HP mass spectrometer 5973 series with ionization energy of 70 eV and mass scan range from 40 to 550 amu at $1.97 \text{ scans s}^{-1}$. The MS source temperature was set at 230°C . Compounds were identified by comparison to commercially available standards or the equipment's library (NIST 98).

2.6. SA application to cacao tissues

SA was applied to young and old leaves, flowers, trunk and fruits (2 months old) of cacao using a handheld sprayer. Plant parts were sprayed until their surface was completely covered with solution. Spray solution contained 20 mM of SA in distilled water and 0.05% Tween 80. Control spray solution contained 0.05% Tween 80 in distilled water.

2.7. Statistical analysis

Statistical analysis of the data was performed using SAS version 9.1 for Windows (SAS Institute, USA).

3. Results and discussion

Plant disease resistance involves activation of a series of defense pathways that try to counterattack the pathogen. However, it may not be desirable for plants to launch defenses that are metabolically costly if they are not effective. Therefore, plants have regulatory mechanisms to activate particular defenses according to the specific stimuli or stresses that they are subjected to. Some of these pathways can be opposing, down regulating each other, while others work in combination where the activation of one triggers another. For some plants, including cacao, it may not be advantageous to activate the SA pathway when attacked by necrotrophic pathogens.

M. perniciosa is a hemibiotrophic fungus, with a biotrophic and necrotrophic phase. By avoiding the SA pathway cacao is preventing cell death. This allows the plant to avoid inducing the phase transition of the fungus from biotroph to necrotroph, which at the same time may

provide for activation of other more effective defenses. This suggestion is supported by the observation that SA levels in young leaves treated with elicitors were not statistically different than control treatments for both cultivars. Furthermore, young cacao leaves maintained the same SA levels as the controls after pathogen inoculation, confirming that the plant's response to *M. perniciosa* infection is likely induced by a pathway other than SA (Fig. 1).

Although SA levels did not increase, caffeine, which is toxic to *M. perniciosa*, was higher in young leaves following pathogen infection or SA application [8]. During infection the plant accumulates caffeine as a defense compound through a pathway that bypasses SA in order to avoid cell death, which would favor the pathogen. Although there was no increase in SA in the inoculated leaves, the molecule could affect nearby tissues since it is mobile in the plant. This was not the case, as there was no increase in SA levels found in non-inoculated leaves on the same flush as the inoculated ones (Fig. 1).

Endogenous SA levels of both tested cultivars including the more disease tolerant SCA and the more susceptible CCO, did not differ statistically (Fig. 1), reinforcing that SA levels are not indicative of resistance to *M. perniciosa* in cacao. In addition, *M. perniciosa* preferentially infects the young meristematic tissue, even though both varieties of cacao had the highest levels of SA in the youngest tissues independent of inoculation (Fig. 1). In both SCA and CCO cultivars, levels of SA were significantly higher in the youngest leaf pair (L1) than in the older pairs (L2 and L3) independent of inoculation. When comparing the two healthy non-inoculated leaf pairs, the younger pair had higher SA levels in both cultivars independent of inoculation. The inoculation had no effect on detected SA levels in inoculated leaves or the adjacent pairs when compared to the controls in both cultivars (Fig. 1).

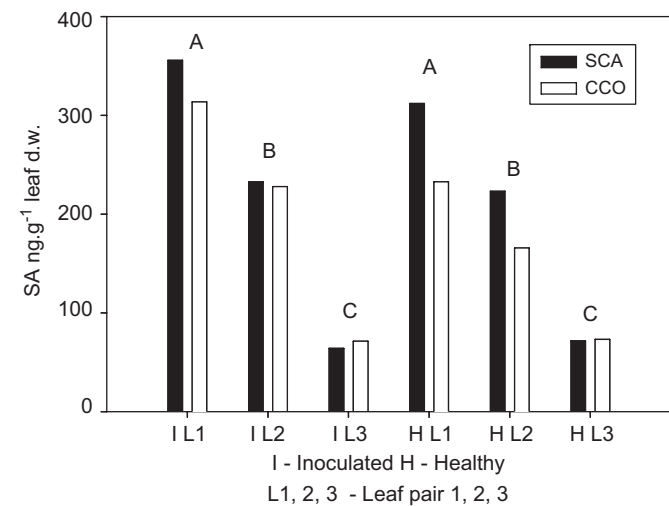


Fig. 1. Salicylic acid levels in young leaves. *Different letters indicate significant difference using Fischer's significance test at $p < 0.01$.

Some pathogens produce plant growth hormones (auxin, ethylene) and derivatives (coronatine, a JA analogue) as virulence factors that may act by disrupting the tightly regulated plant defense metabolism, altering physiological processes and favoring the pathogen to thrive. All *M. perniciosa* isolates, when grown in liquid culture media, produce SA at varying levels (Fig. 2). Freeze-dried culture broths were extracted and analyzed by HPLC following the method of Veberne et al. [9], as previously mentioned. The identification of SA was made by matching the retention time of the standard with the compound in the sample at specific excitation and emission wavelengths. A calibration curve was prepared by injecting different concentrations of the commercially available SA standard. All 10 *M. perniciosa* isolates tested produced SA in the media at levels ranging from about 0.3 to 2.2 ng g⁻¹ of fresh weight of mycelia (Fig. 2). These fungi were collected from diseased cacao tissue. Thus it is possible that non-pathogenic strains do not produce SA and therefore have a symbiotic interaction with cacao.

Our results also confirm the presence of two other aryl compounds, mandelic acid and phenyllactic acid (Fig. 3). In order to confirm SA's identity, samples extracted for the HPLC analysis were examined by GC-MS. SA was identified as its trimethylsilylated derivative obtained after reaction with BSA. The retention time and mass spectra of the compound present in the fungal extract were identical to the trimethylsilylated SA standard. The major fragment ions were m/z 73(78.2), 91(11.4), 135(28.2), 149(7.4), 179(8.6), 209(6.8), 267(100), 268(22.4), 269(10.3) and M^+ 282(<1) (Fig. 3). Another phenolic compound characterized by GC-MS was 2-hydroxy-2-phenylacetic acid (also known as mandelic acid) with the distinctive fragmentation m/z 73(65.3), 147(33.1), 163(11.3), 179(100), 253(15.2), 281(2.5) and M^+ 296(<1) (Fig. 3). A third compound identified was 2-hydroxy-3-phenylpropanoic acid (also known as phenyllactic acid) with the following fragmenta-

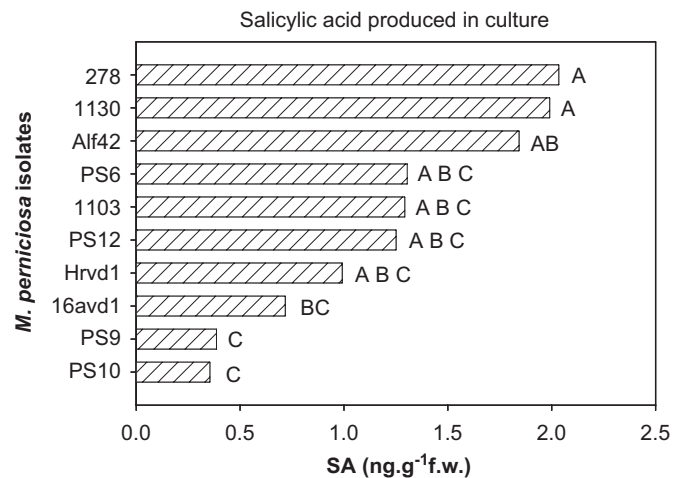


Fig. 2. Salicylic acid produced by *M. perniciosa* isolates in culture media (PDB). *Means followed by the same letter are not significantly different using Fischer's significance test at $p < 0.05$.

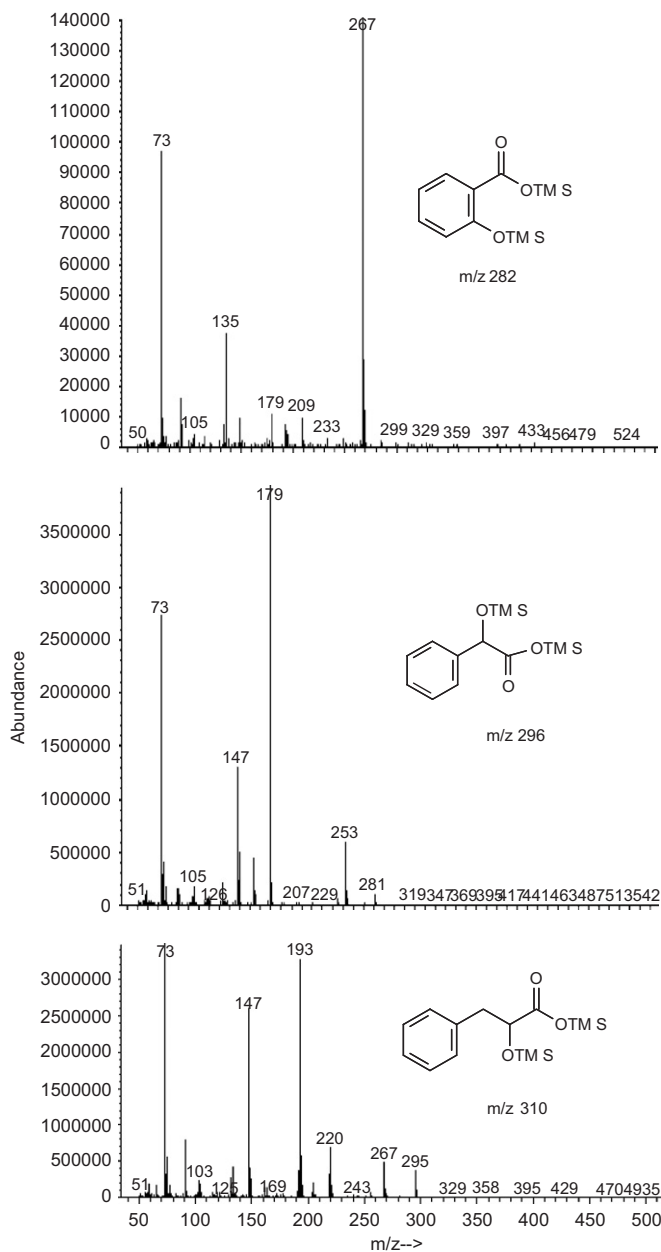


Fig. 3. Mass spectra of salicylic acid, mandelic acid and phenyllactic acid after derivatization with BSA.

tion pattern: m/z 59(5.5), 73(10.0), 147(74.5), 193(93.7), 205(5.9), 220(19.7), 267(14.1), 295(10.8) and M^+ 310(<1) (Fig. 3). Identification of the two latter compounds was based on their fragmentation pattern and by comparison to the equipment's library. These molecules have been previously identified as intermediates in the metabolic pathway of the white rot fungus *Bjerkandera adusta*, leading to synthesis of aryl metabolites [10]. In our case, these metabolites could be intermediates in the synthesis of SA as well as active compounds that could interfere with the plant defenses by mimicking SA.

The jasmonic acid pathway, responsible primarily for defense responses against herbivores, is negatively regu-

lated by SA [11,12]. Therefore, activation of SA may render the plant susceptible to attack from other pathogens by what Cui et al. [13] called systemic induced susceptibility (SIS). In the SIS activated by SA, the pathogen alters a defensive pathway to induce vulnerability to pathogen-vectoring herbivores that would, in one hypothesis, increase the spread of the pathogen. Moreover, *Arabidopsis thaliana* mutants impaired in the JA pathway are more susceptible to necrotrophic fungal pathogens including *Alternaria brassicicola*, *Botrytis cinerea*, and *Pythium* sp. [11]. Following this hypothesis, in cacao, SA blocks the JA pathway rendering the plant susceptible to *M. perniciosa* infection.

There are evolutionary speculations that *M. perniciosa* evolved from an endophyte to a pathogen. It may have acquired the mechanism of producing SA to alter plant defenses to its advantage, and as a consequence, modified itself from a biotrophic and endophytic organism to a hemibiotrophic pathogen. At a certain stage in its



Fig. 4. Salicylic acid treatment of young leaves of *Theobroma cacao*.

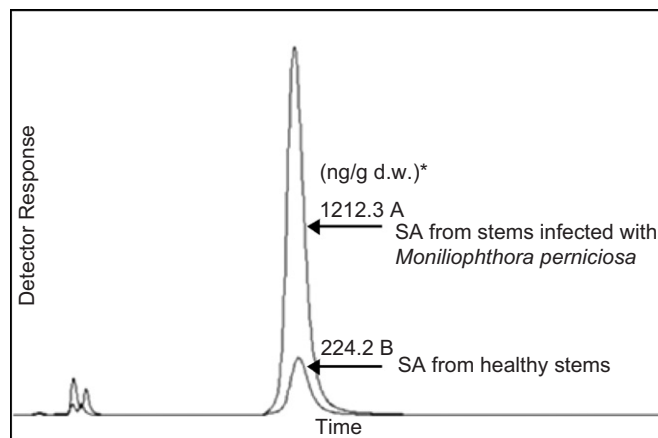


Fig. 5. HPLC chromatogram of salicylic acid in brooms and healthy stems. *Different letters following means indicate significant difference using Fischer's test at $p < 0.01$.

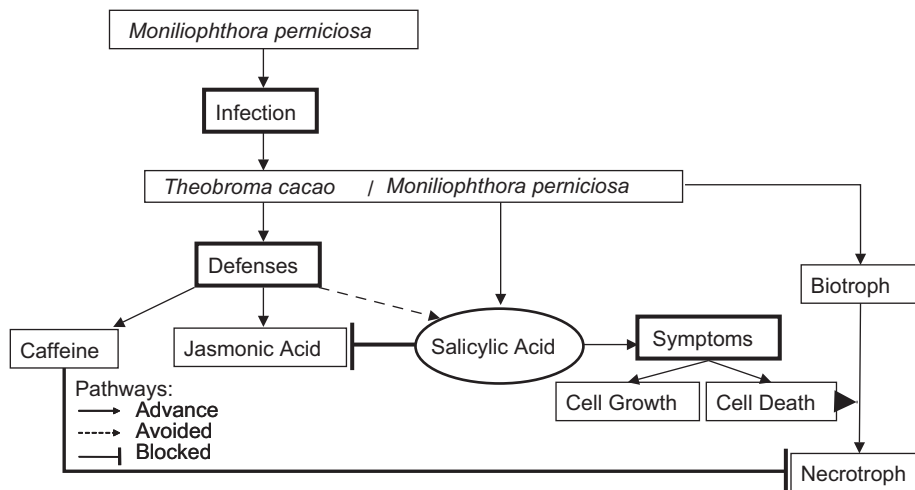


Fig. 6. Flow chart with proposed role of SA in pathogenicity.

development, the fungus may be the source of the SA, which stimulates cell growth and enlargement, producing the broom symptoms. The rising SA levels induce cell death and the brooms become necrotic. The symptoms remain localized in the young tissues due to their sensitivity to SA. When we applied high concentrations of SA to different plant parts, only flowers and young leaves showed necrosis and abnormal growth (Fig. 4). In addition, the analyzed broom tissue had SA levels 5 times higher than healthy tissue (Fig. 5). Healthy shoots had $224.2 \text{ ng SA g}^{-1}$ dry weight stem tissue, while brooms had statistically higher (F , $p < 0.01$) levels of $1212.3 \text{ ng SA g}^{-1}$ dry weight stem tissue. Although we cannot rule out the possibility that the higher levels of SA are produced by the plant, our results suggest that the fungus promotes an increase in SA levels, which may play a role in pathogenesis (Fig. 6) and provide a target for disease control.

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