

Moniliophthora perniciosa produces hormones and alters endogenous auxin and salicylic acid in infected cocoa leaves

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Introduction

The basidiomycete *Moniliophthora perniciosa* is a hemibiotroph and its biotrophic phase causes witches' broom disease (WBD) in *Theobroma cacao*, which decimates cocoa production. The biotrophic (primary) mycelium induces excessive and disorganized growth at the apices and causes the emergence of multiple shoot axes (the green brooms) from vegetative buds (Baker & Holliday, 1957) and abnormal flower development (Andebrhan, 1983). The fungus subsequently undergoes dikaryotization and the host tissues are invaded by secondary, narrower, clamped, and saprophytic mycelium that inhibits growth and causes necrosis, which may result in the death of the host. The completion of a life cycle is achieved by the production of basidiocarps on the surface of desiccated brooms and release of basidiospores (Evans, 1980).

Historically, WBD symptoms such as hypoplasia, hypertrophy, and loss of apical dominance in infected *T. cacao* were assumed to stem from hormonal imbalances (Dudman & Nichols, 1959; Krupasager & Sequeira, 1969; Evans, 1980). Pathogens can alter hormone levels in infected tissues either by releasing hormones into the host tissue or by changing the phytohormone metabolism of the host (Sequeira, 1973;

Abstract

Moniliophthora perniciosa is the causative agent of witches' broom disease in *Theobroma cacao*. Exogenously provided abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA), and salicylic acid (SA) promoted mycelial growth, suggesting the ability of the pathogen to metabolize plant hormones. ABA, IAA, JA, and SA were found endogenously in the mycelium and in the fruiting body of the pathogen. The pathogen contained high amounts of SA in the mycelium ($0.5 \pm 0.04 \mu\text{g g}^{-1}$ DW) and IAA ($2 \pm 0.6 \mu\text{g g}^{-1}$ DW) in the basidiocarps. Growth of the mycelium in the presence of host leaves for 10 days did not affect ABA or JA content of the leaves but IAA and SA increased 2.5- and 11-fold, respectively. The amounts of IAA and SA in infected leaves increased beyond the levels of the uninfected leaves and suggest a synergistic response to host-pathogen interaction. The ability of *M. perniciosa* to produce and sustain growth in the presence of elevated endogenous IAA and SA levels during colonization indicates that these phytohormones contribute to its pathogenicity.

Jameson, 2000). However, few studies were conducted to elicit the role of hormones in the development of WBD. Previous *in vitro* studies reported that the dikaryotic stage of *M. perniciosa* did not produce significant amount of cytokinins, auxins (Krupasager & Sequeira, 1969), or gibberellin-like substances (Dudman & Nichols, 1959) but produced auxin-inactivating enzymes such as IAA-oxidase and laccase (Krupasager & Sequeira, 1969). In contrast, Orchard *et al.* (1994) reported a small increase in zeatin riboside in infected brooms, which correlated with the swelling of terminal and axillary buds. Upon infection with *M. perniciosa*, an increase in ethylene before symptom development and a decline with the death of infected tissues were also reported (Scarpari *et al.*, 2005).

There is increasing evidence that hormones control developmental and physiological processes in fungi. For example, indole-3-acetic acid (IAA) induced filamentation of yeast (Prusty *et al.*, 2004) and gall/tumor formation in tissues infected by *Ustilago* (Banuett, 1995; Chung & Tzeng, 2004). Ectomycorrhizal fungi excrete auxins that affect their interactions with plants (Niemi *et al.*, 2002). Fungi are also capable of abscisic acid (ABA; Crocoll *et al.*, 1991), jasmonic acid (JA; Nakamori *et al.*, 1994), and salicylic acid (SA; Zouchova *et al.*, 1982) biosynthesis; however, their role in

development and pathogenesis is unclear. Exogenous SA has antifungal properties and reduces fungal development including hyphal growth (Amborabé *et al.*, 2002).

Furthermore, the responses of plants to pathogens include changes in hormones. ABA negatively regulates disease resistance by interfering with JA, SA, and ethylene or increases resistance by callose deposition (Mauch-Mani & Mauch, 2005). Similarly, auxins enhance host susceptibility to some pathogens by down-regulating genes involved in plant–defense responses (Dominov *et al.*, 1992). JA induces resistance to necrotrophic pathogens and herbivores (Pozo *et al.*, 2004). SA initiates systemic acquired resistance (SAR) and activates broad resistance against biotrophic pathogens in noninfected parts of the plant (Ryals *et al.*, 1996).

Collectively, these reports suggest an important role for hormones in plant–pathogen interactions. The lack of sensitive methods to quantify hormones may have limited previous attempts to correlate WBD with changes in hormones. The ability of *M. perniciosa* to grow in the presence of exogenous hormones was tested. Using GC-MS, investigation was carried out on the endogenous levels of ABA, IAA, JA, and SA in the mycelium and basidiocarps of *M. perniciosa*, the leaves of *T. cacao* in various developmental stages, and subsequently, on the temporal changes in hormones in the infected host tissue.

Materials and methods

Pathogen

Cultures of *M. perniciosa* (formerly known as *Crinipellis perniciosa*) were obtained from American type culture collection (strain 64190; permit # 53313) and grown and maintained on potato dextrose agar (PDA). Basidiocarps were obtained from *M. perniciosa*-infected *T. cacao* trees in the natural habitat. Freshly developed basidiocarps were harvested from dried broom tissues and lyophilized (gift from Dr Alan Pomella, Almirante Cacao, Itajuípe, Brazil). Dry weight of 20-day-old mycelial cultures grown on PDA and basidiocarps of *M. perniciosa* was determined and their endogenous hormones were analyzed.

To determine the effect of exogenous hormone application on the growth of *M. perniciosa*, single mycelial plugs (1 cm diameter) from the perimeter of PDA cultures were transferred to PDA supplemented with 0.1, 1, or 10 μM of ABA, IAA, JA, or SA. Because dimethyl sulfoxide (DMSO) was used as a solvent, PDA with DMSO (0.3%) served as control.

Cultures were photographed (Nikon Coolpix 4500) 3, 5, 10, and 15 day after inoculation. ImageJ (1.32j) software was used to measure the growth area. The relative growth rate was calculated as difference in the radial growth measurements between two consecutive time points.

Plant material

Seed-grown *T. cacao* plants were maintained in a greenhouse and leaves of four developmental stages were used for the experiments: (1) very young (2–3 cm), (2) young-red (6–8 cm), (3) mature-tender (< 10 cm), and (4) mature-lignified (> 10 cm). In stages (1) and (2) leaves are meristematic and grow rapidly, stage (3) is characterized by leaf expansion followed by maturation and lignification in stage (4). Freshly harvested leaves of each stage were used for dry weight and hormone quantification.

Plant–pathogen interaction

All experiments were conducted *in vitro* and cultures were maintained in a growth chamber (Percival E-54U) at 25 °C in the dark. To reduce contamination, leaves were surface sterilized with 0.1% streptomycin and 1% dithane, and rinsed in 70% ethanol (20 s), before fungal inoculation. Stage (2) leaves were inoculated with the fungus by applying four to five mycelial plugs on the abaxial surface of the leaves. The hormone levels were quantified in infected leaves 3, 5, and 10 days after inoculation. Fungal mycelium and leaves were separately maintained on PDA and used as controls.

Hormone extraction and quantification

About 1–2 g of FW of material was extracted as described previously (Wang *et al.*, 2001). Internal standards (200 ng each of D₃-ABA, ¹³C-IAA, and D₂-JA) were added to the samples before extraction in 30 mM imidazole buffer pH 7 in 70% isopropanol. The samples were extracted overnight at 4 °C and centrifuged. The supernatant was combined with three consecutive extractions (2 mL of 100% isopropanol each), in a 25 mL flask and radioactive tracers (20 000 cpm of ³H-ABA (1.96 TBq mmol⁻¹, Amersham Life Science), ³H-IAA (10 TBq mmol⁻¹, Amersham Pharmacia Biotech), ³H-JA (1.85 TBq mmol⁻¹, American Radiolabeled Chemicals Inc.), and ¹⁴C-SA (433 MBq mmol⁻¹, Sigma # S8644) were added. The organic phase was removed in a rotary evaporator and the aqueous phase was diluted and prepurified on NH₂-columns (Alltech # 211150). The columns were primed with methanol, 5% acetic acid, and water. After sample application, the columns were washed with hexane and methanol and eluted with 2 mL of 5% acetic acid in 75% MeOH followed by 2 mL of 0.3 N HCl in 75% MeOH. The eluant was dried and taken up in 36% MeOH and purified by HPLC (150 × 4.6 mm, ODS, 1 mL min⁻¹, isocratically with 1% acetic acid in 36% MeOH for 20 min and then ramped linearly in 10 min to 54% MeOH, 1% acetic acid). Under these conditions, the retention times for IAA, SA, ABA, and JA were 11, 16, 24, and 30 min, respectively.

The fractions with the highest radioactivity of each compound were combined, dried, methylated with 0.5 mL

of ethereal diazomethane, dried under N_2 , and taken up in 50 μ L of ethyl acetate. For SA, no stable isotope standard was available and the yield was determined by measuring the radioactivity of an aliquot. The methylated samples were analyzed on an HP 6890 GC equipped with an HP 5973 mass-selective detector using selected ion monitoring. Chromatography was carried out on a capillary column (ZB-5; 30 m \times 250 μ m \times 0.25 μ m, Phenomenex) with helium as carrier gas (flow rate 50 $cm\ s^{-1}$). The retention times were 3.87, 3.98, 4.23, and 5.12 min for IAA, JA, SA, and ABA, respectively. The relative abundance of representative fragments for ABA (m/z 190/193), IAA (m/z 130/136), JA (m/z 151/153), and SA (m/z 120) was used for quantification. The data were calculated in $ng\ g^{-1}$ FW or DW \pm SE of four repeats.

Statistical analysis

ANOVA (SAS PROC GLM, SAS Institute Inc., Cary, NC, USA, 1999–2001) was used to test the effects of leaf age on hormone composition. Repeated-measure ANOVA (SAS PROC GLM) was used to test the effects of exogenous hormones on fungal growth rate and the effects of fungal inoculation on endogenous ABA, IAA, JA, and SA levels in infected leaves. Paired comparisons using Tukey-adjusted least-square means were used to test for differences between individual data.

Results

Effect of exogenous hormones on fungal growth

The growth rate of *M. perniciosa* mycelia was significantly affected by ABA, IAA, JA, and SA (Fig. 1; $F_{(52)} = 11.29$;

$P < 0.0001$). Growth depended significantly on hormone concentration ($F_{(13)} = 42.25$; $P < 0.0001$) and on the duration of application ($F_{(4)} = 141.08$; $P < 0.0001$). All four hormones promoted fungal growth for 15 days compared with the control (PDA+DMSO; Fig. 1). The strongest promotion resulted from application of 0.1 μ M hormones, except for SA, which was most effective at 1 μ M ($P < 0.0001$; Fig. 1). Even at 10 μ M concentration of hormones, the growth rate increased from day 3 to day 15 with maximal growth at day 15.

Endogenous hormones in fungus

Moniliophthora perniciosa contained all examined hormones but their levels varied between the mycelium and basidiocarps (Fig. 2). The mycelium contained high SA and low auxin content. SA and JA levels in the mycelium (0.52 ± 0.04 and $0.19 \pm 0.11\ \mu$ g g^{-1} DW, respectively) did not differ significantly from those of the basidiocarps (Fig. 2). However, the auxin content in basidiocarps was 147-fold higher than in the mycelium ($2 \pm 0.55\ \mu$ g g^{-1} DW; $P < 0.0231$). ABA levels were also significantly higher in the basidiocarps ($0.088 \pm 0.01\ \mu$ g g^{-1} DW; $P < 0.0412$).

Hormones in leaves

Leaves of *T. cacao* exhibit an age-dependent hormone profile (Fig. 3; $F_{(9)} = 2.8$; $P = 0.0207$). The hormone content per g DW of stage (1) leaves was significantly higher than in all other stages (Fig. 3; $F_{(9)} = 6.98$; $P = 0.0082$). Maturation of leaves (stages 3 and 4) resulted in a significantly lower content of ABA, IAA, and SA (Fig. 3). Regardless of the stage, the hormonal profile of *T. cacao* leaves was similar. SA and ABA decreased by a factor of five from young leaves (stage 1) to maturity (stage 4). Although much lower in

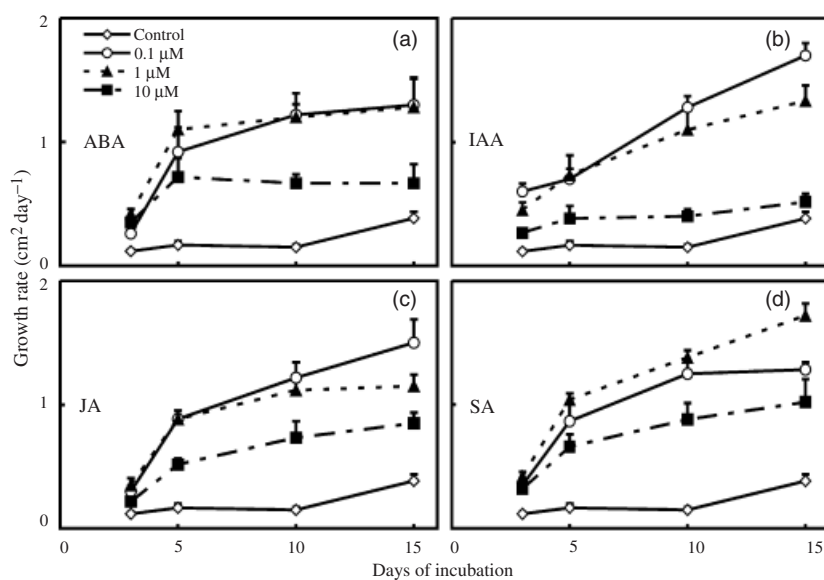


Fig. 1. Growth of *Moniliophthora perniciosa* mycelium on PDA enriched with 0.1, 1, and 10 μ M ABA (a), IAA (b), JA (c), and SA (d) in comparison with controls (0.3% DMSO). The growth rate was calculated from the difference between two consecutive time points divided by the number of days between the measurements. $N = 5 \pm$ SE.

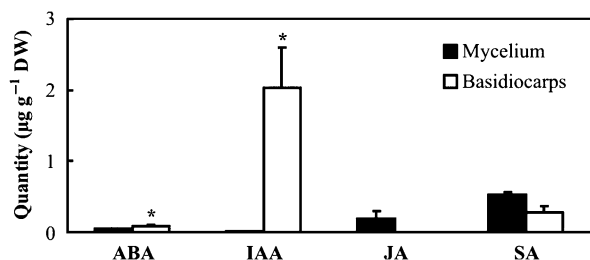


Fig. 2. The content of ABA, IAA, JA, and SA in 20 d-old mycelial cultures and basidiocarps of *Moniliophthora perniciosa*. The mycelial cultures were grown on PDA. The basidiocarps were harvested from infected *Theobroma cacao* and lyophilized before hormone analysis. $N = 4 \pm SE$. *Indicate significantly higher IAA ($P < 0.0231$) and ABA ($P < 0.0412$) levels in the basidiocarps.

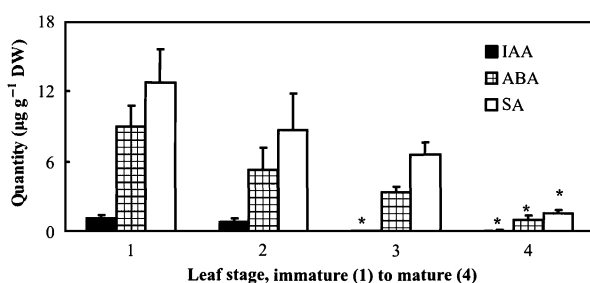


Fig. 3. Hormone content in *Theobroma cacao* leaves. Leaves of stage (1) and (2) were meristematic (2–3 and 6–8 cm long, respectively), stage (3) were maturing (> 10 cm) and lignified (stage 4). $N = 4 \pm SE$. *Indicate significant change ($P = 0.0082$) in hormone level compared with stage (1).

content, IAA was 12-fold higher in meristematic leaves ($1.2 \mu\text{g g}^{-1}$ DW, stage 1) than in mature leaves ($0.1 \mu\text{g g}^{-1}$ DW, stage 4; $P = 0.0123$; Fig. 3).

Hormonal changes during host–pathogen interaction

Hormone levels in infected leaves were quantified during the course of fungal growth and development in the host tissue. Fungal inoculation resulted in friable, necrotic, and brown leaves after 10–12 days, while the noninoculated leaves on PDA appeared healthy and showed leaf expansion (data not shown). The hormone levels changed significantly after infection ($F_{(7)} = 85.48$; $P < 0.0001$) and over time ($F_{(3)} = 3.59$; $P = 0.0202$; Fig. 4) with significant interaction between time and infection ($F_{(21)} = 3.14$; $P = 0.0005$).

Fungal inoculation on leaves did not affect ABA and JA quantities (Fig. 4a and c). The IAA levels decreased significantly over time ($F_{(3)} = 37.77$; $P < 0.001$) and the pattern of changes in IAA was initially similar between control and infected leaves. Five days after incubation, the IAA levels reached a minimum but increased strongly by 10 days such that inoculated leaves contained twice the amount of the controls ($F_{(1)} = 148.8$; $P < 0.001$, Fig. 4b). SA accumulation

in *T. cacao* leaves was significantly affected by time ($F_{(3)} = 21.7$; $P = 0.004$) and pathogen inoculation ($F_{(1)} = 12.9$; $P = 0.012$; Fig. 4d). SA levels increased significantly 10 days after infection ($P = 0.001$; Fig. 4d) and preceded leaf necrosis.

Discussion

The presence of four examined hormones ABA, IAA, JA, and SA in *M. perniciosa* indicates that they can be metabolized and thus are likely to influence growth and development (Figs 1 and 2) and the pathogen's interaction with the host, *T. cacao* (Fig. 4). The effect of these hormones on development and interaction of the fungus with *T. cacao* pertains to the ability of the pathogen to cope with the hormonal environment imposed by the host and to alter either the metabolism or ratio of these hormones to its advantage.

Fungal growth and development

Knowledge on the role of hormones in fungal growth, development, and differentiation is very limited. Few examples, such as the appressorium formation by spores of *Colletotrichum gloeosporioides* in response to ethylene (Kolattukudy *et al.*, 1995) and differentiation of haploid invasive growth and diploid filamentation in yeast, in response to IAA (Prusty *et al.*, 2004), suggest that both host perception and invasion are influenced by hormones. Similarly, the IAA antagonist hypaphorine from the basidiomycete *Pisolithus tinctorius* induced symbiosis-related differentiation and cytoskeletal reorganization in eucalyptus (Ditengou *et al.*, 2003). Auxin is not required for the maintenance of biotrophic phase of *M. perniciosa* (Meinhardt *et al.*, 2006). However, it remains to be established if the high levels of hormones in young leaves of *T. cacao* (Fig. 3), specifically IAA and SA, may facilitate *M. perniciosa* colonization and possibly the transition from the monokaryotic-biotrophic to the dikaryotic-saprophytic phase (Kilaru & Hasenstein, 2005). Sustained mycelial growth in the presence of high auxin levels (Fig. 1) and high auxin levels in the basidiocarps (Fig. 2) suggest that auxin may facilitate the phase transition and development of *M. perniciosa*.

Plant–fungal interactions

Plant–fungal interactions involve mutual recognition and exchange of chemical cues, including hormones. Although host resistance to biotrophs includes SA and the response to necrotrophs involves JA and ethylene (Glazebrook, 2005), the hormonal response to hemibiotrophic pathogens, such as *M. perniciosa*, is unknown.

Understanding the nature of hemibiotrophic pathogens is limited by the ability to maintain their distinctive phases in culture. Previous *in vitro* studies showed that the host tissue

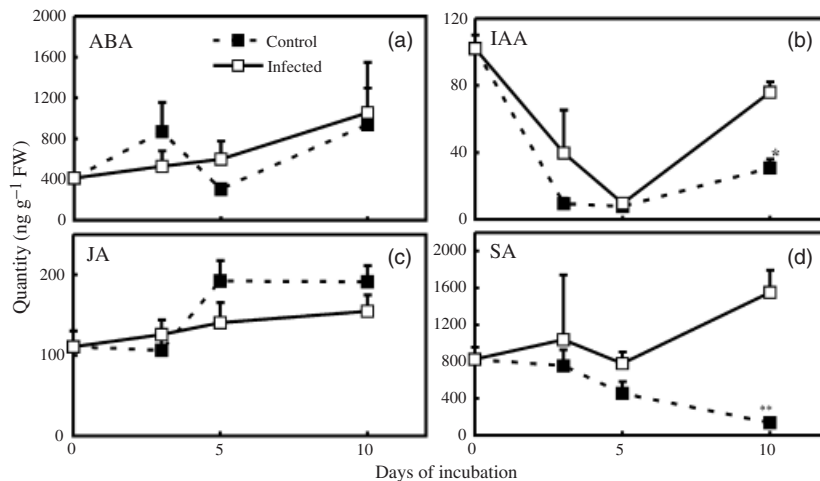


Fig. 4. Temporal profile of phytohormones ABA (a), IAA (b), JA (c), and SA (d) in *Theobroma cacao* leaves (stage (2), ~6–8 cm long) after infection with mycelium of *Monilophthora perniciosa*. $N = 4 \pm SE$. *Indicate significant change in hormone quantities between controls and infected leaves. * $P = 0.0012$; ** $P < 0.001$.

is necessary for phase transition in *M. perniciosa* and that the biotrophic phase can be maintained on young cacao leaves for 10 days before the mycelium showed clamp formation (Kilaru & Hasenstein, 2005). Therefore, the hormonal changes noted in the infected leaves of cacao are indicative of changes induced by the biotrophic phase of the fungus (Fig. 4). Although it is typically the biotrophic phase that is responsible for inducing the disease symptoms and thus more relevant for investigations, it is equally important to understand the subsequent factors that are responsible for the death of the host tissue, often induced by the saprophytic phase. Evans (1980) suggested an auxin-inducing primary phase that causes malformations in *T. cacao* and an auxin-depleting secondary phase that kills the host. *In vitro* studies of the authors show that the increase in auxin coincides with the phase transition after 10 days (Kilaru & Hasenstein, 2005).

Quantification of endogenous hormones in the host leaves at various developmental stages showed that the meristematic leaves (stages 1 and 2) had higher hormone content than mature leaves (stages 3 and 4; Fig. 3). Because *M. perniciosa* infects meristematic tissues of cacao, the fungus seems to benefit or at least is capable of growing in the presence of high quantities of phytohormones. This statement is further supported by the fungus' ability to grow on hormone-enriched media (Fig. 1). The observation of continued mycelial growth on stage (2) leaves suggests the ability of the fungus to tolerate high doses of SA. However, as the interaction between *Theobroma* and *Moniliphthora* progressed, the hormonal content in the host changed (Fig. 4). The most significant changes were observed 10 days after infection where four- and 10-fold higher IAA and SA levels in infected leaves (Fig. 4b and d) suggest a response to the pathogen. Thus far, only auxins have been reported to facilitate invasive fungal growth (Prusty *et al.*, 2004).

Auxin is also related to disease development in *T. cacao*. The increase of IAA in infected cacao leaves (Fig. 4b) could

stem from enhanced IAA biosynthesis, suppression of IAA oxidase activity (Chung & Tzeng, 2004), or excretion of IAA from the pathogen into the host (Niemi *et al.*, 2002). Similar to IAA-elicited tumor formations by *Ustilago maydis* (Banuett, 1995), elevated levels of IAA may result in hyperplasia and hypertrophy of infected cocoa tissue as reported in *in vitro* (Kilaru & Hasenstein, 2005) and *in vivo* (Dabydeen & Sreenivasan, 1989). Abscission of cotyledons depends on the vigor and hormone content of cotyledons, and the delayed abscission after *M. perniciosa* infection suggests that the pathogen affects the hormonal content (Motilal *et al.*, 2003). For example, in *T. cacao* ABA and IAA were shown to delay flower abscission and senescence (Baker *et al.*, 1997) and promote fruit development (Hasenstein & Zavada, 2001). Thus, the increase in IAA after infection may be related to the development of disease symptoms in the host and phase transition in the pathogen.

The occurrence of high SA levels in certain plants has a variety of functions. For example, SA in shoots ($\sim 9 \mu\text{g g}^{-1}$ FW) mediates hyperaccumulation of nickel in *Thlaspi* species (Freeman *et al.*, 2005); similarly, the inflorescence of *Sauromatum guttatum* with $\sim 3 \mu\text{g g}^{-1}$ FW of SA has thermogenic properties (Raskin *et al.*, 1989). The high levels of SA in host and pathogen (Figs 2 and 3) are unexpected because plants typically respond to pathogen attack with enhanced SA production (Ryals *et al.*, 1996) and a role for SA ($\sim 2.5 \mu\text{g g}^{-1}$ FW) in immature leaves of noninfected *T. cacao* has not yet been demonstrated. Although SA and its derivatives are common in antibiotic-producing fungi such as *Crinipellis* (Kupka *et al.*, 1979) and *Oudemansiella mucida* (Zouchova *et al.*, 1982), physiological roles for SA in fungi are unknown. The ability of *M. perniciosa* to produce (Fig. 2) and benefit from SA (Fig. 1) suggests a high tolerance to SA levels of the host (Fig. 4).

The increase in SA after infection (Fig. 4d) suggests that SA induces necrosis in the host tissue similar to SA-induced

senescence (Morris *et al.*, 2000) or cell death (Brodersen *et al.*, 2005), which initiates the transition to the necrotrophic phase of the pathogen. In addition to increase in SA, infections often lead to an increase in JA (Poza *et al.*, 2004), peroxidase, and chitinase B in stage 2 leaves of *T. cacao* (Bailey *et al.*, 2005), and ethylene in infected brooms (Scarpari *et al.*, 2005). Although SA and JA are typically associated with plant-defense reactions, presence of these compounds in the fungus itself (Fig. 2) and enhanced fungal growth in the presence of external SA and JA (Fig. 1c and d) suggest that *M. perniciosus* invasion may not be inhibited by these compounds. Rather, the pathogen tolerates or thrives on these compounds. The lack of significant changes in JA levels during *T. cacao*–*M. perniciosus* interaction suggests that the host response is JA-independent.

This study shows that *M. perniciosus* produces hormones and that its concentrations vary with the developmental stage and that the pathogen causes hormonal changes in *T. cacao* upon interaction. These results suggest that IAA and SA metabolism affect *M. perniciosus*. Further information on changes in other phytohormones as well as auxin antagonists are bound to further characterize the specificities of this host–pathogen interaction.

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