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# Carbon source-induced changes in the physiology of the cacao pathogen *Moniliophthora perniciosa* (Basidiomycetes) affect mycelial morphology and secretion of necrosis-inducing proteins

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**ABSTRACT.** Quantitative and qualitative relationships were found between secreted proteins and their activity, and the hyphal morphology of *Moniliophthora perniciosa*, the causal agent of witches' broom disease in *Theobroma cacao*. This fungus was grown on fermentable and non-fermentable carbon sources; significant differences in mycelial morphology were observed and correlated with the carbon source. A biological assay performed with *Nicotiana tabacum* leaves revealed that the necrosis-related activity of extracellular fungal proteins also differed with carbon source. There were clear differences in the type and quantity of the secreted proteins. In addition, the expression of the cacao molecular chaperone BiP increased after treatment with secreted proteins, suggesting a physiological response to the fungus secretome.

 

We suggest that the carbon source-dependent energy metabolism of *M. perniciosa* results in physiological alterations in protein expression and secretion; these may affect not only *M. perniciosa* growth, but also its ability to express pathogenicity proteins.

**Key words:** Protein secretion; Virulence; Enzyme activity; Hyphal morphology; Molecular chaperones

## INTRODUCTION

Fungal secreted proteins (secretome) are a known key feature during the plant-microbe interaction, since they manipulate host signaling and metabolic pathways in order to provide a suitable environment for completion of the pathogen's life cycle (Hegedus and Rimmer, 2005; Kamoun, 2007). The broad spectrum of secreted proteins in a fungus-plant interaction ranges from structural proteins to enzymes, the majority being hydrolytic enzymes that enable the fungus to penetrate and infect host tissue (Knogge, 1996; Hegedous and Rimmer, 2005). Also, these secreted proteins can act as elicitors of host defense or, depending on the stage of the fungal life cycle, can be involved in the acquisition of nutrients (Feng et al., 2005; Kamoun, 2007).

Nutrient utilization pathways increase metabolic versatility and enable fungi to use a variety of complex compounds as nutrient sources (Keller and Hohn, 1997; Medina et al., 2004). During infection, the nutritional offer of the plant to a phytopathogenic fungus may change, e.g., during *Moniliophthora perniciosa* x *Theobroma cacao* interaction there is a clear correlation between fungal differentiation and plant responses (Evans and Prior, 1987; Frias et al., 1991), confirmed by biochemical and nutritional alterations that occur during *in vivo* infection (Orchard et al., 1994; Scarpari et al., 2005; Kilaru and Hasenstein, 2005; Kilaru et al., 2007).

The phytopathogen *Moniliophthora* (=*Crinipellis*) *perniciosa* (Stahel) Aime & Phillips-Mora is a hemibiotrophic basidiomycete that infects cacao (*Theobroma cacao* L.) in all producing countries of the Americas and Caribbean Islands, causing witches' broom disease (WBD). Interactions in this pathosystem are complex and include sequential alterations or differentiation in the fungus (germination, penetration, colonization, dikaryotization, and fructification; Silva et al., 2002; Kilaru and Hasenstein, 2005) and in the plant (hypertrophy, hyperplasia, branching, activation of defense responses, and cell death; Orchard et al., 1994; Scarpari et al., 2005; Ceita et al., 2007). Recently, in-depth studies of this fungus have been published: *M. perniciosa* was found to produce hormones and alter endogenous auxin and salicylic acid levels in infected cocoa leaves (Kilaru et al., 2007), as well as necrosis-inducing proteins during infection, i.e., *Mp*-NeP1 and *Mp*-NeP2, which are differentially secreted during the course of infection *in planta* (Garcia et al., 2007); Rincones et al. (2008) found that genes expressed in the biotrophic phase of *M. perniciosa* are under catabolite and nitrogen repression. Glycerol seems to play several roles in *M. perniciosa* biology; Santos et al. (2008) showed that *M. perniciosa's* resistance to stress depends on glycerol, and Pungartnik et al. (2009) showed that small amounts of hydrogen peroxide in glycerol medium induce the formation of clamp connections *in vitro* and that basidiospores are the most resistant life form of this fungus when compared to mono- and dikaryotic cells.

In this study, we evaluated the relationship between the general carbon metabolism of the dikaryotic life form of *M. perniciosa* with fungal morphology, the composition of its

 

secretome and the secretome's ability to induce plant cell death. While mycelial morphology seemed to be related to general energy metabolism, the secretory activity and secretome composition varied depending on the specific carbon source provided. Moreover, there was enhanced cell death in the plant by secretome activity when mycelia had been grown in glycerol.

## MATERIAL AND METHODS

### *Moniliophthora perniciosa* culture and growth conditions

*Moniliophthora perniciosa* isolate 553 was obtained from the CEPLAC/CEPEC (Cacao Research Center, Ilhéus, BA, Brazil) culture collection. Dikaryotic mycelia were grown on solid and in liquid mineral media (0.1% ammonium phosphate, 0.02% potassium chloride, 0.02% magnesium sulfate, 0.5% yeast extract, 0.01% copper sulfate, and 0.01% zinc sulfate) supplemented with 20 mM of one of the following carbon sources: glucose (GLU), glycerol (GLY), mannitol, galactose, fructose, mannose, or sucrose. We also tested the addition of two different carbon sources to the mineral medium (GLU + GLY, GLU + galactose, GLU + mannitol, GLU + sucrose; 10 mM of each carbon source) and mineral medium with no addition of carbon source. Mycelia were grown in the dark, at 25°C for 4, 7, 10, or 14 days, with no shaking.

### Monitoring growth of *M. perniciosa* and microscopic analysis

Fungal growth on solid media was determined as the diameter of the mycelium derived from one mycelium disc of 1 cm, after 10 days. The biomass of mycelium grown in liquid was determined by isolation via filtration; the mycelium was freeze-dried (Labconco) for 48 h and then weighed. Results are reported as dry weight.

Light microscope analyses were performed of apical tips from 4- or 7-day-old *M. perniciosa* cultures. Photographs were taken of either fresh hyphae (liquid culture) visualized with a Leica microscope (DM RA2, Germany, 100X) or apical tips (solid medium) with an Olympus microscope (DX40, phase contrast, 40X).

### Protein quantification of secretome and SDS-PAGE

The secretome (30 mL) was separated from 10-day-old mycelium of *M. perniciosa* liquid cultures by filtration (0.45 µm, Millipore), freeze-dried (Labconco, USA) for 48 h, and resuspended in 5 mL phosphate buffer (PB, 10 mM, pH 5.5). Protein quantification was performed with a 2-D quantification kit, according to manufacturer recommendations (GE Healthcare). Proteins were resolved on SDS-gels (12.5%) and visualized after staining with Colloidal Coomassie G-250 solution (Neuhoff et al., 1988).

### Biological activity assay of secretome

#### *Peroxidase activity*

From each secretome of *M. perniciosa*, 2.5 µg extracellular proteins was used. Samples were added to an ELISA plate and mixed (1:1, v/v) with a peroxidase activity buffer (50 mM

acetate buffer, $H_2O_2$ and guaiacol). Plates were immediately read on a kinetic dosage program (4 reads with 30-s intervals), at 570 nm, on a microplate reader (Molecular Device). Peroxidase activity was indicated by the consumed guaiacol/min (nmol guaiacol·mg protein$^{-1}$·min$^{-1}$).

### *Infiltration in tobacco leaves*

Secretome from a 14-day-old *M. perniciosa* culture was desalted in a PD-10 Sephadex™ G-25M column (GE Healthcare), and proteins were recovered in PB (10 mM, pH 6.0), quantified using the Bradford assay (Bradford, 1976), and adjusted to a final concentration of 0.5 mg/mL (w/v). One hundred microliters of each protein suspension obtained was allowed to infiltrate *N. tabacum* (variety Havana) leaves. Controls were infiltrated with PB and also with sterile medium. The plants were maintained in a greenhouse and visually evaluated daily until necrotic symptoms were detected (up to 7 days). Images were acquired with a loupe, at 10X magnification (EZ4 D, Leica, Germany).

### *T. cacao* meristem infiltration and induction of binding protein

Total proteins were isolated from a mix of 20 *T. cacao* meristems, 3 days after infiltration with either a GLY-grown *M. perniciosa* secretome, *Mp*-NeP2 (*M. perniciosa* necrosis-inducing protein; Garcia et al., 2007), PB (10 mM, pH 5.5) or a suspension of *M. perniciosa* basidiospores. The meristems were frozen in liquid nitrogen and immediately submitted to protein extraction according to Pirovani et al. (2008). Precipitated protein was resuspended in sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM dithiothreitol). Protein concentration was determined using a 2-D quantification kit according to manufacturer recommendations (GE Healthcare).

Equivalent amounts of *T. cacao* meristematic proteins (20 μg) were resolved with SDS-PAGE (12.5%) and transferred to nitrocellulose membranes using the Mini-Protean blot apparatus II XI cell (Bio-Rad) according to manufacturer recommendations. BiP (binding protein) was detected with a polyclonal antibody at a 1:1000 dilution (Figueiredo et al., 1997) followed by an anti-rabbit IgG antibody conjugated to alkaline phosphatase at a 1:5000 dilution (Promega). The activity of alkaline phosphatase was assayed using 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium (Promega) as recommended by the manufacturer.

### Statistical analyses

All results are the means of at least 3 independent experiments and error bars represent standard deviation; when necessary, data were analyzed using two-way ANOVA and the Tukey test for analysis of variance as calculated by the Instat program® and set at $P < 0.01$.

## RESULTS

*M. perniciosa* growth, determined as the mycelial radial growth on solid media, was impaired when fungi grew with mannitol as carbon source or in the absence of carbon. In comparison, GLY, mannose and fructose accelerated *M. perniciosa* growth. The highest growth rate was observed when *M. perniciosa* was grown with galactose, GLU or a mixture of GLU

and GLY (Figure 1A). Biomass production of *M. perniciosa*, determined as mycelium dry weight, was smaller in all non-fermentable, in comparison to fermentable carbon sources (Figure 1B). Growth on medium with no carbon was lowest and growth on GLU, fructose and mannose produced the highest *M. perniciosa* biomass. Moreover, growth on a mixture of GLU and GLY resulted in *M. perniciosa* biomass increment, in comparison to GLY alone.

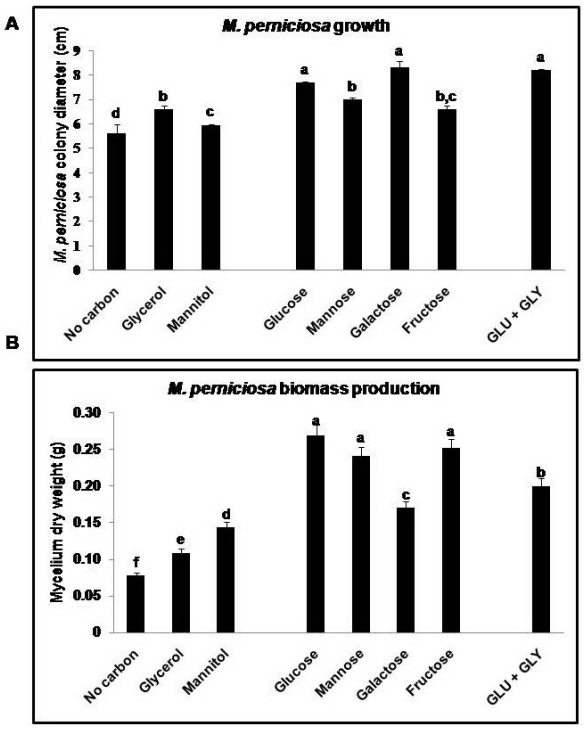


**Figure 1.** *Moniliophthora perniciosa* growth and biomass production is modified depending on the carbon source. Growth on a non-fermentable or with no carbon source impairs *M. perniciosa* growth (A) and biomass production (B) in comparison to growth on fermentable carbon source. Graphs are representative of 3 biological experiments, 10 days of growth. GLU = glucose; GLY = glycerol. Same letters do not differ statistically.

Growth on solid media on Petri dishes revealed an initial difference in *M. perniciosa* mycelial phenotype that persisted throughout the period of observation (Figure 2). Typically, growth with fermentable carbon sources, such as GLU, mannose and fructose, induced a more compact (C)-mycelium, in comparison to growth on non-fermentable carbon sources. C-mycelium is dense, shows high adherence between hyphae and has low amount of aerial hyphae. In contrast, growth on a non-fermentable carbon source, such as GLY or mannitol, or without added carbon source induced a flocculent (FC)-mycelium phenotype. Characteristically, hyphae were more dispersed as they did not have much adherence to each other or to the solid culture medium. Besides that, aerial hyphae were abundant in this growth type. Galactose and sucrose were the only fermentable carbon sources tested that induced the FC-mycelium phenotype (Table 1). Moreover, *M. perniciosa* grown in the presence of a mixture of GLU

and GLY, as well as GLU and galactose, GLU and mannitol or GLU and sucrose, showed a C-mycelium phenotype, the same observed when growth was in GLU alone (Table 1).

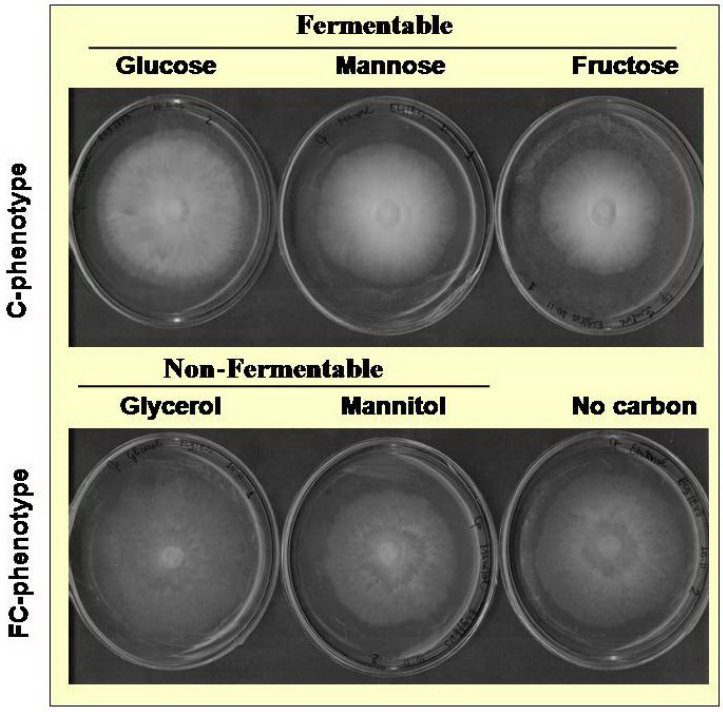


**Figure 2.** Carbon source affects *Moniliophthora perniciosa* mycelial phenotype. Fermentable carbon source induced a more compact *M. perniciosa* growth pattern (C-phenotype) whereas non-fermentable or no additional carbon source (No carbon) induced a less compact and more aerial (flocculent) mycelial phenotype (FC-phenotype). Photographs are representative of 10 replications, 10 days of growth.

**Table 1.** Carbon sources induce differences in *Moniliophthora perniciosa* mycelium phenotype.

| Carbon source | C | FC |
|---|---|---|
| No carbon | | + |
| Non-fermentable | | |
| GLY | | + |
| Mannitol | | + |
| Fermentable | | |
| GLU | + | |
| Mannose | + | |
| Galactose | | + |
| Fructose | + | |
| Sucrose | | + |
| Fermentable + non-fermentable | | |
| GLU + GLY | + | |
| GLU + mannitol | + | |
| Fermentable + fermentable | | |
| GLU + galactose | + | |
| GLU + sucrose | + | |

Depending on the carbon source provided in culture medium, *M. perniciosa* developed a compact mycelium phenotype (C) or a flocculent mycelium phenotype (FC). GLY = glycerol; GLU = glucose.

Microscopic evaluation revealed that *M. perniciosa* FC-mycelium phenotype has thin and branched hyphae (Figure 3A, C, and E). In comparison, C-mycelium showed thicker and less branched hyphae (Figure 3B, D, F, and H). However, the fermentable carbon source galactose that induced the FC-mycelium produced thick hyphae (but less dense) like those observed for the C-mycelium phenotype (Figure 3G). A microscopic evaluation of *M. perniciosa* mycelium apical tips revealed that there is translocation of cell components from older to younger hyphae in all non-fermentable carbon source phenotypes, including galactose (Figure 4).

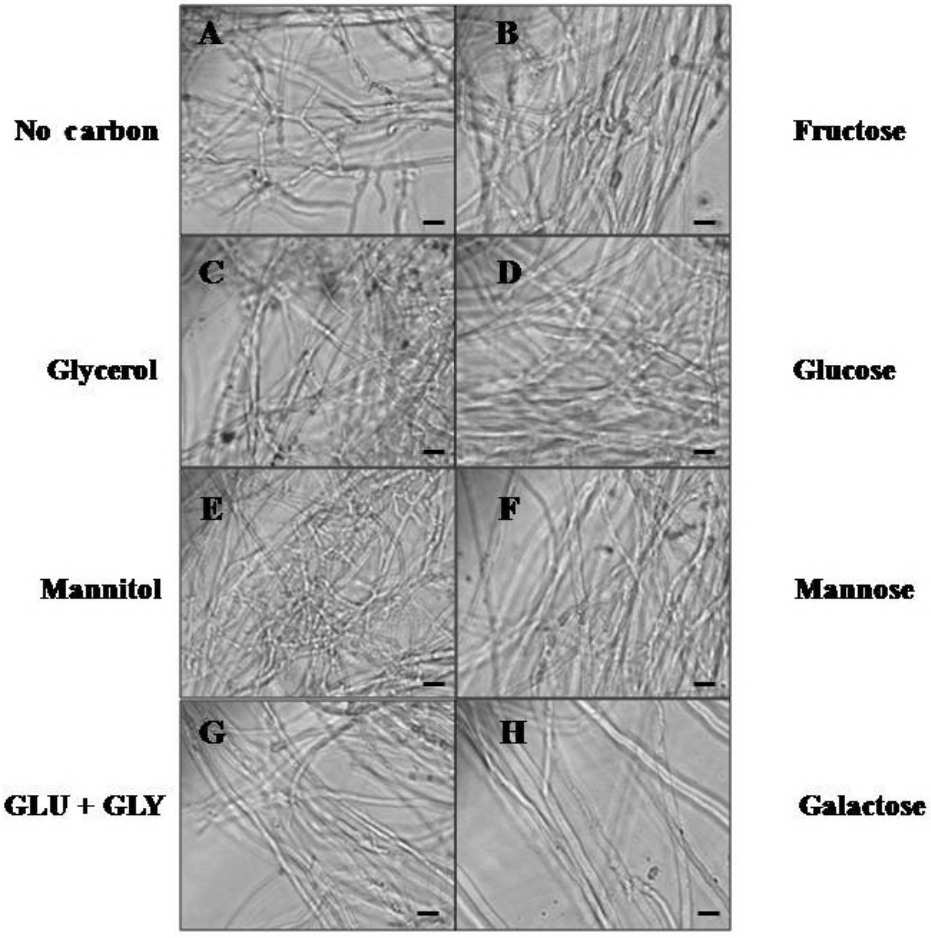


**Figure 3.** Hyphae of *Moniliophthora perniciosa* on non-fermentable carbon sources were thinner and highly branched. *M. perniciosa* grown with no additional carbon source (A) or non-fermentable carbon (C, E) showed thinner and more branched hyphae. Growth on fermentable carbon source (B, D, F) induced a thicker and more compact mycelium. In the presence of a fermentable + non-fermentable (G) carbon source, morphology was similar to the observed on fermentable growth. Galactose (H) induced a thick but less dense mycelium. Photographs are representative of 3 biological replications, 4 days of growth. GLU = glucose; GLY = glycerol. Bars = 10 µm.

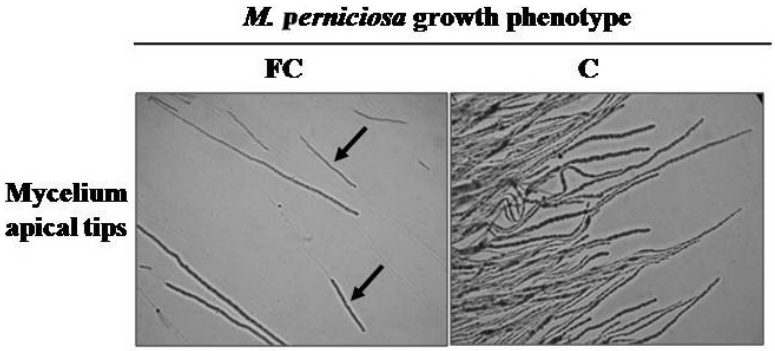


**Figure 4.** Remobilization of hyphal cellular components. Microscopic evaluation of *Moniliophthora perniciosa* mycelium revealed a translocation of cellular components from older to apical hyphae in flocculent (FC)-mycelium phenotype (arrows on left photograph). Compact (C)-mycelium were all filled (right). Photographs are representative of 3 independent biological replications, 4 days after inoculation. 40X magnification.

The offered carbon source also influenced *M. perniciosa* secretory activity, judged by the amount of secreted protein per gram of dry mycelium. Growth on medium with no carbon source other than yeast extract (no carbon) induced the highest secretory activity, in comparison to medium with the addition of a carbon source. Of all carbon sources tested, mannitol was the most efficient in inducing secretory activity (Figure 5A). The

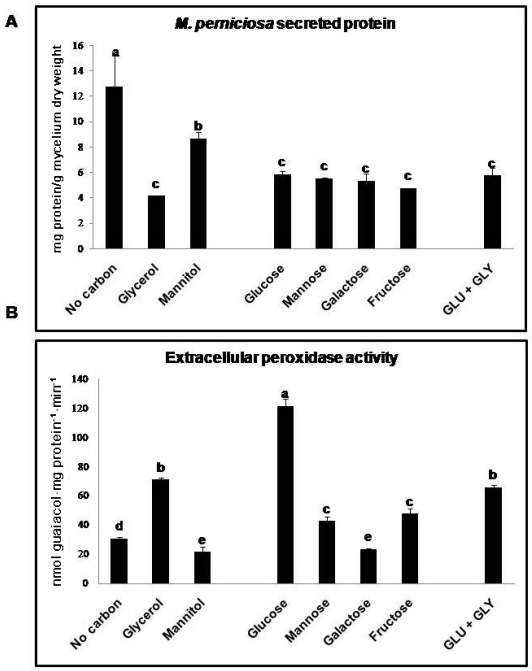


**Figure 5.** Carbon source affects *Moniliophthora perniciosa* secretory activity and secretome composition. Ten days after inoculation, *M. perniciosa* secretome was analyzed for extracellular protein (A) and peroxidase activity (B). Graphs are representative of 3 independent biological replications. GLU = glucose; GLY = glycerol. Same letters do not differ statistically.

addition of GLU to the mannitol carbon supplement resulted in the same lower secretory activity as observed when GLU was the only carbon source (data not shown). In contrast to secretome induction by mannitol, all other carbon sources resulted in a lower *M. perniciosa* secretory activity with no significant difference between them.

Extracellular proteins of *M. perniciosa* were resolved on SDS-PAGE gels in order to investigate possible changes in the composition of secretory proteins derived from growth in different carbon sources. Multiple protein bands were visualized in all secretomes derived from different growth conditions. The size of most of the proteins ranged from 60 to 20 kDa. We could identify changes in many protein bands according to the carbon source provided in the culture medium (Figure 6). We further conducted an activity assay of secreted enzymes to establish that modifications in the composition of the secretome's crude extracellular protein extract depend on the carbon source of the growth media. Extracellular peroxidase activity was quantified and indeed showed significant carbon source-dependent variation. Among the samples, secretome from GLU-grown *M. perniciosa* mycelia had the highest extracellular peroxidase activity, whereas the peroxidase activities from galactose- and mannitol-grown mycelia were the lowest. Secretome isolated from GLU + GLY-grown hyphae showed the same peroxidase activity as the one isolated from GLY-grown hyphae.

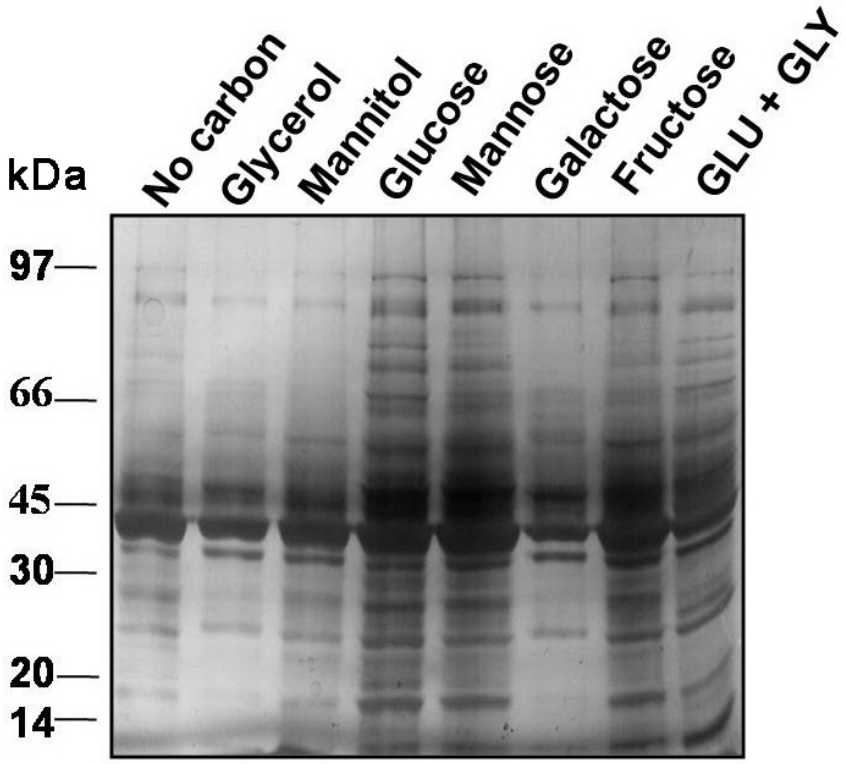


**Figure 6.** Different carbon sources affect *Moniliophthora perniciosa* extracellular protein profile. *M. perniciosa* secreted proteins were resolved on SDS-PAGE (12.5%) and visualized after Coomassie blue staining. Molecular weight marker (kDa) and carbon sources are indicated. GLU = glucose; GLY = glycerol.

We also evaluated *M. perniciosa* secretome ability to promote cell death in a biological activity assay in *Nicotiana tabacum*. All secretomes, independent of the carbon source used during fungal growth, were capable of inducing leaf necrosis. However, secretome of GLY-grown *M. perniciosa* was the most potent, as it caused the appearance of necrosis within five days (Figure 7), whereas all other secretomes required ten days.

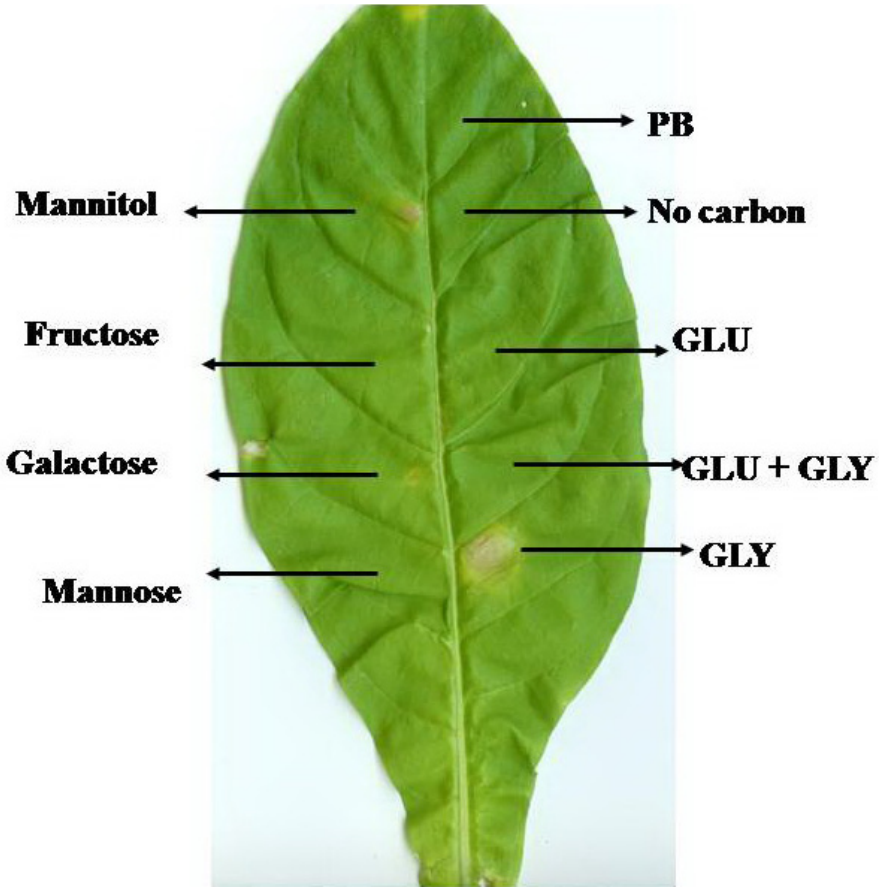


**Figure 7.** Necrosis symptoms induced by *Moniliophthora perniciosa* secreted proteins in *Nicotiana tabacum*. *M. perniciosa* was grown for 14 days with different carbon sources. Secreted proteins were desalted and recovered in phosphate buffer (PB). Same amounts of proteins were placed on *N. tabacum* leaves. Appearance of necrosis was observed daily. Photographs were taken 7 days after infiltration. GLU = glucose; GLY = glycerol.

An immunoblot assay performed with equal amounts of cacao proteins revealed that extracellular proteins of GLY-grown *M. perniciosa* secretome could up-regulate the expression of BiP in infiltrated cacao meristem (Figure 8). BiP levels also increased in response to *Mp*Nep2 (Garcia et al., 2007) infiltration and *M. perniciosa* spore germination. However, the latter two applications were less effective as compared to the proteins from the secretome of GLY-grown fungus (Figure 8).

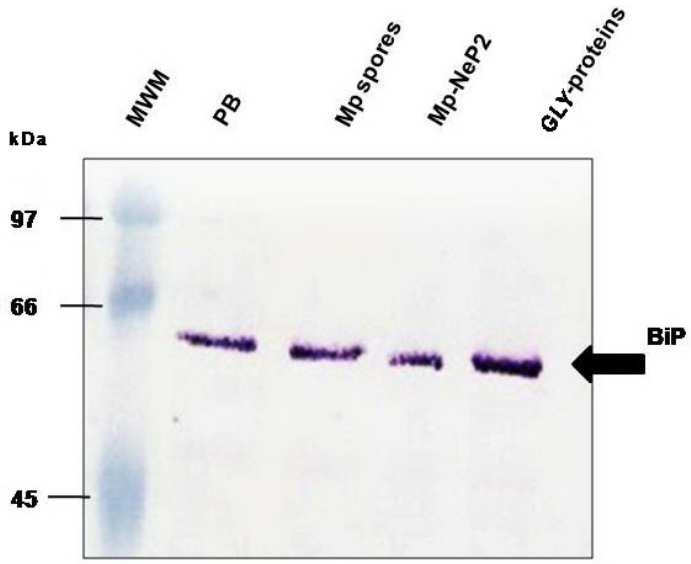


**Figure 8.** Induction of binding protein (BiP) in infiltrated meristems of *Theobroma cacao* by glycerol-grown *Moniliophthora perniciosa* secretome. Equivalent amount of total protein (20 µg per lane) extracted from *T. cacao* (TSH 1188) meristems infiltrated with 10 mM phosphate buffer (PB), *M. perniciosa* spores (Mp spores), *Mp*-NeP2 and glycerol-grown *M.* perniciosa extracellular proteins (GLY-protein) were fractionated by 12.5% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-soybean BiP antibody (1:5000), revealing different levels of BiP. Protein was extracted 4 days after infiltration. Pre-stained molecular weight marker (MWM) is indicated on the left in kDa.

## DISCUSSION

Carbon source induces differences in *M. perniciosa* growth, mycelium phenotype and hyphal morphology.

Microorganisms are often exposed to a broad range of variations, especially regarding the quality and availability of nutrients, which influence the progression of their life cycle, alter physiological and morphological patterns, and may influence the profile of secreted proteins (Thevelein et al., 2000; Oliver et al., 2002; Medina et al., 2004; Vanoni et al., 2005; Bouws et al., 2008). In yeast, the correlation of nutrient deprivation and these responses is well described. Usually, growth with GLU, or a related rapidly fermentable sugar, stimulates rapid growth and the development of larger cells (Thevelein et al., 2000; Vanoni et al., 2005). In the filamentous fungus *M. perniciosa*, rapid growth judged by biomass production was observed when mycelia grew in the presence of GLU or similar fermentable sugars, e.g., fructose and mannose, which enter the glycolytic pathway directly (Figure 1B). Fermentable carbon sources that are not promptly used by the cell, such as galactose, a non-cellulosic polysaccharide that is part of the plant cell wall structure, hindered fungal biomass production (Figure 1B). Besides growth, the facility in metabolizing different carbon sources also seems to affect *M. perniciosa* morphology. Growth in the absence of a carbon source, with non-fermentable carbon sources

or with galactose, all representing nutritional limitations in comparison to the addition of GLU, induced the development of branched and thinner mycelia; it also showed the translocation of components between hyphae (Figures 2, 3, and 4), a characteristic already reported for other fungi grown under nutritional limitation (Shoji et al., 2006). This phenotype correlates with a smaller biomass production and, with the exception of galactose, is related to a slower radial growth of mycelia (Figures 1A,B and 2). Probably, it is the intensive branching of mycelia induced by non-fermentable carbon sources that led to the observed slower growth (Figures 1A, and 2). The addition of GLU to the medium containing GLY (Figure 3), mannitol, sucrose or galactose (Table 1) reversed the mycelial phenotype, indicating that GLU is the preferentially used carbon source of this filamentous fungus. Since only 10 mM of this sugar was sufficient to reverse the mycelial phenotype, GLU may also act as a signal molecule.

The differences in *M. perniciosa* morphology were not related to intense nutritional limitation stress, as yeast extract, present in all culture media, contains many components including carbohydrates. In addition, growth with sucrose produced almost the same biomass as that with GLU (data not shown) but induced the non-fermentable carbon source phenotype (Table 1). Taken together, these data reinforce the hypothesis that mycelial morphology is not directly related to a nutritional stress situation but more likely to a cell sensing machinery that triggers pathways related to morphological changes. Indeed, in *S. cerevisiae*, GLU and galactose are sensed differently (Brown et al., 2009). Moreover, galactose inhibited the expression of genes that are commonly induced by GLU, mannose and fructose (e.g., hexose transporters; Brown et al., 2009). Carbon sources are well-known biochemical signals in other fungi (Gupta and Kaur, 2005), and special attention has been given to GLU, usually a preferred metabolized sugar (Verstrepen et al., 2004; Thevelein et al., 2005; Vanoni et al., 2005; Gancedo, 2008). The correlation of GLU with known signal cascades involved in fungal morphology and pathogenicity has been previously described for yeast (Vanoni et al., 2005; Santangelo, 2006; Cipollina et al., 2008) and for filamentous fungi (Oliver et al., 2002; Lee et al., 2003). In WBD, carbohydrate content undergoes alterations in *T. cacao* (Scarpari et al., 2005); hence, this may influence the life cycle and pathogenesis of *M. perniciosa*.

We further determined if the carbon source induces changes in *M. perniciosa* secretome composition. This has been described for other fungi that especially modify the set of secreted enzymes to adapt their metabolism to varying carbon sources (Bouws et al., 2008). In a first attempt, we analyzed the secreted protein profile on SDS-PAGE and detected some differences in specific protein bands (Figure 6). To better characterize and quantify the modification induced by different carbon sources in extracellular protein composition we performed a peroxidase activity assay. The production of this enzyme in *Coprinus* species is known to be affected by GLU concentration (Ikehata et al., 2004). The GLU-grown secretome of *M. perniciosa* exhibited the highest peroxidase activity (Figure 5B), and this activity declined with the depletion of GLU, as suggested by the results found in GLU + GLY-derived secretome (where GLU was only 10 mM). Taken together, the results reveal that carbon source indeed influenced the extracellular protein composition; however, in the particular case of peroxidase, enzyme activity was not correlated with general energy metabolism but it was influenced by the concentration of GLU in the medium.

## Carbon source affects *M. perniciosa* secretory activity, secretome composition and ability to induce plant cell death

Beside protein composition, carbon source also influenced *M. perniciosa* secretory

capacity, as observed by protein quantification (Figure 5A). Nutrient deprivation, such as in the absence of carbon source or presence of mannitol, a hexose that is not produced in the plant and is usually used by fungi as a carbohydrate reserve (Solomon et al., 2007), induced the highest protein secretion in comparison to all other carbon sources tested. This was not related to a low specific growth rate, as in *Trichoderma reesei* (Pakula et al., 2005), or to the general energy metabolism of *M. perniciosa*, as suggested by our results with GLY (Figure 5A). Here, both non-fermentable carbon sources, mannitol and GLY, impaired growth (Figure 1), but only mannitol could induce high secretory activity in *M. perniciosa*. The observation that the addition of GLU to mannitol-supplemented medium reduced *M. perniciosa* secretory activity to the same level as that found for the secretome of GLU-grown hyphae suggests that secretory activity is to some extent related to the presence of GLU in the culture medium. This again reinforces the hypothesis that in *M. perniciosa*, as in other organisms, GLU may act as a signal molecule, apart from its role in nutrition.

The observed carbon source-induced modifications of the morphology of *M. perniciosa* mycelia together with changes in secretome composition could be correlated to the pathogenesis of this fungus, as observed for other microorganisms (Hegedus and Rimmer, 2005; Perez-Martin et al., 2006; Kamoun, 2007; Klosterman et al., 2007). Specifically, for hemibiotrophic and necrotrophic fungi, the induction of host cell death, a characteristic of pathogenesis, is related to the secretion of pathogenesis proteins into the inter- or intra-cellular space (Kamoun, 2007). Indeed, in WBD, the thinner and branched *M. perniciosa* hyphae, such as the ones observed after growth on non-fermentable carbon sources, are more abundant in a phase that slightly precedes necrosis of infected cacao tissues (Ceita et al., 2007). Thus, we speculate that all secretomes isolated from thin and branched *M. perniciosa* mycelia could have enhanced necrosis-inducing activity. However, a biological activity assay performed using *N. tabacum* leaves revealed that this is not the case. The morphology of *M. perniciosa* mycelium was not related to the necrosis-inducing potency of the secretomes, since only GLY growth-derived secretome showed an increment in necrosis activity (Figure 7). This suggests again that in *M. perniciosa*, like in other pathogens, carbon sources behave as signaling molecules in addition to their nutritional role (Oliver et al., 2002; Thevelein et al., 2005; Vanoni et al., 2005). Indeed, GLY was previously related to acquired resistance to paraquat and oxidative stress in *M. perniciosa* (Santos et al., 2008) and to maintenance of the monokaryotic life form of this fungus *in vitro* (Meinhardt et al., 2006).

## Glycerol growth-derived extracellular proteins induce unfolded protein response in meristem of *T. cacao*

To further evaluate the differences in the activity of *M. perniciosa* secretomes, we tested the differences of the most effective secretome (GLY growth-derived) on tissues of *T. cacao*. The host response to the GLY growth-induced secretome was biochemically evaluated by analyzing the BiP levels in infiltrated meristems before the onset of necrosis (Figure 8). BiP is a molecular chaperone and resident of the endoplasmic reticulum of eukaryotic cells, which acts as a major regulator of the unfolded protein response (UPR) and whose level has a direct correlation with biotic or abiotic stress sensing (Alvim et al., 2001; Irsigler et al., 2007). Recent studies have connected cell apoptosis with endoplasmic reticulum stress that induces the activation of the UPR pathway (Lai et al., 2007). When

the UPR is perturbed or not sufficient to overcome the stress, it triggers pro-apoptotic pathways (Iwata and Koizumi, 2005). The increased BiP levels in meristems of *T. cacao* (TSH 1188) treated with GLY-grown *M. perniciosa* proteins, before the appearance of necrosis, suggest that the induction of endoplasmic reticulum stress takes place after secretome application (Figure 8) or that the plant secretory machinery is being prepared to secrete proteins involved in defense (Jelitto-Van Dooren et al., 1999). Indeed, levels of cystatine, a family of protease inhibitors that are at the front-line of defense against pathogens (Shen and Bogyo, 2008), increase in response to this GLY growth-induced extracellular protein extract before the appearance of necrosis in the infiltrated cacao meristem (Pirovani CP, personal communication). The higher BiP level in response to the GLY growth-induced secretome sample, in comparison to *Mp*-Nep2 infiltration or to *M. perniciosa* spore germination, indicates a more severe stress caused by this extracellular protein extract.

Taken together, our results demonstrate that *M. perniciosa* morphology and growth rate are regulated by the general energy metabolism. However, the secretory capacity, protein composition and expression of pathogenicity genes are more related to specific carbon sources, which may act as regulatory signals. Moreover, the results indicate that glycerol is involved in the signal cascade related to the expression of pathogenicity genes.

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