

Carbon source-dependent variation of acquired mutagen resistance of *Moniliophthora perniciosa*: Similarities in natural and artificial systems

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Abstract

The basidiomycete *Moniliophthora perniciosa* causes Witches' Broom disease in *Theobroma cacao*. We studied the influence of carbon source on conditioning hyphae to oxidative stress agents (H_2O_2 , paraquat, 4NQO) and to UVC, toward the goal of assessing the ability of this pathogen to avoid plant defenses involving ROS. Cells exhibited increased resistance to H_2O_2 when shifted from glucose to glycerol and from glycerol to glycerol. When exposed to paraquat, cells grown in fresh medium were always more resistant. Apparently glycerol and/or fresh media, but not old glucose media, up-regulate oxidative stress defenses in this fungus. For the mutagens UVC and 4NQO, whose prime action on DNA is not via ROS, change of carbon source did not elicit a clear change in sensitivity/resistance. These results correlate with expression of fungal genes that protect against ROS and with biochemical changes observed in infected cacao tissues, where glycerol and high amounts of ROS have been detected in green brooms.

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1. Introduction

The basidiomycete fungus *Moniliophthora perniciosa* [formerly *Crinipellis perniciosa*] is a major pathogen of *Theobroma cacao* that is presumed to have arisen concomitantly with its host (Purdy and Schmidt, 1996). Basidiospores of *M. perniciosa* are not very resistant, but over short distances within the humid canopies of cacao plantations they disseminate the disease most effectively (Griffith et al., 2003). The fungus has a hemibiotrophic life-cycle: basidiospores germinate to form a monokaryotic biotrophic mycelium infecting young stems, meristems or any other actively growing tissue leading to a loss of apical dominance, abnormal axillary bud growth, proliferation of lateral shoots, and hypertrophy [green broom]; during the

second, necrotrophic stage of fungal growth, the infected plant tissue turns necrotic and *M. perniciosa* displays a dikaryotic saprophytic mycelium that spreads rapidly in the dying plant tissue [dry broom] and develops basidiomas (Evans and Bastos, 1980; Rincones et al., 2003; Ceita et al., 2007). Green brooms of infected *T. cacao* contain a high concentration of glycerol (Scarpari et al., 2005) and increased levels of hydrogen peroxide [H_2O_2] (Cascardo, J.C., personal communication) the latter presumably a breakdown product of calcium oxalate (Ceita et al., 2007).

Plant defenses against fungal pathogens consist of both a localized response that is often associated with an oxidative burst and a more generalized systemic response mediated by signaling molecules (Mayer et al., 2001; Gesteira et al., 2007). The oxidative burst generates reactive oxygen species (ROS) i.e. anion superoxide [$O_2^{\bullet-}$], hydroxy radical [OH^{\bullet}] and H_2O_2 (Lamb and Dixon, 1997) that form a toxic barrier to pathogen invasion. To overcome or neutralize this plant defense, fungal pathogens, including *M. perniciosa*, have evolved several strategies that either limit the formation, or directly overcome the effects of plant

Abbreviations: GLU, glucose; GLY, glycerol; *Mp*, *Moniliophthora perniciosa*; UVC, UV_{254nm} radiation; 4NQO, 4-nitroquinoline-1-oxide; PAQ, paraquat; H_2O_2 , hydrogen peroxide; ROS, reactive oxygen species.

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cell-generated ROS (Hegedus and Rimmer, 2005; Ceita et al., 2007), thus leading to a successful infection and usually to the death of the plant.

Cellular ability to survive and to induce resistance after an oxidative challenge depends in part on molecular sensing and efficient signaling aimed at enhancing anti-oxidant defense mechanisms that either eliminate/decompose ROS (Maris et al., 2000; Ikner and Shiozaki, 2005) or repair the ROS-caused DNA oxidative damage (Boveris, 1998). ROS can interact with RNA, DNA, proteins, and lipids. Up to a normal, endogenously generated level (e.g., caused by incomplete O₂ reduction during respiration) this oxidative potential can be counteracted or tolerated by the cell's neutralization and repair mechanisms. Amongst the most evolutionarily conserved defense mechanisms against ROS are the primary barriers known as superoxide dismutases (SOD) and catalases (Boveris, 1998). SODs are responsible for dismutating O₂^{•-} into H₂O₂ and O₂, and catalases are responsible for detoxifying H₂O₂ by converting it to H₂O and O₂. Although efficient, these processes lead, via Fenton reaction, to formation of another type of ROS, the extremely reactive and hence deleterious OH[•], against which the cell lacks a specific quencher (Boveris, 1998).

At higher concentrations, ROS induce oxidative stress which leads to signaling of a different pattern of gene expression, favoring production of more proteins indirectly or directly involved in neutralizing and removing ROS or in repairing ROS-caused DNA damage (Boveris, 1998). Gene expression is induced by stress-specific transcription factors like Yap1 in yeast (Maris et al., 2000) or CreA in *Aspergillus nidulans* (Mogensen et al., 2006) which, in turn, are responsible for activating not only genes encoding proteins involved in protection against ROS (e.g., *SOD1*, *SOD2*, *CTA1*, and *CTT1*) but also loci encoding inducible transport/export permeases, e.g., *SNQ2*, *PDR5*, *YOR1* (Decottignies et al., 1998), and repair genes (*OGG1*), which would help to detoxify cells or repair oxidative DNA damage.

Alternatively, exogenous chemicals may also produce ROS or direct DNA lesions. H₂O₂, for instance, may produce OH[•] via Fenton reaction or it may directly damage DNA bases; other chemicals like Paraquat [PAQ, Peter et al., 1992] and 4-nitroquinoline oxide [4NQO, Yano et al., 1995] can be metabolically activated by oxygen via redox cycle and generate either O₂^{•-}, oxidated bases or DNA adducts (Kappus and Sies, 1981; Nunoshiba and Demple, 1993; Dizadaroglu and Karakaya, 1999). UVC may also form ROS via radiolysis of water, a process that, however, only contributes weakly to cellular damage as compared to directly radiation-induced lesions (Friedberg et al., 1995).

In large concentrations ROS may thus be deleterious to living cells, leading to cell death and/or to many diseases. However, when present in lower concentrations, ROS and ROS-producing enzymes are known to play an important role in cell signaling, especially in fungi and other

microbial eukaryotes, where they are responsible for initiation of cell differentiation (Aguirre et al., 2005).

Recent advances have been made in the study of the physiology of *M. pernicioso*, i.e., a transformation system (Lima et al., 2003), a quantitative toxicity assay (Filho et al., 2006), and a rapid molecular test to verify identity of *M. pernicioso* cultures (Melo et al., 2006) are available. Also, the switch from mono- to dikaryotic growth of this hemi-biotrophic fungus *in vitro* can now be postponed by maintaining basidiospore-derived mycelium on glycerol media whereas glucose media allow a rapid switch to the dikaryotic phase (Meinhardt et al., 2006).

Filamentous fungi are ubiquitous organisms able to obtain energy from different substrates (Delgado et al., 2003). While most microorganisms can utilize a variety of carbon sources, many go to great lengths to ensure that they use up the available glucose before turning to alternative fuels (Johnston, 1999). Glucose, the most abundant monosaccharide in nature, is the primary energy provider for microorganisms; it is the preferred carbon and energy source for most cells and is a substrate for biosynthesis of cell components (Towle, 2005). Also, it can act as a “growth hormone” in regulating several aspects of cell growth, metabolism, and development (Ozcan and Johnston, 1999). Glycerol is also an important substrate in several species' energy metabolism and lipid synthesis (Holms, 1996). It also plays an important role in the balance of cellular redox potential and inorganic phosphate recycling (Alonso-Monge et al., 2003). Glycerol metabolism is also involved in response to oxidative stress, caused by high intracellular concentrations of free oxygen radicals that may result from several metabolic reactions or from external stress agents, i.e., radiation, metals and drugs (Ames et al., 1993). Yeast and fungal cells have developed adaptive mechanisms to maintain glycerol flux at levels suitable for growth. These mechanisms include modulation of glycerol production, dissimilation, conservation and increased retention, as well as glycerol transport regulation across the plasma membrane (Kayingo et al., 2001).

In yeasts utilization of various carbon and phosphate sources is highly regulated by gene expression induced by the putative substrates. This ensures correct enzyme levels for optimal utilization (Carlson, 1987). With abundant glucose, *Saccharomyces cerevisiae* can satisfy most of its energy requirements from glycolysis/fermentation (exponential phase of growth, LOG) as the genes necessary for LOG growth are induced by glucose (de-repressed) while a large number of genes involved in respiratory metabolism (stationary phase of growth, STAT) are repressed (glucose repression). After glucose depletion *S. cerevisiae* will change the pattern of gene expression [during diauxic shift (Santangelo, 2006)], promoting the de-repression of genes needed for respiratory ethanol metabolism, which also include those providing higher protection from oxidative stress (phenotypes include better survival of heat, pH, osmotic or oxidative stress, amongst others). Other yeasts and fungi show a similar response, called “glucose repres-

sion” or “the glucose effect”, discovered over 100 years ago (Dienert, 1900).

Our present study shows that, by mimicking the natural oxidative conditions within infected tissues (Scarpari et al., 2005; Ceita et al., 2007) using the cytotoxicity of four well-known agents (for the purpose of this paper, the three chemicals, 4NQO, PAQ, H₂O₂, and one physical agent UVC, though exerting cytotoxicity via different mechanisms, will be named mutagens as all are able to induce mutations via DNA damage) in the dikaryotic life form of *M. pernicioso* grown either in glycerol or glucose, different levels of resistance to ROS are observed *in vitro* and that this is paralleled by a change in expression of genes encoding anti-ROS defense proteins.

2. Material and methods

2.1. Growth conditions

Moniliophthora pernicioso hyphae were always grown at 25 °C in complete media (CPD, 2% glucose, 2% peptone; or CPG, 2% glycerol, 2% peptone; 2% agar added for solid media). Since contamination of cultures is frequent in slow-growing filamentous fungi we regularly controlled the identity of *M. pernicioso* culture by the highly species-specific PCR-amplification of the actin gene (Melo et al., 2006) and also verified its dikaryotic state by microscopic observation of clamp connections, typical for the dikaryotic phase of fungal growth (Fig. 1A and B). Broken hyphae were obtained according to Filho et al. (2006). One to three milliliters of the broken hyphae suspension was transferred to 10 mL of fresh liquid media and incubated for 7 days without agitation. Cultures were grown either in CPD (GLU) or CPG (GLY, as carbon source). After 7 days CPD media of cultures were replaced or not by the same amount of fresh media (either CPD, GLU→GLU or CPG, GLU→GLY) and further incubated for 24 h. CPG-grown cultures were manipulated accordingly (GLY→GLU, GLY→GLY). The broken hyphae (1 mL) were then used for toxicity assays.

2.2. Mutagen exposure and cell survival of *M. pernicioso*

Sensitivity of broken hyphae to different mutagens was determined on solid media (CPD/CPG) supplemented

either with 4NQO (2 to 8 μM), PAQ (100 to 400 μM), or H₂O₂ (1 to 4 mM). One mL of broken hyphae suspension was applied to three plates of CPD or CPG containing each mutagen, and the absolute number of grown “pseudo-colonies” was counted after 7 days of incubation at 25 °C. Thus, the three plates yielded the surviving broken hyphae able to form “pseudo-colonies”/mL of suspension. UVC treatment was performed by irradiation of *M. pernicioso*-inoculated plates with exposure doses between 0 and 96 J/m² (Spectro-linker[®], Spectronics Corp., Westburg, NY). Results are expressed as the percentage of survival related to the untreated controls and are the mean of at least three independent experiments; the error bars represent standard deviation as calculated by the GraphPad Prism[®] program (GraphPad Software Inc., San Diego, CA).

2.3. Catalase assay

Three disks of *M. pernicioso* grown in solid CPD for 15 days were added to 100 mL of water containing H₂O₂ [1 M]. The oxygen production was measured with a Bernauer (F1550A) oxymeter over 30 min. Results are expressed as oxygen produced in mg/L and are the mean of two independent experiments; the error bars represent standard deviation as calculated by the GraphPad Prism[®] program (GraphPad Software Inc., San Diego, CA). Sodium azide (NaN₃, 10 mM) was used to inhibit enzyme reactions.

2.4. Primer design

Primers (Table 1) were designed based on genome sequences of *M. pernicioso* (The Genome Project, <http://www.lge.ibi.unicamp.br/vassoura/>). The sequences showed homology with seven genes, and encode following presumed proteins: *ACT1*, actin; *CTA1*, peroxisomal/mitochondrial catalase; *CTT1*, cytosolic catalase; *SOD2*, superoxide dismutase; *SNQ2*, a 4NQO export permease (ABC transporter); *PST2*, a gene of unknown function, highly inducible by Yap1 in *S. cerevisiae*; *YORI*, plasma membrane ATP-binding cassette (ABC) transporter conferring multi-drug resistance. GenBank[®] accession numbers (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) of putative *M. pernicioso* genes are given in Table 1.

2.5. Gene expression

Extraction of mRNA and further handling of the samples was as described by Melo et al. (2006). Briefly, samples were collected after following incubation (i) either in CPD or in CPG for 7 days; and (ii) 7 days of growth and 24 h of incubation in fresh media (CPD or CPG). RNAs were isolated with RnaPure kit (QIAGEN[®]). Analysis of RNA extraction efficiency and gene expression was carried out in 1% agarose gel (PROMEGA[®]). RNA was purified from DNA by DNase treatment (FERMENTAS[®]). The first-strand cDNAs were synthesized by the following protocol: 6 μL of reverse transcription reaction

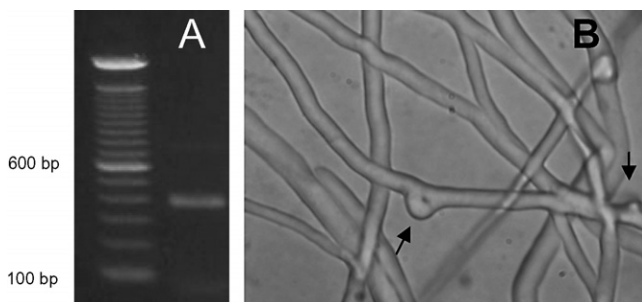


Fig. 1. *Moniliophthora pernicioso* species confirmation: (A) PCR-actin gene amplification; (B) clamp connection (100×).

Table 1
Moniliophthora perniciosa primers used for RTPCR gene expression

ORF ^a	Primer (5'→3')	Annealing temperature (°C)
<i>ACT1</i> EF066485	F CCACAATggAggACgAAgTCg R CCCgACATAggAgTCCTTCTg	52
<i>SOD2</i> EY221171	F TgCTCTCgAgCCCTACATT R AACgCTTAGTgTCCggTTTg	60
<i>CTT1</i> ET165041	F gTTgTTCCTggCATCgATTT R ATAgACgAATTggCCCACAg	60
<i>CTA1</i> ET218451	F CTCCACTCAAgCCTCCTgTC R CgCTCAgTAATCTCgCTCAA	60
<i>PST2</i> EU477412	F gTCTgCTggTggTgCTgTTA R CTAggTTgCAAgAgTCgAgA	58
<i>SNQ2</i> ET218450	F CgACTTgCTgAgAATggACA R ggAAAAGAggCACTgCAAAC	58
<i>YORI</i> EU503540	F gCTCgTgAACggCCCTCAAAGAgCg R CgTTCAGTgCgTCggTAGCCTCTgCg	56.5

^a Predicted: *ACT1*, Actine; *SOD2*, superoxide dismutase; *CTA1*, catalase A; *CTT1*, catalase T; *PST2*, protoplast-secreted; *SNQ2*, sensitivity to 4-nitroquinoline-*N*-oxide; *YORI*, yeast oligomycin resistance. GenBank accession number under gene symbols.

mixture containing total cellular RNA, 5 µM of oligo (dT), 5× RT buffer (260 mM Tris–HCl, 200 mM KCl, 25 mM MgCl₂, 2.5% Tween 20), 10 mM dNTPs, and 20 U/mL of RNase inhibitor were incubated at 37 °C for 5 min. After adding 200 U of Reverse Transcriptase (Invitrogen[®]) tubes were incubated at 42 °C for 60 min, heated to 70 °C for 10 min and then quick-chilled on ice. The cDNA was then used for amplification the samples by PCR. Non-inducible Mp*ACT1* gene was used as internal standard of gene expression. Mp*CTA1* cDNA was diluted 1:20. PCR was performed in 25 µL reaction volumes containing 20 ng cDNA, 1.25 µL dNTPs (2.5 mM), 1 µL MgCl₂ (50 mM), 2.5 µL of buffer PCR 10× (Tris–HCl 10 mM, KCl 50 mM, MgCl₂ 1.5 mM, pH 8.3), 0.2 µM of each primer pair and 0.2 µL of *Taq* DNA polymerase (5 u/µL) (Invitrogen[®]) and distilled water to complete. Cycling conditions in a thermocycler (Eppendorff Master-Cycler[®]) consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 95 °C for 50 s, annealing temperature of each primer for 50 s, and 72 °C for 1 min. PCR was completed by a final extension of 7 min at 72 °C. Products were resolved in 1% agarose gel and were visualized with ethidium bromide and ultraviolet illumination. Images were taken and stored by using the Kodak-EDAS[®] system.

3. Results

3.1. Glycerol-induced ROS resistance phenotype

In all media conditions and combinations presented, dikaryotic broken hyphae of *M. perniciosa* growing in

GLU were much more sensitive than their GLY-grown counterparts to all four well-known mutagens (Figs. 2 and 3). The sensitivity differences to the four tested mutagens between GLU and GLY-grown cells ranged between a maximum of two (H₂O₂) and a minimum of one (UVC) orders of magnitude of reduction in survival. Cells grown in GLY displayed a clear enhancement in acquired resistance. GLU→GLU cells acquired higher resistance to all mutagens (Figs. 2 and 3, each A and C) while GLY→GLU cells showed highest sensitivity to all tested mutagens (Figs. 2 and 3B and D) except for 4NQO (Fig. 3D). GLU→GLY or GLU→GLU cells acquired the same level of induced resistance to the mutagens (Fig. 2B–D), except for H₂O₂ (Fig. 2A) where GLU→GLY cells had a much higher level of induced resistance (better survival by two orders of magnitude). When cells were shifted from GLY→GLY they did not show induced resistance, but on the contrary, increased sensitivity to the mutagens UVC (Fig. 3B) and PAQ (Fig. 2D), except for H₂O₂ (Fig. 2B) where they acquired higher resistance. In all experimental conditions, 4NQO resistance/sensitivity was not affected by type/amount of nutrients, except when cells were grown only in GLU (Fig. 3C and D).

3.2. Expression of genes involved in ROS protection

A molecular analysis of medium-induced variations on expression of selected *M. perniciosa* genes was performed via RT-PCR using the non-inducible actin gene (Mp*ACT1*) as an internal control (Fig. 4A). We could successfully amplify cDNA of three different putative Mp*ORFs* that are homologs to genes involved in oxidative stress protection: Mp*SOD2* (manganese superoxide dismutase), Mp*CTT1* and Mp*CTA1* (cytoplasmic and mitochondrial catalase, respectively).

Mp*SOD2* gene did not show significant alterations in gene expression at any of the medium variations (Fig. 4A, lanes 1 to 6). The two putative *M. perniciosa* catalase genes yielded different patterns of expression. There was a 20-fold higher expression of Mp*CTA1* than that of Mp*CTT1* and the basal level of expression of Mp*CTA1* was already so high that induced expression could only be seen when the cDNA was diluted 1:20 before performing PCR (Fig. 4A, lanes 1 to 6). While expression of Mp*CTT1* was repressed after a GLY→GLU medium shift, Mp*CTA1* was induced. After a GLY→GLY shift only a slight induction of Mp*CTA1* could be seen. A medium shift from GLU→GLY induced both putative catalase genes while GLU→GLU slightly increased gene expression only of Mp*CTT1*. Catalases directly catalyze decomposition of H₂O₂ to ground-state O₂ and can be non-specifically inhibited by sodium azide. Production of O₂ was successfully measured in *M. perniciosa* using O₂-electrode method with excess amount of H₂O₂ (1M) and could be inhibited by addition of, or totally suppressed by pre-incubation with, NaN₃ (Fig. 4B).

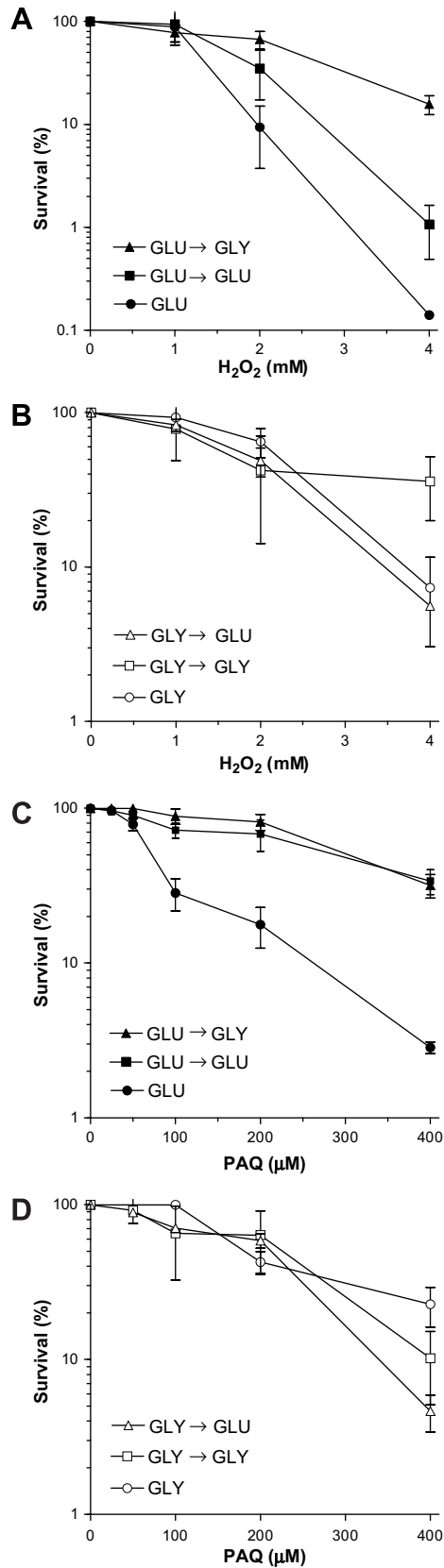


Fig. 2. Survival of *M. perniciosa* exposed to H₂O₂ or paraquat (PAQ). Survival increased when cells exposed to H₂O₂ were (A) shifted from glucose to glycerol or (B) from glycerol to glycerol. Survival of cells exposed to PAQ was highest after shift from glucose to fresh media (C) and most sensitive after shift from glycerol to glucose (D).

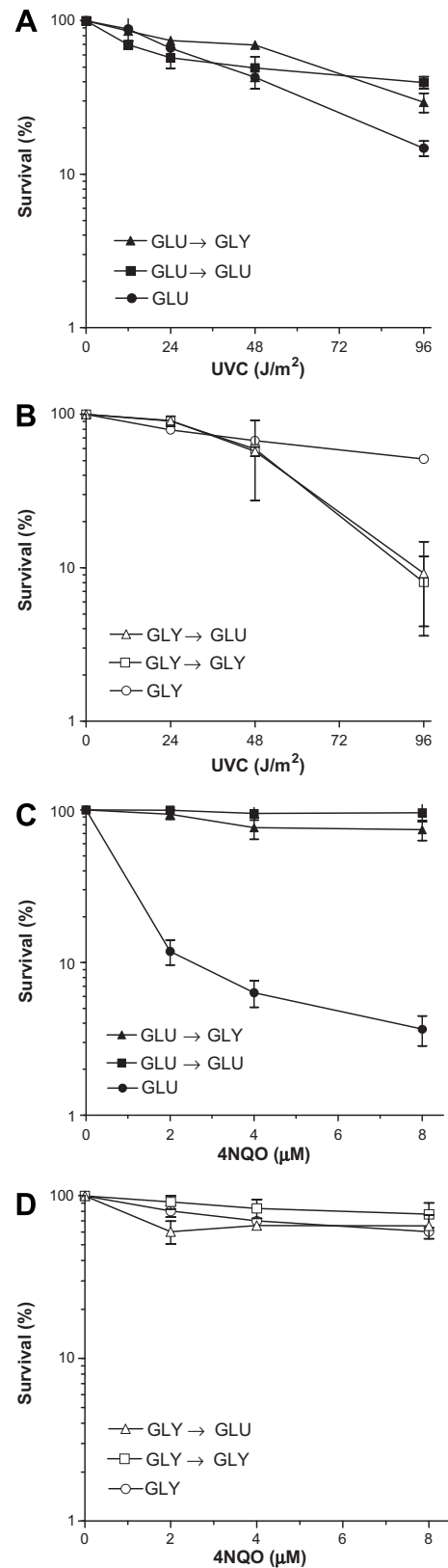


Fig. 3. Survival of *M. perniciosa* exposed to UVC or 4NQO. When exposed to UVC (A) cells were more resistant when shifted from glucose to fresh media or (B) cells were more sensitive when shifted from glycerol to fresh media. Survival of cells exposed to 4NQO was highest after shift from glucose to fresh media (C) and cells were always resistant when pre-grown in glycerol (D).

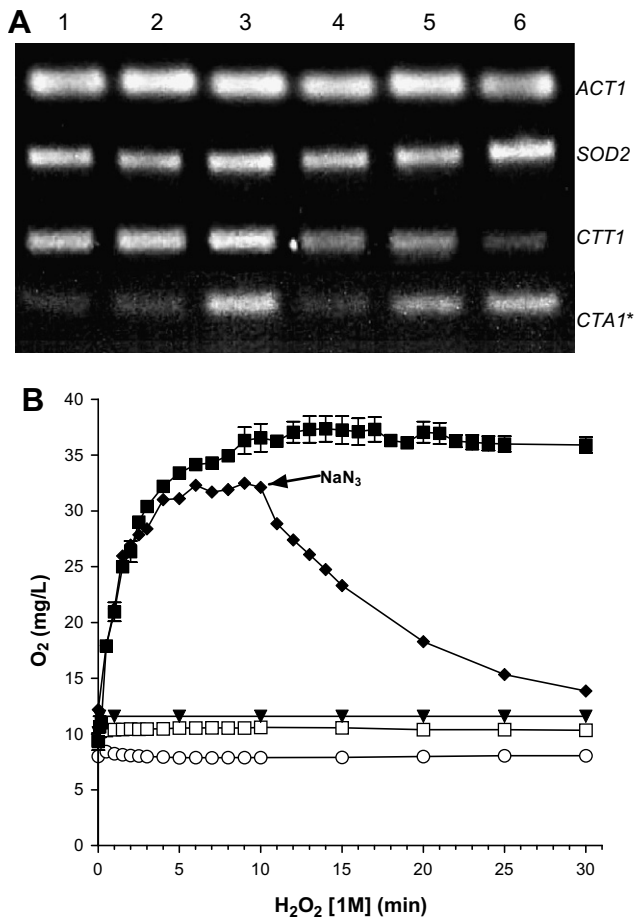


Fig. 4. *Moniliophthora perniciosa* basal oxidative protection genes. (A) RT-PCR of selected genes after medium change. Lanes: (1) glucose; (2) shifted from glucose to glucose; (3) shifted from glucose to glycerol; (4) glycerol; (5) shifted from glycerol to glycerol; (6) shifted from glycerol to glucose; MpSOD2, expression unchanged; MpCTT1, expression up-regulated in 2 and 3 as compared to 1, down-regulated in 6 as compared to 4; MpCTA1, 20-fold higher expression when compared to MpCTT1 and highly induced in 3 and 6 as compared to 1 and 4, respectively. (B) H₂O₂ breakdown measured as oxygen production: (■) *M. perniciosa* rapid production of oxygen in the presence of 1M H₂O₂; (◆) NaNO₃ (10 mM, added after 10 min) blocking oxygen production of *M. perniciosa* in presence of 1M H₂O₂; (▼) *M. perniciosa* in presence of both 1M H₂O₂ and NaNO₃ at start, without production of oxygen; internal controls without oxygen production: (○) water; (□) water + 1 M H₂O₂.

3.3. Expression of genes involved in transport and transcription activation

Dikaryotic broken hyphae were exposed to 4NQO and gene expression was measured in a time-dependent manner before [Fig. 5A (GLU) and B (GLY)] and after medium shift [Fig. 5C (GLU→GLU) and D (GLY→GLY)]. While expression of MpPST2 was induced in GLU (Fig. 5A) and was highly expressed in GLY→GLY cells (Fig. 5D), it was not induced in either GLY or GLU→GLU (Fig. 5B and D). ABC transporter homologs MpSNQ2 and MpYORI were differentially expressed. MpSNQ2 was not induced either in GLU, GLU→GLU or GLY→GLY (Fig. 5A, C, and D, respectively) and its expression was repressed in

GLY (Fig. 5B) after 4NQO exposure. There was a double band amplified with MpYORI primers and both gene products displayed transient gene expression in GLU (Fig. 5A) whereas only one of them (upper band) was induced in GLY and highly induced in both GLU→GLU and GLY→GLY (Fig. 5B–D, respectively).

4. Discussion

The physiological, biochemical, and genetical characterization of the basidiomycete *M. perniciosa* is challenging because, though its genome has been sequenced (<http://www.lge.ibi.unicamp.br/vassoura/>), only little biological data is available, and most information is related to details of plant–pathogen interactions (Ceita et al., 2007). However, we know that *M. perniciosa* keeps its monokaryotic life form and grows intercellularly during the initial phase of plant infection [green broom] and that the conversion to a dikaryon is correlated to necrotrophic intracellular growth [dry broom] (Ceita et al., 2007; Garcia et al., 2007). The prolonged monokaryotic phase *in vitro*, when glycerol is the sole carbon source, and the conversion to dikaryon in the presence of glucose (Meinhardt et al., 2006) suggest that the availability of a defined carbon source could also influence this differentiation *in vivo*.

In yeast, carbon source-specific resistance to ROS is a natural phenomenon (Maris et al., 2000) and there is increasing evidence that ROS play an important physiological role in microbial development or in cell expansion during the morphogenesis of plants (Aguirre et al., 2005; Georgiou et al., 2006). Could a carbon source-induced resistance to ROS, via a similar mechanism, also explain the behavior of the two different life forms of *M. perniciosa* during colonization of the plant host? In a first approach to this question we grew dikaryotic cells of this basidiomycete fungus in different media (GLY or GLU as carbon source) and then exposed the cells to four treatments that generate different types and intensities of ROS. Thus we proposed to find out if the carbon source had a significant influence on cellular sensitivity/resistance to ROS. Indeed, growth in different carbon sources (GLU or GLY) elicited different physiological responses in dikaryotic *M. perniciosa*, observable (1) as variable sensitivity phenotypes [cytotoxicity of H₂O₂, PAQ, UVC, and 4NQO (Figs. 2 and 3)] and (2) in parallel, via sqRT-PCR analysis of gene expression (Figs. 4 and 5A).

4.1. Oxidative stress response induced by glycerol in dikaryotic cells

Filho et al. (2006) had shown the pronounced sensitivity of dikaryotic *M. perniciosa* to PAQ and H₂O₂ and interpreted this as a probable consequence of a weak protection from oxidative stress-inducing agents. The results of our present work (Figs. 2 and 3) show that experimental conditions chosen at that moment (GLU, Filho et al., 2006) yielded, by chance, the most sensitive cells and that

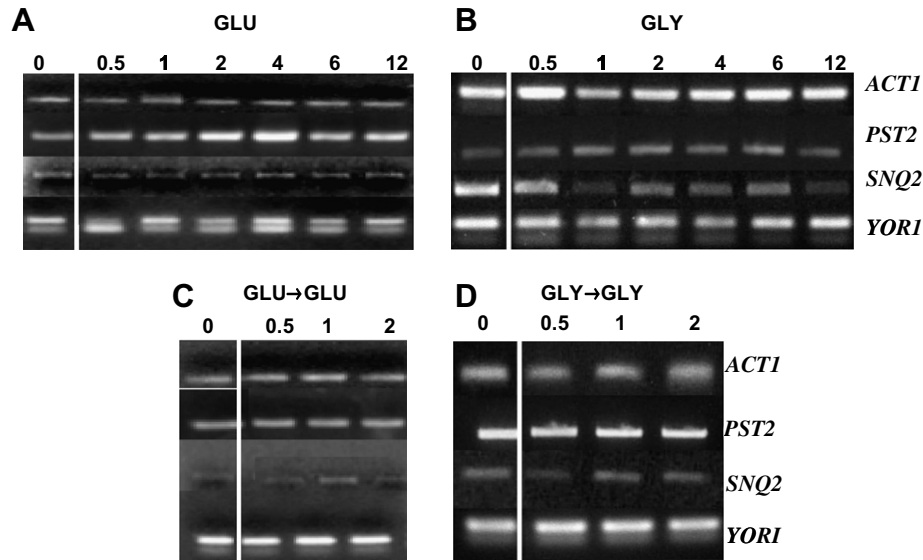


Fig. 5. *Moniliophthora perniciosa* RT-PCR of selected genes after 4NQO exposure [4 μ M]. Numbers above lanes correspond to exposure time (h). MpPST2 up-regulated in (A), with peak at 4 h, down-regulated in (B), expression unchanged in (C), and highly expressed in (D); MpSNQ2 unchanged in (A and D), down-regulated in (B and C); MpYORI shows two bands: in (A) upper band down-regulated at 0.5, 2, and 6 h and up-regulated at 4 h; lower band up-regulated at 0.5 and 4 h, other times unchanged expression; in (B) upper band unchanged expression and lower band down-regulated; in (C and D) upper band up-regulated, lower band down-regulated.

GLY-grown dikaryotic *M. perniciosa* gains an overall acquired resistance to oxidative stress and to DNA-damaging mutagens (Figs. 2 and 3). This resembles the phenotypic response of respiring *S. cerevisiae* cells (an intrinsic resistance) against H₂O₂-imposed oxidative stress when compared to their fermenting counterparts (Jamieson, 1992). The induction of oxidative stress triggers expression of stress-defense and DNA repair enzymes in yeast, and glycerol metabolism is also involved in generating oxidative stress (Ames et al., 1993; Maris et al., 2000; Schüller, 2003).

GLY-grown dikaryotic *M. perniciosa* has a similar response since genes encoding oxidative stress protective defenses, i.e., manganese superoxide dismutase (MpSOD2) and both catalases (MpCTT1 and MpCTA1) are induced in GLY cells (Fig. 4A, lanes 4–6). Infected tissues (green brooms) presented high levels of glycerol (Scarpari et al., 2005) and increased accumulation of H₂O₂ (Ceita et al., 2007). The constant basal expression, albeit at different levels, of the two specific catalases (Fig. 4A) are the main contribution of this phytopathogenic fungus in protection against oxidative burst of *T. cacao*. Production of O₂ was immediate after addition of H₂O₂ [peroxidases do not decompose H₂O₂ into O₂ and pseudocatalase would not be inhibited by sodium azide (Fig. 4B)] (Gutteridge and Halliwell, 2000). Therefore, we can assume a clear correlation between glycerol levels, differential expression of catalases and resistance to ROS, both *in vivo* and *in vitro*.

4.2. Is there catabolite repression in *M. perniciosa*?

The presence of a non-fermentable carbon source like glycerol leads to high expression levels of yeast genes involved in stress resistance (Maris et al., 2000), and this

could also be true for the GLU \rightarrow GLY acquired resistance phenotype of *M. perniciosa* where resistance to all four tested mutagens coincides with induced expression of oxidative stress-protective genes (Fig. 4A, line 3). Inversely, the increased sensitivity to mutagens (other than 4NQO) of *M. perniciosa* GLY \rightarrow GLU shifted cells could be interpreted as a result of catabolite repression (Figs. 2 and 3); however, at the level of gene expression only MpCTT1 is down-regulated (Fig. 4A, lane 6). Also, catabolite repression is only true for the H₂O₂ response (Fig. 2A), because GLY \rightarrow GLY cells exposed to UVC and PAQ also become more sensitive (Fig. 3B and D). Catabolite repression is also not indicated because GLU \rightarrow GLU cells become more resistant as compared to GLU only cells (Fig. 2) and MpCTA1 is apparently de-repressed (Fig. 4A, lanes 2 and 3). *M. perniciosa* grows rather slowly and thus converts only a fraction of the offered carbon source into biomass (F. Alvim, personal communication). Accordingly, glucose consumption during the first seven days of growth *in vitro* is less than 25% of the offered amount (data not shown). The natural low growth rate of *M. perniciosa* in infected tissues, where it is barely detectable by microscopy (Ceita et al., 2007), should not be necessarily interpreted as due to low offer of carbohydrates.

Many putative *M. perniciosa* homologs of yeast genes involved in stress response and under control of glucose repression have already been detected, including MpCTT1 (Table 2). This makes them candidates for regulation via catabolite repression in *M. perniciosa*. However, we might not obtain simple answers by just switching media. Mogensen et al. (2006) showed expression profiles of *A. nidulans* indicating that the carbon catabolite repression is not a simple on/off switch in this

Table 2
Moniliophthora perniciosa putative gene homologs of glucose repressible *S. cerevisiae* genes involved in stress response^a

Yeast ^b	<i>Moniliophthora perniciosa</i>
<i>MDR1</i>	5e–177
<i>PPZ2</i>	4e–132
<i>SSA3</i>	1e–88
<i>SSA4</i>	1e–88
<i>UBC5</i>	5e–76
<i>CTA1</i>	6e–57
<i>SSE2</i>	3e–46
<i>CDC55</i>	5e–42
<i>PRE1</i>	2e–40
<i>TRX2</i>	4e–37
<i>GRX2</i>	2e–35
<i>SSD1</i>	6e–27
<i>STO1</i>	4e–06
<i>GTS1</i>	4e–05
<i>HSP30</i>	0.086

^a Santangelo, 2006.

^b Yeast genome database proteins blasted against *M. perniciosa* READS (BLASTtx).

filamentous fungus, as it depends not only on the carbon source but also on the presence or absence of the transcription factor CreA. We should also consider that *M. perniciosa* is a hemibiotrophic filamentous fungus with two very distinct phases during its life cycle, and is phylogenetically distant from yeast.

4.3. Catabolite repression interferes with active transport

While *M. perniciosa* has a specific and clear response to H₂O₂, resembling that of glucose (catabolite) repression in *S. cerevisiae* (Maris et al., 2000), it has a rather unusual and atypical resistant response to 4NQO. 4NQO is a pro-mutagen, and has to be metabolized to 4HAQO which forms DNA adducts and also generates ROS, mainly O₂^{•−} that is readily converted to H₂O₂ by SOD (Yano et al., 1995). The vast amount of catalase initially present (*MpCta1p*, Fig. 4A and B) could very efficiently neutralize the H₂O₂ formed by metabolization of 4NQO (Fig. 4B) so that neither of the ROS formed would contribute to cell damage (Nunoshiba and Demple, 1993) and hence increased sensitivity might not be expected.

Mycelia of filamentous fungi are efficient “secretion machines”, sometimes exporting into the medium more than half of the proteins that they make (Knowles et al., 1987) because of their unique mode of growth that only occurs in the hyphal apex (Wessels, 1999). There must be a vast number of export permeases, a fact that could account for an increase of exportation of 4NQO in *M. perniciosa*. Although in yeast 4NQO or 4HAQO is exported via stress-inducible Snq1p/Atr1p (Gömpel-Klein and Brendel, 1990) and Snq2p (Servos et al., 1993) the same is not true for *M. perniciosa* where putative *MpSNQ2* shows unchanged expression (Fig. 5A, C, and D) or is repressed (Fig. 5B). It is interesting to note

that the amplification of *MpYOR1* homolog yielded two bands, apparently due to amplification of a conserved region of yet another ABC transporter, which could be *PDR5*, because the primers may also anneal to that sequence (bioinformatics analysis, data not shown). After 4NQO, both genes showed transient expression induction in GLU (Fig. 5A) and induced expression in GLU→GLU (Fig. 5C); therefore, one of the amplified genes may be a better candidate for a 4NQO export permease than Snq2p, and responsible for 4NQO resistance (Fig. 3D) which would explain the sensitivity of *M. perniciosa* in GLU medium (Fig. 3C). Although all three transporters, Snq2p, Yor1p, and Pdr5p, are regulated by the same transcription factors (Decottignies et al., 1998) and have overlapping specificities for xenobiotics transport in yeast (Rogers et al., 2001) it cannot be expected that their regulation would be the same in a distant fungus as *M. perniciosa*.

4.4. Transcription activation as response to stress?

The large group of *M. perniciosa* genes homologous to glucose-repressible yeast genes that are involved in stress response (Table 2) suggests that transcription factors responsible for this response may also exist in this fungus. In *S. cerevisiae*, one important regulator in oxidative stress signaling is transcription factor Yap1p, which is activated when cells are exposed to oxidative stress agents, like H₂O₂ and PAQ. Yap1p plays an important, but indirect role in the H₂O₂-dependent regulation of *GSH1* transcription (Maris et al., 2001; Dormer et al., 2002). Many yeast genes are regulated in a Yap1p-like manner and *ScPST2* (function of *ScPst2p* still unknown) is one of them. So far no *ScYAP1* homologs could be found in the genomic library of *M. perniciosa* (>90% sequenced, J.C.M. Cascardo, personal communication). However, a homolog of transcription factor creA of *Aspergillus niger* (a catabolite repressor) has been found in *M. perniciosa*. The *MpPST2* transcript was successfully detected in a cDNA library and used for designing primers to monitor this gene's expression after 4NQO exposure (Fig. 5). *MpPST2* shows exposure dose-dependent induction in GLU (repressed cells) and is highly induced in GLY→GLY cells (oxidative metabolism); it is repressed in GLY and GLU→GLU cells. That could mean, if there is a Yap1-like system in *M. perniciosa*, other genes involved in oxidative stress resistance, like *GSH1* [which is one of the genes that are up-regulated during the shift from fermentative (GLU) to oxidative metabolism (GLY, Maris et al., 2000, 2001)], would also be induced and this could explain the GLY acquired resistance of *M. perniciosa* to the four mutagens. However, the sensitivity of GLY→GLY cells to PAQ and UVC (Figs. 2 and 3D,B) cannot be explained by these arguments. And the expression profile of *MpSnq2p* certainly rules out this possibility, especially considering its response to 4NQO.

5. Conclusions

In summary, our results show that the two different carbon sources (glucose and glycerol) in fact elicit different physiological answers in dikaryotic *M. perniciosa* as they clearly lead to the following carbon source-dependent variation in acquired mutagen resistance: (1) there is an oxidative stress response to the non-fermentable carbon source GLY, i.e., respiring cells show intrinsic resistance to oxidative stress when grown on the non-fermentable carbon source GLY; (2) If glucose de-repression is functioning at all, it cannot be solely responsible for acquired mutagen resistance because only resistance to H₂O₂ is consistent with the pattern expected from glucose de-repression; (3) Expression of anti-ROS defense genes *MpSOD2*, *MpCTT1* and *MpCTAI* show carbon source-dependent regulation; (4) *T. cacao* oxidative burst protection mechanisms can be bypassed by the “ready-to-use” and efficient ROS defense mechanisms of *M. perniciosa*. Further specific studies on carbon catabolite repression are needed to better understand how *M. perniciosa* reacts to differences in carbon source (molecular analyses of general transcription factors) and why it responds differently to glucose and glycerol.

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