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Differential responses of genes and enzymes associated with ROS protective responses in the sugarcane smut fungus

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ABSTRACT

The fungal pathogen *Sporisorium scitamineum* causes sugarcane smut disease. We have previously shown that resistant sugarcane plants induce ROS, coinciding with a delay in fungal colonization. Here, we investigated whether the fungus modifies the enzymatic antioxidant system in vitro and when colonizing sugarcane tissues in response to ROS. In vitro, the exposure to ROS did not affect cell integrity, and a combination of superoxide dismutases (SOD) and catalases (CAT) were active. In vitro, the fungus did not alter the expression of the transcriptional regulator Yap1 and the effector Pep1. The fungus activated distinct enzymes when colonizing plant tissues. Instead of CAT, *S. scitamineum* induced glutathione peroxidase (Gpx) expression only when colonizing smut-resistant plants. Yap1 had an earlier expression in both smut-susceptible and -resistant plants, with no apparent correlation with the expression of antioxidant genes sod, cat, gpx, or external redox imbalance. The expression of the effector pep1 was induced only in smut-resistant plants, potentially in response to ROS. These results collectively suggest that *S. scitamineum* copes with oxidative stress by inducing different mechanisms depending on the conditions (in vitro/in planta) and intensity of ROS. Moreover, the effector Pep1 is responsive to the stress imposed only by the sugarcane resistant genotype.

1. Introduction

The basidiomycete fungus *Sporisorium scitamineum* (Syd.) Piepenbr and Oberw. 2002 (= *Ustilago scitaminea* Sydow & P. Sydow) (Piepenbring et al., 2002) is a biotrophic pathogen and the causal agent of sugarcane smut disease responsible for considerable losses to the crop worldwide (Sundar et al., 2012). The life cycle of *S. scitamineum* can be described in three distinct phases: haploid sporidia, dikaryotic hyphae, and diploid teliospores. Sporidia propagate easily as yeast-like haploid cells in lab conditions (Izadi and Moosawi-Jorf., 2007) and the fusion of two haploid sporidia belonging to opposite mating-types results in dikaryotic hyphae essential to infect host tissues (Alexander and Ramakrishnan, 1980). Fungal colonization of sugarcane tissues induces the development of a whip-like structure composed of host and fungal cells where sporogenesis takes place to produce billions of teliospores (Marques et al., 2016; Trione, 1990).

Sugarcane genotypes are evaluated for resistance by submitting buds to artificial inoculation, and the percentage of whipped plants is considered as a measure of disease susceptibility in a given clonal population (Latiza et al., 1980; Lemma et al., 2015). Rates and patterns of sugarcane tissue colonization also differ between susceptible and resistant genotypes (Carvalho et al., 2016; Lloyd and Pillay, 1980; Peters et al., 2017).

Reactive Oxygen Species (ROS), such as superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are continually produced during healthy development but also play a significant role in plant responses to pathogen recognition (Heller and Tudzynski, 2011). The

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oxidative burst may act not only as toxins, capable of directly killing or slowing down pathogen growth but also as part of a signaling cascade activating hypersensitive response, cell wall modifications, synthesis of antimicrobial compounds and changes in overall gene expression (Thordal-Christensen et al., 1997; Torres et al., 2006; Smirnoff and Arnaud, 2018).

The overcoming of host-derived ROS is essential for fungal virulence and can be achieved by either suppressing the basal defense reaction or by scavenging ROS built up at the early stages of infection (Heller and Tudzynski, 2011; Komalapriya et al., 2015). Additionally, the regulation of cellular levels of ROS is critical for fungal developmental differentiation (Chang et al., 2018; Gessler et al., 2007; Halliwell, 2006; Lushchak, 2011). The mechanisms involved in response to ROS are, in general, divided into enzymatic and non-enzymatic defenses (Gratão et al., 2005; Mittler et al., 2004; Soares et al., 2019). The non-enzymatic defense consists of the synthesis of small soluble molecules that are oxidized by ROS, such as glutathione (GSH) and other antioxidant compounds - phenolics, polysaccharides, tocopherols, flavonoids, carotenoids, glycosides, ergothioneine and ascorbic acid - a combination of which is unique of specific fungal species (Sánchez, 2017). The enzymatic system includes superoxide dismutases (SOD), and peroxidases such as glutathione peroxidase (GPx) and catalase (CAT) (Apel and Hirt, 2004). Some Basidiomycota members present other extracellular oxidoreductases, such as laccases involved in scavenging of free radicals (Arnstadt et al., 2016; Janusz et al., 2013).

It is known that some fungal transcriptional activators and effector proteins can act during ROS exposure contributing to development and pathogenicity (Doehlemann et al., 2009; Hemetsberger et al., 2012; Molina and Kahmann, 2007). For instance, Yap1 has a significant role in directing the oxidative response to activate the expression of genes encoding catalases, superoxide dismutases, glutathione peroxidases, among other ROS detoxifying enzymes (Aguirre et al., 2005; Kuge et al., 1997; Moye-Rowley et al., 1989). Yap1 orthologs, for instance, are present in several microorganisms, including *Candida albicans* (Raymond and Alarco, 1999), *Schizosaccharomyces pombe* (Toone et al., 1998), *Kluyveromyces lactis* (Billard et al., 1997), and plant pathogens such as *Cochliobolus heterostrophus* (Lev et al., 2005), *Alternaria alternata* (Lin et al., 2009); *Botrytis cinerea* (Temme and Tudzynski, 2009) and *Ustilago maydis* (Molina and Kahmann, 2007). In this later, Yap1 has been associated with pathogenicity. Additionally, smuts harbor a defense strategy mediated by the core effector Pep1, which counteracts plant class III peroxidases to prevent increasing H_2O_2-mediated defense responses and signaling (Doehlemann et al., 2009; Hemetsberger et al., 2012).

In a previous study, we demonstrated that during *S. scitamineum* and sugarcane interaction, an increased H_2O_2 concentration produced by the plant negatively impacted fungal colonization (Peters et al., 2017). The waves of oxidative burst detected in resistant genotypes suggested pathogen recognition and delayed (not impaired) fungal colonization (Peters et al., 2017). To investigate the mechanisms used by *S. scitamineum* to cope with ROS, we investigated components of the antioxidant enzymatic system to identify biochemical and molecular changes. We considered the effects of exogenous H_2O_2 and Paraquat on fungal haploid cells cultured *in vitro* and that on the dikaryon colonizing smut susceptible and resistant sugarcane genotypes. We observed distinct responses of the *S. scitamineum* antioxidant system and its regulation to *in vitro* and *in planta* stresses.

2. Material and methods

2.1. Biological material

Teliospores SSC39 (39) were used to inoculate sugarcane plants. The *S. scitamineum* SSC39A haploid cells previously isolated by Taniguti et al. (2015) were used in the *in vitro* experiments. The two sugarcane genotypes used in this study were: the clone IAC66-6 (susceptible to smut) and the cultivar SP80-3280 (resistant to smut) (Peters et al., 2017). The plants were provided by the Instituto Agronômico (IAC), Centro de Cana, Ribeirão Preto, Brazil. The resistant cultivar SP80-3280 can be colonized by the fungus occasionally producing whip (Carvalho et al., 2016).

2.2. Sequence analyses of fungal proteins

BLASTp analysis with default parameters and the non-redundant protein sequence database were used to identify *S. scitamineum* encoded protein sequences of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and GPx (EC 1.11.1.9) isoenzymes. The encoded protein sequences were analyzed for domain conservation by InterProScan (Zdobnov and Apweiler, 2001) and subcellular location using WoLF PSORT (www.genscript.com/wolf-psort.html). Additionally, the molecular weights of the SOD, CAT, and GPx isoenzymes were calculated using the ExPASy ProtParam tool (Gasteiger et al., 2005). The UniProt protein sequence collection and Peroxibase database were used to select sequences to produce dendrograms (protein sequences from fungi of different phylum - Ascomycota, Basidiomycota, Glomeromycota, Zygomycota, Chytridiomycota, and Microsporidia) (Table S1) (Mir et al., 2015). The protein sequences were aligned using Clustal W software using default parameters. Dendrograms were constructed using the neighbor-joining method in the MEGA 6.0 software (Tamura et al., 2013).

Orthologs of Yap1 (Yeast activator protein), Pep1 (Protein essential during penetration-1), Ap18B (Clathrin coat assembly protein AP180A) protein sequences were manually inspected for functional domains based on the features described in Molina and Kahmann (2007); Hemetsberger et al. (2015) and Wendland and Emr (1998), respectively. Protein sequence alignments were performed using PRALINE multiple sequence alignment platform (Bawono and Heringa, 2014). The identification of each gene used in this study is presented in Table 1 and Table S2.

2.3. Sensitivity curves of S. scitamineum to ROS

To evaluate the effect of hydrogen peroxide (H_2O_2) on *S. scitamineum in vitro*, haploid cells (optical density, $OD_{600} = 0.1$) were grown to early-exponential phase (5×10^7 - $OD_{600} = 0.8$) in Yeast Universal Medium (YM) (3 g of yeast extract, 3 g of malt extract, 5 g of soybean peptone and 10 g glucose per L of distilled water) incubated at 28 °C in an orbital shaker set at 200 rpm for 6 h. At this point, H_2O_2 (Merck Millipore) was added at different concentrations (0, 2, 4, 6 and 8 mM) and the growth measured by optical density (OD_{600}) every 2 h until growth reached the end-exponential phase (12 h). The H_2O_2 concentrations were chosen based on studies with smut *U. maydis* (Molina et al., 2007) and other phytopathogenic fungi such as *Fusarium oxysporum*, *Alternaria solani* (Angelova et al., 2005); *Mycosphaerella fijiensis* (Beltrán-García et al., 2009); *Sclerotinia sclerotiorum* (Yarden et al., 2014) and *Rhizoctonia solani* (Samsatly et al., 2016). Also, peroxide was added in the cell culture medium 6 h after *S. scitamineum* growth because of its reactivity in the presence of transition metal

Table 1
List of genes and primers used in this study for quantitative RT-qPCR.

Gene ID[a]	Gene	Sequences
g2980_chr07_Ss	*sod1*	5′ CAACCCTAAGAACTCCAAGCTC 3′ 3′ AAGTTGATGACCGACCAGATG 5′
g651_chr01_Ss	*sod2*	5′ TGTGCTGAAGGATGCTATTGAC 3′ 3′ CCTTGACGTTCTGGTACTGGAG 5′
g3299_chr08_Ss	*sod3*	5′ TGATCAGTAACCAGCCAAACC 3′ 3′ GCACTCAACAACAGCTTCTTCC 5′
g1075_chr02_Ss	*katE*	5′ CCCGTCCCAACTACATCTCG 3′ 3′ GTTGCCAATCGTCTGCTTCC 5′
g4614_chr13_Ss	*katG*	5′ CTTGAGGTAGAGGTTGGTCCAC 3′ 3′GTAAGACTCACGGTGCTGGTAAC5′
g1608_chr03_Ss	*gpx1*	5′ AGGAAGTACGCAGACCAAGG 3′ 3′ CTTAGTGTTGGAGCCGTATCG 5′
g691_chr01_Ss	*GAPDH*	5′ GGTCAACATCGGTATCAACGG 3′ 3′ CTCGAGGTCAATGAAGGGGTC 5′
g1237_chr02_Ss	*Tubulin beta chain*	5′ CAGTGTGACATTCCTCCTCGTG 3′ 3′ CCTCGGTGAACTCCATCTCG 5′
g4200_chr11_Ss	*yap1*	5′ CGAACGCAAGCAATCTTACCTC 3′ 3′ CGCTCAATGTGGGCAAACTT 5′
g3636_chr09_Ss	*ap18B*	5′ TTTGGCGGTATGGGTATGGG 3′ 3′ CAGTCGCTTGTGGCTGAATG′ 5′
g1816_chr03_Ss	*pep1*	5′ GCACGCATCAGGAAAAGTACG 3′ 3′ GCAGGCTGGATAGTATTGAACG 5′

[a] Gene ID as described in Taniguti et al. (2015) and Material Supplementary Table S2.

ions (Halliwell et al., 2000). The experiment was kept in the dark. The measurements were performed in triplicate, and the results scored as the mean and standard deviation (SD).

We used Paraquat (SIGMA-ALDRICH), an organic compound often used to catalyze the formation of ROS to evaluate *in vitro* stress induced by superoxide anions (Matsuo et al., 2017; Wallace et al., 2005). The *S. scitamineum* haploid cells (OD_{600} = 0.1) were inoculated in YM medium containing Paraquat at final concentrations of 1 and 5 mM incubated at 28 °C in an orbital shaker set at 200 rpm. Similar to the H_2O_2 concentration, these concentrations were selected based on previous studies with phytopathogenic fungi (Angelova et al. 2005; Beltrán-García et al., 2009; Samsatly et al., 2016). Fungal growth was measured (OD_{600}) every 2 h until growth reaching the end-exponential phase (12 h). The measurements performed in triplicate were scored as the mean and standard deviation (SD). For biochemical analysis, the cells were exposed to Paraquat 1 mM until reaching early-exponential phase (Fig. S1).

2.4. H_2O_2 stability in culture medium

We measured the persistence of H_2O_2 in YM medium under the following conditions: YM; YM + H_2O_2 and YM + H_2O_2 + fungus incubated at 28 °C in an orbital shaker set at 200 rpm for 30 and 180 min. 200 μL of the media in each of the above conditions were added to 200 μL of 100 mM potassium phosphate buffer (pH 7.0) and 800 μL of 1 M potassium iodide (KI) (Alexieva et al., 2001). The absorbance was measured at 390 nm for all samples (PerkinElmer Lambda 40), and the H_2O_2 content determined using known concentration as a standard curve. The results were expressed in μmol ml^{-1}.

2.5. Viability assays of S. scitamineum cells exposed to H_2O_2

We analyzed the viability of *S. scitamineum* exposed to H_2O_2 for 30 and 180 min (Fig. S1). Cells inoculated in YM medium were incubated at 28 °C in an orbital shaker set at 200 rpm until reaching early-exponential phase (5×10^7 - OD_{600} = 0.8) when different concentrations of H_2O_2 (2, 4, 6 and 8 mM) were added (Fig. S1). After 30 and 180 min in the presence or absence of H_2O_2, cells were diluted in fresh liquid YM to a concentration of 1×10^4 cells/mL and 20 μL plated in triplicate on solid YM plates. Viable cell counts were performed after incubation for 3 days at 28 °C. Tests were performed in biological triplicates. The concentration of 2 mM of H_2O_2 was chosen for further *in vitro* analysis (enzymatic activity assay and RT-qPCR) (Fig. S1).

2.6. Sporisorium scitamineum MDA and intracellular H_2O_2 content determination

For lipid peroxidation rate, we applied the method described by Heath and Packer (1968), where malondialdehyde (MDA) content is measured by the content of the thiobarbituric acid reactive substance (TBARS). *Sporisorium scitamineum* cells were centrifuged at 8000 rpm and 4 °C for 10 min. Frozen powdered cells (100 mg) of each independent treatment was homogenized in 1 mL of 0.1% (w/v) TCA solution and centrifuged at 12,000 *g* for 10 min at 4 °C. An aliquot of 250 μL of the supernatant from TCA extraction was added to 1 mL of a solution containing 20% (w/v) TCA and 0.5% (w/v) TBA. The samples were incubated for 30 min at 95 °C and then centrifuged for 5 min at 12,000 *g*. MDA content was monitored by absorbance measurements at 535 and 600 nm in a PerkinElmer Lambda 40 spectrophotometer, and the concentration calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. The results were expressed in nmol fresh weight g^{-1}.

To determine the content of intracellular H_2O_2, 100 mg of frozen powdered cells were homogenized in 1 mL of 0.1% (m/v) TCA. The homogenates were centrifuged at 12,000 *g* for 10 min at 4 °C, and 200 μL of the supernatant was added to 200 μL of 100 mM potassium phosphate buffer (pH 7.0) and 800 μL of 1 M potassium iodide (KI) (Alexieva et al., 2001). The absorbance was measured at 390 nm for all samples (PerkinElmer Lambda 40) and the H_2O_2 content was determined using a known concentration as a standard curve. The results were expressed in μmol fresh weight g^{-1}.

2.7. Superoxide dismutase and catalase activities

SOD and CAT activity assays were measured by grounding 400 mg of cells to a fine powder in liquid nitrogen and homogenized (3:1, buffer volume: fresh weight) in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol (DTT), 2 mM β-mercaptoethanol and 5% (w/w) polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged at 12,000 *g* for 30 min at 4 °C. The protein concentration was determined using bovine serum albumin as standard (Bradford, 1976).

SOD total activity was measured as described by Giannopolitis and Ries (1977) by determining the enzyme's ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction solution (3 mL) was composed of 75 μM NBT, 2 μM riboflavin, 13 mM methionine, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.8) and 50 μL enzyme extract. Negative control and enzyme extract were irradiated under light (15 W fluorescent lamps) for 5 min. Enzyme activity was determined spectrophotometrically (560 nm) and expressed as SOD units mg^{-1} protein. SOD activity staining was determined as described by Beauchamp and Fridovich (1971) and Garcia et al. (2006). The 12% non-denaturing polyacrylamide gels (PAGE) were loaded with 50 μg of protein extract, and one unit of bovine liver SOD (Sigma) was used as a positive control of the activity. The gel was incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium and 0.3% N,N,N',N'-tetramethylethylenediamine. After 30 min under gentle shaking, the gels were rinsed with distilled deionized water,

maintained in water and exposed to white light until the development of achromatic bands of SOD activity on a purple-stained gel.

CAT total activity was assayed as described by Gratão et al. (2012) at 25 °C in a reaction mixture of 1 mL 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 μL H_2O_2 (3% solution). The reaction was initiated by the addition of 25 μL of protein extract, and the activity was determined by following the decomposition of H_2O_2 according to changes in absorbance at 240 nm. CAT activity is expressed as μmol min^{-1} mg^{-1} protein. CAT activity following non-denaturing PAGE was determined as described by Boaretto et al. (2014). Gels were incubated in 0.003% H_2O_2 for 10 min and subsequently in a 1% (w/v) ferric chloride ($FeCl_3$) and 1% (w/v) potassium hexacyanoferrate III ($K_3Fe(CN_6)$) solution for additional 10 min. One unit of bovine liver CAT (Sigma, St. Louis, USA) was used as a positive control.

2.8. Statistical analysis for biochemical and sensitivity/viability assays

All experiments were performed in a completely randomized design. The significance of the observed differences was verified by using a one-way analysis of variance (ANOVA) followed by the Tukey's test ($p < 0.05$) for yeast cells exposed to H_2O_2 *in vitro* or by the Student's t test ($p < 0.05$) for significant differences between the control and yeast cells exposed to Paraquat 1 mM. All statistical analyses were performed using R software (URL http://www.r-project.org). In this study, all comparisons described in the results section were carried out between the treatment and its control (H_2O_2-30 min vs. control-30 min and H_2O_2-180 min vs. control-180 min).

2.9. Expression levels of S. scitamineum genes: yeast cells exposed to H_2O_2 in vitro

Total RNA was obtained from *S. scitamineum* sporidial cultures after 30 min ($OD_{600} = 0.85$) and 180 min ($OD_{600} = 1.0$) of H_2O_2 exposure using Trizol reagent (Invitrogen) and treated with DNase I (Thermo Scientific). RNA quality was verified both by agarose gel electrophoresis and by nanoCell spectrophotometer (Thermo Scientific). Primers manually designed were analyzed using Gene Runner (http://www.generunner.net/) and Beacon Designer™ Free Edition (http://www.premierbiosoft.com) (Table 1). All reactions were conducted in the 7500 Fast Real-Time PCR System (Applied Biosystems) using GoTaq® One-Step RT-qPCR System Kit (Promega). A reaction mixture containing 2 μL of a 25 ng solution of RNA, 6.5 μL of GoTaq® qPCR Master Mix, 0.2 μM of each primer, 0.25 μL of GoScript™ RT Mix and nuclease-free water to a final volume of 12.5 μL were used for three biological and two technical replicates. Cycling conditions were as follows: 37 °C for 15 min, 95 °C for 10 min; 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s. The *S. scitamineum* endogenous gene encoding GAPDH was used to normalize expression signals. PCR efficiencies and Ct values were obtained using the LinReg PCR program (Ramakers et al., 2003). Relative changes in gene expression ratios were calculated by REST software (Pfaffl et al., 2004). Control samples were used as calibrators.

2.10. Expression profile of fungal genes in sugarcane infected plants

Single-bud sets of 10-month-old healthy plants of IAC66-6 (susceptible to smut) and SP80-3280 (resistant to smut) genotypes were superficially disinfected (Carvalho et al., 2016) and a teliospore suspension ($7x10^7$ teliospores mL^{-1}) was drop inoculated according to Peters et al. (2017). Six buds of each genotype were collected in triplicates at 0, 24, 48 and 72 h post-inoculation (hpi) (Fig. S1). The samples were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using Trizol® Plus Direct-zol RNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions and treated with DNase I (Sigma Aldrich). RNA quality was verified by agarose gel electrophoresis and nanodrop spectrophotometer (Thermo Scientific). The cDNA synthesis was performed from 800 ng of total RNA and oligo (dT) primers using GoScript Reverse Transcription System (Promega). The cDNA was diluted (10-fold) and 2 μL was used as a starting sample to set up the reactions.

The qPCR reactions were conducted in the StepOnePlus System (Applied Biosystems) in a final volume of 12.5 μL using GoTaq® qPCR Master Mix (Promega). Thermal cycling parameters consisted of an initial preheating step for 2 min at 95 °C followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s for data collection and real-time analysis; then, the set point temperature was increased after cycle 2 from 60 to 95 °C in 0.3 °C increments to construct a melting curve. PCR efficiencies and Ct values were obtained using the LinReg PCR program (Ramakers et al., 2003) and control samples were used as calibrators. Relative changes in gene expression ratios of *sod*, *cat* and *gpx* were calculated by REST software (Pfaffl et al., 2004). *Sporisorium scitamineum* genes encoding for D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tubulin beta chain were used to normalize expression signals and the data is presented as the fold changes of the expression between resistant/susceptible plants. For *pep1*, *yap1* and *ap18b* the relative gene expression rates were calculated by the ΔCt method normalized with the *GAPDH* endogenous gene expression. The significance of the observed differences was verified using a one-way ANOVA followed by Tukey's test ($p < 0.05$). The expression of ap18 was used to monitor gene expression *in vitro* and in plant tissues. Ap18 is involved in normal vesicle trafficking (Wang and Shen, 2011).

3. Results

3.1. SOD, CAT and GPx encoded enzymes by the S. scitamineum genome

We first defined a set of antioxidant enzymes encoded by the *S. scitamineum* genome (Taniguti et al., 2015). Sequence analyses revealed three SOD (*g2980_chr07_Ss, g651_chr01_Ss,* and *g3299_chr08_Ss*); two CAT (*g1075_crh02_Ss and g4614_chr13_Ss)* and one GPx (*g1608_chr03_Ss*) protein (Table 2).

The evolutionary relationships of these enzymes encoded by various fungi revealed that SODs of *S. scitamineum* grouped with other smut fungi (*Sporisorium reilianum, U. maydis,* and *Ustilago hordei*) (Fig. 1A). Reference fungal SODs, such as those described for *C. albicans* and *Saccharomyces cerevisiae* (Mir et al., 2015), were used to classify *S. scitamineum* isoenzymes (Fig. 1A). All three isoforms (*g2980_chr07_Ss, g651_chr01_Ss*, and *g3299_chr08_Ss*) had domains described for Mn/Fe proteins (PF: PF00081: alpha N-terminal domain; PF02777: alpha/beta C-terminal domain) (Fig. S2). The SOD3 had two Mn/Fe C-terminal domains divided by an interval of 49 amino acids (Fig. S2). None of the enzymes encoded by *S. scitamineum* had a signal peptide.

CAT sequences separated into two distinct groups. The CAT encoded by g1075_chr02_Ss, closer to *U. hordei* UHOR 03771, is a member of this first group, classified as mono-functional CATs. The second group includes bifunctional peroxidase/catalase proteins, also present in *U. hordei*, but the *S. scitamineum* enzyme is closer to the *S. reilianum* sr14389 protein (Fig. 1B). Based on sequence similarity, we proposed that the genes *g1075_crh02_Ss* and *g4614_chr13_Ss* encode KatE (peroxisomal CAT) and KatG (cytoplasmic CAT), respectively. Sequence feature analysis of KatE

Table 2
Superoxide dismutase, catalase and glutathione peroxidase isoenzymes predicted of *S. scitamineum*.

Gene ID	Gene name	Protein domain[a]	Predicted Subcellular localization[b]	Molecular weight (kDa)[c]	Protein Description
g2980_chr07_Ss	*sod1*	Mn/Fe SOD	Cytoplasmic	26.6	Superoxide dismutase
g651_chr01_Ss	*sod2*	Mn/Fe SOD	Mitochondrial	27.9	Superoxide dismutase
g3299_chr08_Ss	*sod3*	Mn/Fe SOD	Cytoplasmic	32.7	Superoxide dismutase
g1075_crh02_Ss	*katE*	Immune-responsive domain/Haem domain	Peroxisomal	63.8	Catalase
g4614_chr13_Ss	*katG*	Haem domain/Haem domain	Cytoplasmic	82.8	Peroxidase/catalase
g1608_chr03_Ss	*gpx1*	–	Cytoplasmic	18.41	Glutathione peroxidase

[a] Protein domain was predicted using InterProScan (Zdobnov and Apweiler, 2001). Fe/MnSOD are SOD isoenzymes manganese co-factored (Mn/SOD) or iron co-factored (Fe/SOD).
[b] Subcellular localization was predicted using WoLF PSORT (https://wolfpsort.hgc.jp) (see Supplementary Table S3).
[c] Molecular weight was predicted using ExPASy ProtParam tool (Gasteiger et al., 2005).

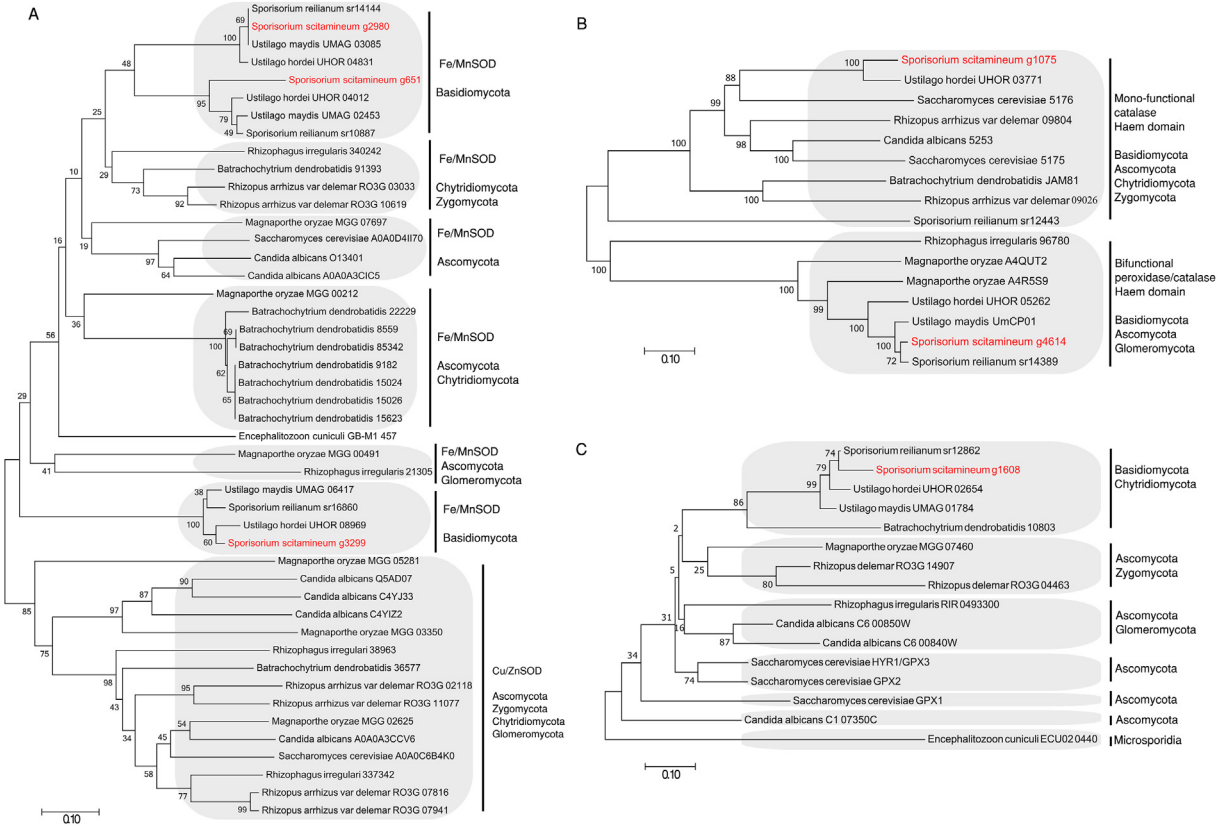

Fig. 1. Dendrogram of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) proteins in selected fungi of various phylum (Basidiomycota, Ascomycota, Chytridiomycota, Zygomycota, Glomeromycota, and Microsporidia). A neighbor-joining tree was constructed based on the sequences of these proteins using MEGA 6.0 software. Numbers at nodes represent bootstrap confidence values or percentage of clade occurrence in 2000 bootstrap replicates (A) *Sporisorium scitamineum* SODs proteins and that of other fungi are represented in red or black, respectively. Fe/MnSOD are SOD isoenzymes manganese co-factored (Mn/SOD) or iron co-factored (Fe/SOD). Cu–Zn/SOD are SOD isoenzymes copper-zinc co-factored (Cu–Zn/SOD) (B) CAT genes and characterized genes from other fungi are depicted in red or black, respectively (C) GPx protein and that of other fungi are represented in red or black, respectively. The scale bar represents the number of amino acid differences per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(*g1075_chr02_Ss*) revealed the presence of an immune response domain and a haem domain, while KatG (*g4614_chr13_Ss*) displayed two haem domains (Fig. 1B and S2, Table 2).

S.porisorium scitamineum encodes only one GPx (*g1608_chr03_Ss)* sharing 74.8% and 72.7% similarities with *S. cerevisiae* GPx2 and Hyr1/GPx3, respectively. GPx is clustered with orthologs of other smut fungi and is mostly related to *S. reilianum* (Fig. 1C).

3.2. Sequence analysis of Yap1 and Pep1

As described for the *S. cerevisiae* ortholog, *S. scitamineum* Yap1 (*g4200_chr11_Ss*) contains 2 cysteine-rich domains (CRDs) at the N-distal (N-CRD) and C-proximal (C-CRD) termini essential for its activation and localization (Lin et al., 2009) (Fig. S3). *Sporisorium scitamineum* Pep1 (*g1816_chr03_Ss*) shares 71% of sequence similarity with the well-characterized protein of *U. maydis* (Hemetsberger et al., 2012) involved in interfere with a maize class III peroxidase function to impair hydrogen peroxide (H_2O_2) production and defense responses leading to plant susceptibility.

3.3. ROS effects on the in vitro growth of S. scitamineum cells

3.3.1. Sporisorium scitamineum sensitivity to oxidative stress

We analyzed the *S. scitamineum* sensitivity to oxidative stress by exposing the fungal haploid cells to increasing concentrations of

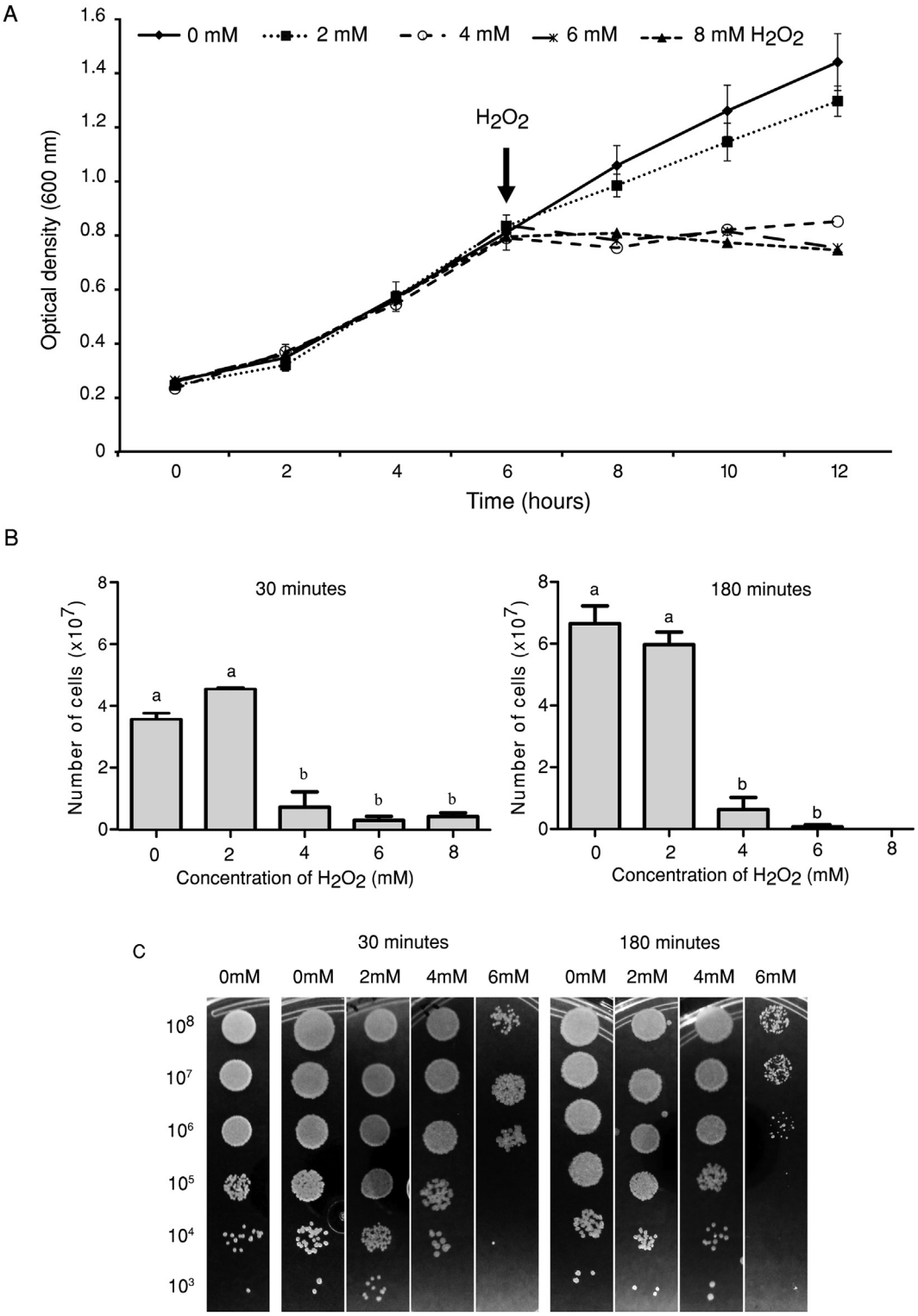

Fig. 2. Evaluation of *S. scitamineum* sensitivity to oxidative stress when exposed to H_2O_2 (A) *S. scitamineum* growth after exposition to increasing concentrations of H_2O_2 (2, 4, 6 and 8 mM - growth to the early-exponential phase). Error bars correspond to the standard deviations (SD) of measurements performed in triplicate (B) Cultures of 1×10^4 viable cells of *S. scitamineum* grown for 30 and 180 min in the presence of 0, 2, 4, 6 and 8 mM H_2O_2. Values represent the means from three replicates. Numbers with different letters are significantly different ($p < 0.05$) by one-way analysis of variance (ANOVA) and Tukey's test (C) *S. scitamineum* yeast cells grown in YM media for 30 and 180 min in the presence of H_2O_2 at 0, 2, 4 and 6 mM. For both times no cell growth was observed in the presence of H_2O_2 at 8 mM.

H_2O_2 and Paraquat (Fig. S1). Both treatments reduced cell growth in a dose-dependent manner (Fig. 2 and Fig. S4). At 2 mM H_2O_2, fungal colony morphology was slightly changed (Fig. S5) but were unaffected (Fig. 2). We chose this dose for the biochemical assays and gene expression analysis in order to understand how H_2O_2 is dealt with by cells growing *in vitro*. The addition of Paraquat reduced cell growth slightly at 1 mM and drastically 5 mM (Fig. S4).

3.3.2. Destination of exogenous H_2O_2 concentration

We evaluated the destination of exogenous H_2O_2 added into the YM medium during *S. scitamineum* growth in the presence of 2 mM H_2O_2. Fungal cells decomposed H_2O_2 that was absent in the medium after both 30 and 180 min of incubation (Fig. S6). H_2O_2-supplemented YM medium incubated without fungus for the same period did not alter peroxide concentrations (Fig. S6). Additionally, exogenous H_2O_2 levels did not change when the fungus was grown in a non-supplemented YM medium (Fig. S6).

3.3.3. Intracellular H_2O_2 concentration and membrane lipid peroxidation

The intracellular H_2O_2 levels of cells exposed to exogenous peroxide decreased to 28% after 30 min when compared to the control at 30 min (no H_2O_2), and were similar after 180 min (Fig. 3A). The same analysis performed with Paraquat (1 mM) led to significant changes in H_2O_2 intracellular concentration (decreased by 40%) when compared to the control (no Paraquat) (Fig. S7).

The oxidative damage of the cell membranes was measured indirectly through determining the malondialdehyde (MDA) content followed the same treatments described above (Fig. 3B). The results revealed altered MDA content (68%) at 30 min after H_2O_2 exposure, returning to levels similar to the control in a more extended period (180 min) (Fig. 3B). As expected, Paraquat 1 mM did not alter the MDA level of *S. scitamineum* yeast-like cells (Fig. S7). The prompt damage detected with hydrogen peroxide is symptomatic of the oxidative capacity in the presence of transition metals. However, Paraquat did not change SOD total activity, increased CAT activity and decreased the amount of hydrogen peroxide at the moment analyzed, suggesting that lipid peroxidation would not be affected readily.

3.3.4. ROS alter the antioxidant enzyme activities of S. scitamineum cells

We determined SOD and CAT activities in response to H_2O_2 and Paraquat (Fig. 4 and Fig. S8). Total SOD specific activity increased by 26% in fungal cells treated for 30 min with H_2O_2 in comparison to the control 30 min (no H_2O_2), while after 180 min, no significant changes were observed (Fig. 4A). SOD activity staining following non-denaturing PAGE revealed the presence of only one isoenzyme (I) in all treatments with H_2O_2 (Fig. 4B), whereas exposure to Paraquat revealed the presence of three isoenzymes (Fig. S8B). Our experiments were conducted at 4 °C, preventing partial protein degradation and confirming the presence of three genes encoding SODs in the *S. scitamineum* genome.

Cells exposed to H_2O_2 for 30 min (28% increase) and 180 min (30% increase) increased total CAT specific activity compared to its control (Fig. 4C). The assessment of CAT activity by non-denaturing PAGE revealed the presence of 2 isoenzymes (I and II) in all treatments. Further, for CAT, bands I and II increased in intensity when comparing treatments 30 min and 180 min (Fig. 4D). We observed similar results with Paraquat stress (Figs. S8C and D). However, the total CAT specific activity of *S. scitamineum* cells exposed to Paraquat, increased by 178% in comparison with its control.

3.4. Gene expression assays for in vitro and in vivo analyses

3.4.1. The expression of sod, cat and gpx

It was detected the upregulation of *sod1*, *sod2*, *katE* and *katG* in the presence of H_2O_2 *in vitro* after 30 min and returned to control levels 180 min after the exposure (Fig. 5). The expression data (Fig. 5A) were in agreement with the results of the biochemical activity of the *in vitro* assays (Fig. 4). Conversely, the fungus reduced *gpx1* gene expression when growing *in vitro*.

We observed different results analyzing each gene during infection of smut-susceptible and -resistant sugarcane plants 0, 24, 48, and 72 h post-inoculation (hpi) (Fig. 5B). The expressions of the *sod2* (predicted to encode a mitochondrial enzyme), *sod3* (predicted to encode a cytoplasmic protein), and *gpx* raised during fungal colonization of smut-resistant plants (Fig. 5B). Otherwise, *cat* genes did not alter their expression.

3.4.2. Expression pattern of fungal genes responsive to plant oxidative burst

The *yap1*-ortholog of *S. scitamineum* expressed 0 hpi, in both smut susceptible and resistant sugarcane genotypes (Fig. 6A). Colonization resulted in a decreased but regular expression (24, 48, and 72 hpi). Resistant and susceptible genotypes did not alter *yap1* expression regardless of the resistance level. There was no variation of *yap1* expression *in vitro* (Fig. 6B). However, *pep1* gene expression

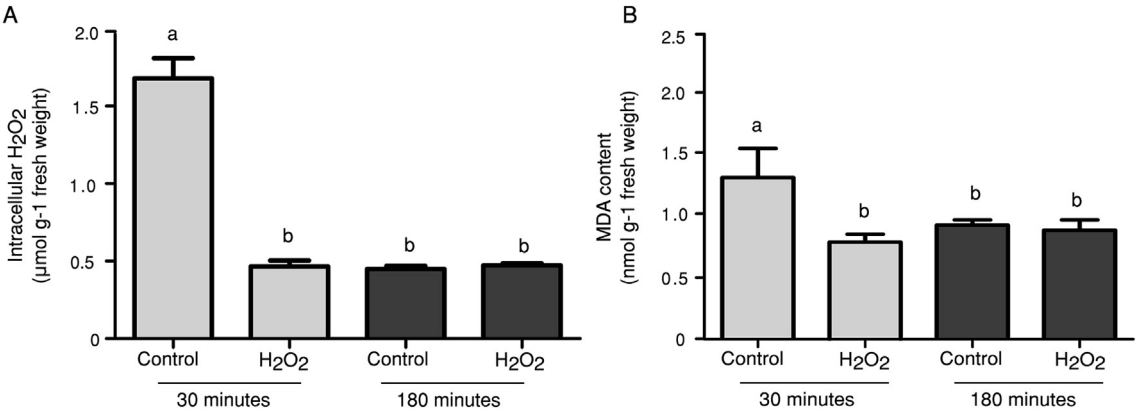

Fig. 3. Detection of the intracellular H_2O_2 and malondialdehyde (MDA) content of *S. scitamineum* yeast cells (A) The intracellular H_2O_2 content of yeast cells exposed to H_2O_2 for 30 and 180 min (B) MDA content of yeast cells exposed to H_2O_2 for 30 and 180 min. Values represent the means from three replicates. Letters show significance ($P < 0.05$) by one-way analysis of variance (ANOVA) and Tukey's test.

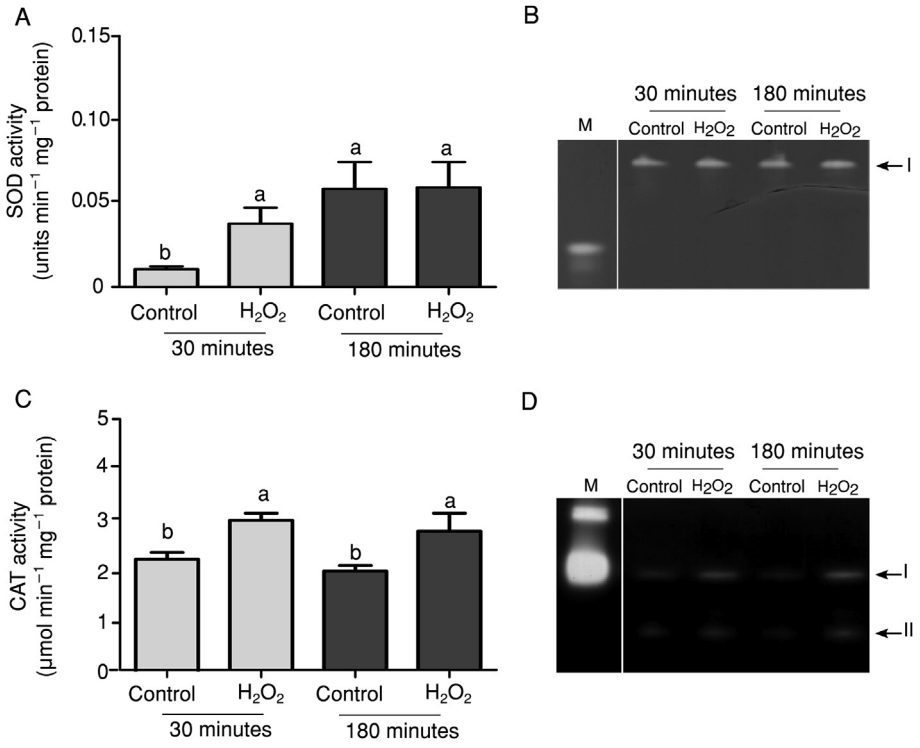

Fig. 4. SOD and CAT specific activity (A) The total specific activity of superoxide dismutase (SOD) (units min^{-1} mg^{-1} protein) of *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 min (B) Activity staining for SOD (units min^{-1} mg^{-1} protein) following non-denaturing PAGE of *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 min. First lane (M) is a bovine SOD standard and other lines are *S. scitamineum* yeast cells grown in the treatments: control (30 min), H_2O_2 (30 min), control (180 min) and H_2O_2 (180 min), respectively. Arrow indicates numbered SOD band for *S. scitamineum* cells (I) (C) The total specific activity of catalase (CAT) (µmol min^{-1} mg^{-1} protein) of *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 min (D) Activity staining for CAT (µmol min^{-1} mg^{-1} protein) following non-denaturing PAGE of *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 min. First lane (M) is a bovine CAT standard and other lines are *S. scitamineum* yeast cells grown in the treatments: control (30 min), H_2O_2 (30 min), control (180 min) and H_2O_2 (180 min), respectively. Arrows indicate numbered CAT bands for *S. scitamineum* cells (I and II). Values of total specific activity SOD and CAT represent the means from three replicates. Letters show significance ($P < 0.05$) by one-way analysis of variance (ANOVA) and Tukey's test.

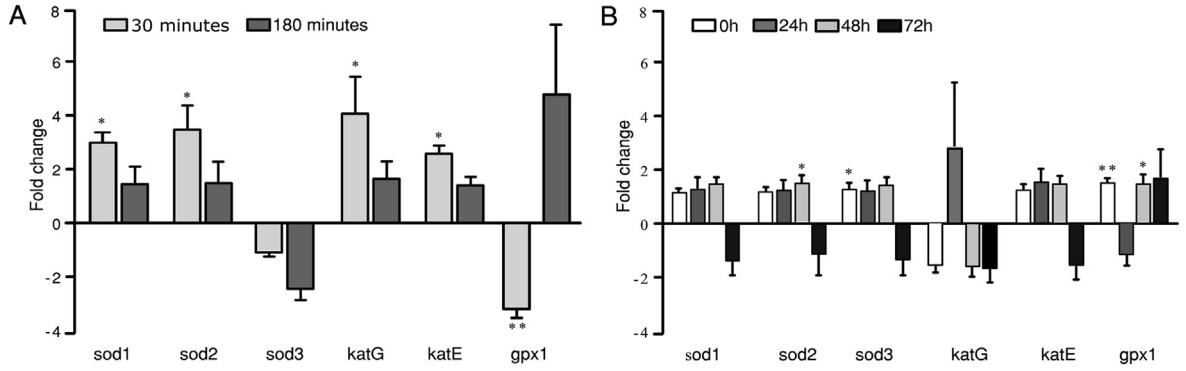

Fig. 5. Gene expression of *sod1* (g2980_chr07_Ss), *sod2* (g651_chr01_Ss), *sod3* (g3299_chr08_Ss), *katG* (g1075_crh02_Ss), *katE* (g4614_chr13_Ss) and *gpx1* (g1608_chr03_Ss) in *S. scitamineum* by RT-qPCR analysis (A) *In vitro* expression profiling of antioxidant genes in *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 min (B) *In planta* expression profiling of antioxidant genes in *S. scitamineum* infecting sugarcane plants resistant and susceptible at 0, 24, 48 and 72 h post-inoculation (hpi). Statistical analysis performed in REST® software. Asterisks represent genes differentially expressed in RT-qPCR reactions (** *p*-value < 0.01; * *p*-value < 0.05). *In planta* data is presented as the fold changes of the expression between resistant/susceptible plants.

was modulated in a genotype-dependent manner. The expression increased 24 hpi, reaching six times higher 48 hpi in the smut resistant genotype. In the susceptible genotype, *pep1* expression raised 48 hpi (Fig. 6A).

The gene *ap18B* encoding for a protein involved in vesicle assembly of endo and exocytosis pathways (Wendland and Emr, 1998) had an expression pattern increased with fungal colonization of plant tissues independent of the plant genotype (Fig. 6A). Otherwise, the expression did not change *in vitro* (Fig. 6B). The gene ap18B indicated fungal growth in all conditions.

4. Discussion

One of the first plant responses to a pathogen attack is the oxidative burst as a result of pathogen recognition (Torres et al., 2006). The effect of ROS accumulation in different plant pathosystems involving fungal infections is not entirely understood.

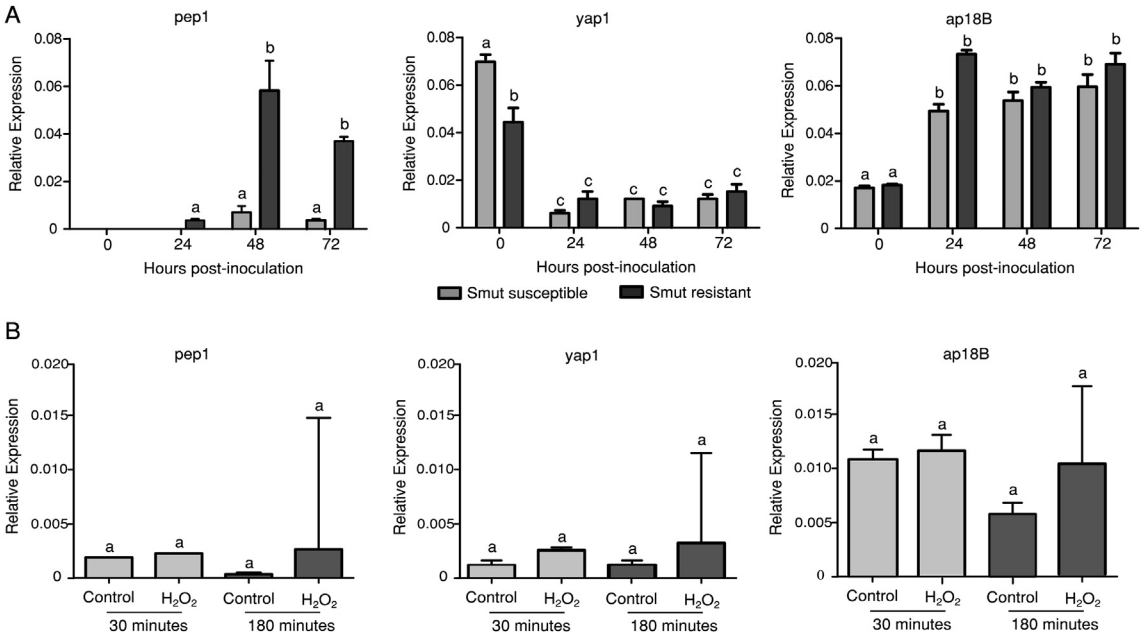

Fig. 6. Gene expression of *pep1* (g1816_chr03_Ss), *yap1* (g4200_chr11_Ss) and *ap18B* (g3636_chr09_Ss) in *S. scitamineum* by RT-qPCR analysis (A) *In planta* expression profiling of *pep1*, *yap1* and *ap18B* genes in *S. scitamineum* infecting sugarcane plants resistant and susceptible at 0, 24, 48 and 72 h post-inoculation (hpi) (B) *In vitro* expression profiling of *Pep1*, *yap1* and *ap18B* genes in *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 min. The delta Ct method was used. Different letters show significant differences at $P < 0.05$ after a one-way analysis of variance (ANOVA) followed by Tukey's test. Error bars represent the standard deviation of three biological replicates.

Notably, for biotrophic pathogens, there are distinct responses considering the effect of ROS over pathogen growth and virulence (Barna et al., 2012; Chang et al., 2018; Glazebrook, 2005; Mellersh et al., 2002). Previously, we observed distinct patterns of ROS accumulation between smut-susceptible and -resistant plants infected with *S. scitamineum* (Peters et al., 2017). The waves of increased levels of hydrogen peroxide (H_2O_2) in resistant plants co-occurred with teliospore germination and appressorium formation (Peters et al., 2017). To provide further insights into these early moments of contact between *S. scitamineum* cells and sugarcane tissues, we set out to examine how the fungus reacts in the presence of exogenous sources of ROS.

4.1. Sporisorium scitamineum *catalases are responsive to H_2O_2-stress* in vitro

The fungal antioxidant system studied *in vitro* showed that *S. scitamineum* cells overcame stress imposed by peroxide (2 mM) at levels above those encountered during colonization of sugarcane tissues (Peters et al., 2017). The meristem tissues of smut-infected plants increase H_2O_2 concentration from 23 μM to 90 μM depending on the resistance status of the genotypes tested (Peters et al., 2017). The assay performed to ascertain the stability of the peroxide concentration *in vitro* revealed that *S. scitamineum* deals with high concentrations of exogenous stress (Fig. S6). The results showed an ability to decompose H_2O_2 as fast as described for necrotrophic pathogens such as *M. fijiensis* (Beltrán-García et al., 2009) and *B. cinerea* (Gil-ad and Mayer, 2002).

Non-denaturing PAGE-gel staining for enzyme activity revealed three and two isoforms for SODs and CATs, respectively, which is in agreement with the number of genes identified in the genome. As expected, the activities were differentially detected when the fungus was growing under stress imposed by either peroxide or Paraquat (Apel and Hirt, 2004; Gessler et al., 2007; Halliwell, 1991). Only the exposure to Paraquat revealed all the SOD isoforms.

Sporisorium scitamineum has previously shown a differential response to peroxide when exposed to 2.5 mM of peroxide in mating conditions compared to growth as single mating-type cells (Yan et al., 2016). Haploid cells were more resistant to peroxide than the dikaryon growing *in vitro*. The reason for the sensitivity of hyphal extension to H_2O_2 is unclear. However, *S. scitamineum* hyphae delay growth and visibly accumulate peroxide vesicles when infecting sugarcane resistant plants (Peters et al., 2017). Recently, a fungal elevated intracellular ROS was associated with its differentiation, and virulence (Chang et al., 2018). Some ROS catabolic enzymes (catalases and thioredoxin peroxidase) of *S. scitamineum* were shown to be under the control of components of the conserved cAMP/PKA pathway. The authors also showed that the elevation of intracellular ROS levels is required for virulence since ROS seemed to induce fungal developmental changes.

Our results indicated that *S. scitamineum* activates distinct antioxidant mechanisms according to the fungal developmental stages; when growing *in vitro* and when colonizing plant tissues; and of smut-susceptible or -resistant genotypes. Although the expression of CAT genes was affected during *in vitro* growth, they were not responsive when the fungus colonized sugarcane tissues. *In vitro* growth mostly changed CAT activity (mainly CAT I, Fig. 4D).

None of the enzymes produced by *S. scitamineum* had signal peptides and they are probably not secreted by conventional processes. Possibly, *S. scitamineum* converts the H_2O_2 intracellularly. Based on sequence similarity, the *S. scitamineum* CAT I is a cytoplasmic peroxidase/catalase (82.4 kDa) product of the katG gene expression. Catalase/peroxidases are relevant for the tolerance to exogenous H_2O_2 (Bienert et al., 2007; Gao et al., 2018). The intracellular content of H_2O_2 decreased when *S. scitamineum* cells were exposed to H_2O_2 for 30 min, which coincides with increased CAT activity, whereas after 180 min, H_2O_2 content entered in redox homeostasis restoring the initial environment. These results suggested an adjustment of the antioxidant system in the first 30 min and that an adaptive response occurs after 180 min of exposure (González-Párraga et al., 2003; Jamieson et al., 1996; Komalapriya

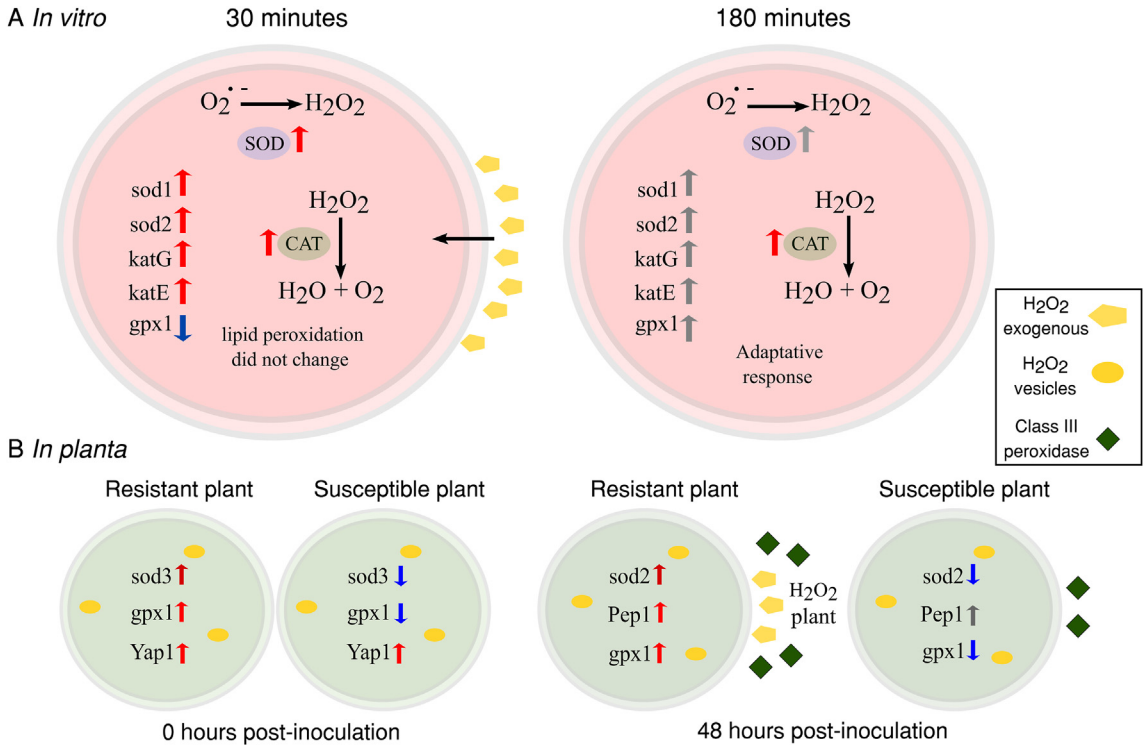

Fig. 7. Proposed model for ROS catabolic enzymes response of *S. scitamineum* exposed to exogenous stress (A) *Sporisorium scitamineum* under exogenous H_2O_2 stress for 30 and 180 min (B) *Sporisorium scitamineum* infecting sugarcane at 0 and 48 h post-inoculation. Red arrows represent increases in enzymatic activity and gene expression, whereas blue arrows show decreases in enzymatic activity and gene expression. Gray arrows indicate no alterations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2015; Marschall et al., 2016; Sethiya et al., 2019). There was no increase in the internal peroxide concentration or significant lipid damage after prolonged exposure with both stress-agents, which is probably essential for fungal survival *in vitro* (Beltrán-García et al., 2009).

4.2. *Cells growing* in vitro *and colonizing plant tissues induce a different combination of genes related to ROS defense*

To investigate which genes of the *S. scitamineum* genome were involved, we first selected the complete set of SOD, CAT, and GPx-encoding genes using sequence similarity searches (BLAST). We determined the expression profile of each gene upon H_2O_2 exposure. *In vitro,* the cells responded to H_2O_2 up-regulating gene expression of *katG*, *katE*, *sod1*, and *sod2*, but not *sod3*, whereas *gpx1* was down-regulated 30 min after the exposure. CAT and SOD activity assays supported the results of gene expression.

Sporisorium scitamineum colonizing plant tissues did not alter either catalase gene expression but induced in the resistant genotype *gpx1* and *sod2* at 48 hpi. Previous work determined that the oxidative burst only occurs in resistant plants infected with *S. scitamineum* (Peters et al. 2017). In the resistant genotype, the second peak of peroxide increase began at 48 hpi reaching higher levels at 72 hpi, by which time 50% of the spores had germinated and had produced an appressorium. In the susceptible genotype, no oxidative burst was detected, and appressorium formation was initiated at 12 hpi (Peters et al. 2017).

In bacteria and fungi, the induction of distinct types of peroxide-degrading enzymes enables them to detoxify H_2O_2 at a wide range of concentrations as well as other organic peroxides (Hansberg et al., 2012; Jittawuttipoka et al., 2009). Gpx is known to convert reduced glutathione and H_2O_2 to oxidized glutathione and water and is more effective than catalase for detoxifying H_2O_2 at low levels (Blondet et al., 2018), which seems to be the case in *S. scitamineum* colonized tissues of sugarcane resistant plants (Peters et al., 2017). Our work suggests that *S. scitamineum* orchestrates different mechanisms of peroxide detoxification, and plant colonization requires *gpx1* instead of *katE* or *katG*.

Although we did not assess all the components of the *S. scitamineum* antioxidant system, the results so far indicate that the regulatory network underlying the defense response *in vitro* and *in planta* have different switches. Other non-enzymatic antioxidants may also take part in the process that were not considered in our work.

4.3. *Other elements of the antioxidant system may be involved in ROS detoxification*

The protein Gpx encoded by *S. scitamineum* genome is closely related to Gpx2 and Gpx3 of *S. cerevisiae* (Paulsen and Carroll, 2009). The protein sequences of Gpx2 and Gpx3 of *S. cerevisiae* are very similar, varying by a few amino acids. GPx3 has been extensively studied and proven to form a redox relay system with the transcription factor Yap1 (Paulsen and Carroll, 2009). Orthologs of Yap1 show distinct roles in the various pathosystems studied regarding pathogenicity (Temme and Tudzynski, 2009) and fungal development all stages, including spore germination (Guo et al., 2011). In *U. maydis*, YAP1 is relevant for virulence due to its role in ROS defense (Molina and Kahmann, 2007) and in regulation of intracellular ROS levels essential for fungal development in host tissues (Egan et al., 2007; Giesbert et al., 2008). Here we show that *S. scitamineum yap1* was up-regulated upon germination in plant tissues independently of the plant genotype and remained constant in all *in vitro* treatments. *Yap1* of *S. scitamineum* appears to be

related to ROS-related internal redox associated with fungal development rather than with the stress provoked by external sources. Our data suggest no correlation of *yap1* expression and other genes associated with ROS defense, as shown for other systems (Lev et al., 2005; Lushchak et al., 2010). In some filamentous fungi, cell development can be related to the production of ROS, the *yap1* gene expression and enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) (Mendoza-Martínez et al., 2019). These enzymes transfer electrons from NADPH to produce superoxide, which can consequently produce H_2O_2. Studies showed that *A. alternata* mutants lacking NoxB or its regulatory subunit NoxR had a drastic decrease in conidiation and the *yap1* expression was reduced (Yang and Chung, 2013, 2012).

We did not explore the actual role of *yap1* and *pep1* in our studies. However, here we detected that *yap1* and *pep1* expression behavior *in vitro* were not responsive to external H_2O_2 stress in the conditions we used. Nevertheless, *pep1* showed induced expression during tissue colonization of resistant plants at 48 hpi. We hypothesized that, as described for other smut fungi, there is an attempt of the fungus to control type III peroxidases and, consequently, ROS production in the resistant genotype (Doehlemann et al., 2009; Peters et al., 2017). More detailed analysis and functional experiments need to address the roles of *yap1* and *pep1* in the sugarcane-smut pathosystem.

5. Conclusions

The extent of ROS influence in fungal development is still under investigation but seems to play important roles in the biotrophic lifestyle previously unanticipated. Herein we presented some of the players involved in the antioxidant system and how they act when the fungus is challenged growing *in vitro* and during colonization of plant tissues (Fig. 7). ROS activated a different combination of the *S. scitamineum* enzymes SOD, CAT and Gpx when growing *in vitro* and in plant tissues to face environment and internal changes in redox state. Previously identified genes responsive to ROS in other smut fungi, were also responsive in *S. scitamineum* but showing a slightly different pattern of expression. *Yap1* seems to be related to internal fungal stress and it was not correlated to *cat*, *sod* and *gpx* gene expression.

Pep1, however, was induced in smut resistant plants suggesting a role in host tissue colonization. Neither *yap1* nor *pep1* had their expressions modulated *in vitro* in the presence of ROS. The mechanisms involved in the expression of these two genes remain to be established but are likely to be regulated in a host-dependent manner.

Author contributions

L.P.P., G.C. and C.B.M-V. designed the experiments. L.P.P., N.S.T-S., A.P.B., M.M.L.S., N.M., G.S.C. and G.C. conducted the experiment. L.P.P., N.S.T-S., A.P.B., G.C. and C.B.M-V. interpreted the data and prepared the manuscript. R.A.A., G.C., and C.B.M−V supervised the study and edited the manuscript.

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Declaration of competing interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2020.09.009.

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