University of São Paulo
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A more detailed view of reactive oxygen species metabolism in the sugarcane and *Sporisorium scitamineum* interaction

Leila Priscila Peters

Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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To my loves…

Odilon and Dircéa;

Bethânia, Diogo, Danilo, Ana and Moisés.

With love I dedicate this work.

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Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

(Albert Einstein)

CONTENTS

RESUMO

Uma visão mais detalhada do metabolismo de espécies reativas de oxigênio na interação cana-de-açúcar e *Sporisorium scitamineum*

Cana-de-açúcar (*Saccharum* spp) é uma importante cultura comercial amplamente cultivada em países tropicais e subtropicais. A cana-de-açúcar é principalmente utilizada para produzir açúcar e recentemente é considerada uma valiosa fonte para produção de bioetanol, biodiesel, bioplásticos e bioeletricidade. O carvão é uma das doenças mais graves da cana-de-açúcar e ocorrem em canaviais do mundo inteiro. A doença é causada pelo fungo biotrófico *Sporisorium scitamineum*. Este fungo induz mudanças metabólicas na planta, levando a formação de uma estrutura chamada chicote, onde ocorre a esporogênese. O objetivo desse estudo foi analisar a produção de espécies reativas de oxigênio (EROs), atividade de enzimas antioxidantes e a expressão de genes associados ao metabolismo de EROs em genótipos de cana-açúcar susceptível (IAC66-6) e resistente (SP80-3280). Além disso, este trabalho avaliou a relação entre as enzimas antioxidantes e sensibilidade de *S. scitamineum* a peróxido de hidrogênio (H_2O_2) exógeno. Esta tese está apresentada no formato de 2 capítulos (capítulos 2 e 3). No segundo capítulo, os resultados revelaram que ocorreram alterações no sistema antioxidante, bem como na produção de EROs no genótipo resistente, enquanto que poucas mudanças ocorreram no genótipo susceptível inoculado com *S. scitamineum*. Análises de microscopia revelaram que a germinação de teliósporos e a formação de apressórios de *S. scitamineum* atrasou durante o início da infeção no genótipo resistente ao carvão, coincidindo com o acúmulo de H_2O_2. No capítulo 3, os resultados demonstraram que *S. scitamineum* é altamente resistente a H_2O_2 exógeno. O fungo crescendo na concentração de 2 mM de H_2O_2 apresentou um eficiente sistema antioxidante em resposta a produtos secundários do estresse oxidativo. Além disso, quando *S. scitamineum* foi exposto a 2 mM de H_2O_2 exógeno, ele pode adquirir uma resposta adaptativa ao H_2O_2. Os resultados obtidos neste estudo contribuíram para aumentar o entendimento dessa complexa interação entre cana e *S. scitamineum* e será útil para a compreensão de quais aspectos estão envolvidos na resistência a este fungo. Estas informações são importantes para criar estratégias para o melhoramento de cana a essa doença.

Palavras-chave: Peróxido de hidrogênio; Enzimas antioxidante; Estresse biótico; Fitopatógeno; Explosão oxidativa; Estresse oxidativo

ABSTRACT

A more detailed view of reactive oxygen species metabolism in the sugarcane and *Sporisorium scitamineum* interaction

Sugarcane (*Saccharum* spp) is an important commercial crop cultivated widely in tropical and subtropical countries. Primarily sugarcane is used to produce sugar and recently it is proven to be a valuable resource for bioethanol, biodiesel, bioplastic and bioelectricity. Smut is one of the most serious sugarcane disease and occurs in sugarcane fields all over the world. The disease is caused by the biotrophic fungus *Sporisorium scitamineum*. The fungus induces metabolic changes in the plant leading to the production of a whip-like structure where fungal sporogenesis take place. The objective of this study was to analyse the reactive oxygen species (ROS) production, antioxidant enzymes activity and expression of genes associated with the ROS metabolism in smut susceptible (IAC66-6) and resistant sugarcane genotypes (SP80-3280). In addition, this work assessed the relationship between antioxidant enzymes and sensitivity of *S. scitamineum* to exogenous hydrogen peroxide (H_2O_2). This thesis is presented in the format of two chapters (chapters 2 and 3). In the second chapter, the results revealed that there were variations in the antioxidant system as well as in the ROS production in resistant sugarcane genotype, whereas few changes occurred in the susceptible genotype inoculated with *S. scitamineum*. Microscopic analysis revealed that *S. scitamineum* teliospore germination and appressorium formation were delayed during early infection in the smut resistant genotype, which coincided with H_2O_2 accumulation. In chapter 3, the results demonstrated that *S. scitamineum* is highly resistant to exogenous H_2O_2. At 2 mM exogenous H_2O_2 concentration the fungus presented an effective antioxidant system in response to the secondary products of oxidative stress. Furthermore, *S. scitamineum* when exposed for a long time at 2 mM exogenous H_2O_2 concentration it can acquire an adaptive response to H_2O_2. The results obtained in this study contributed to increase the understanding of this very complex interaction between sugarcane and *S. scitamineum* and it will be helpful toward understanding which aspects are involved in the resistance to *S. scitamineum*. These informations are important to create strategies for improving smut resistance in sugarcane.

Keywords: Hydrogen peroxide; Antioxidant enzymes; Biotic stress; Phytopathogen; Oxidative burst; Oxidative stress

1 INTRODUCTION

The modern sugarcane (*Saccharum* spp.) is derived from crosses between *Saccharum officinarum*, a domesticated sugar-producing species and *Saccharum spontaneum*, a wild species and it became one of the most economically important crop worldwide (ARRUDA, 2012). In 2013, sugarcane ranked fifth in production worldwide, after rice, wheat, soybeans and tomatoes (FAOSTAT, 2013), and it is found in 90 tropical and subtropical countries. The importance of this crop is associated with its multiple applications, from its use *in natura* as forage for animal feeding to produce alcoholic beverages, sugar, ethanol, bioplastic and biodiesel. Furthermore, the byproducts of the sugarcane industry might be used for the development of synthetic fibers for the textile industry, as well as for the production of second generation ethanol (COSTA et al., 2013). Brazil stands out as the world's largest sugarcane producer, reaching approximately 659 million tons in the 2014/15 harvest. The state of São Paulo is the main producer accounting for 51.82% among the Brazilian states (CONAB, 2013).

Sugarcane productivity as any other cultivated plants may be affected by several abiotic and biotic factors. Diseases such as sugarcane rust, ratoon stunting disease, leaf scald and smut represent are among the main biotic stresses of this crop (BARBASSO et al., 2010; ROTT; GIRARD; COMSTOCK, 2013; TANIGUTI et al., 2015; CARVALHO et al., 2016). Smut is a disease caused by the biotrophic fungus *Sporisorium scitamineum*, which leads to reduced culm diameter and development, reduced number of tillers that can be industrialized, losses in sucrose content, and causes a restriction in the use of highly productive sugarcane varieties (LEE-LOVICK, 1978; RAGO; CASAGRANDE; MASSOLA-JÚNIOR, 2009).

S. scitamineum grows within the host meristem tissues and induces the formation of reproductive structures, the teliospores, in the apical region of the plant (whip – main disease symptom) (SANTIAGO et al., 2012; SUNDAR et al., 2012). The life cycle of the pathogen involves teliospore germination in the surface of sugarcane buds (SANTIAGO et al., 2009; TANIGUTI et al., 2015). During germination, the diploid teliospore undergo meiosis generating four sporidial cells (haploid) (Figure 1). The anastomosis of two sexual compatible sporidial cells creates an infective dikaryotic hyphae. This process depends on the type of sexual reaction (*mating-type)*, which consists in the fusion of two sporidia belonging to opposite sexual groups (*a* and *b*). Subsequently, fungal hyphae differentiate appressorium structures to penetrate plant tissues (TANIGUTI et al., 2015; YAN et al., 2016). This set of events usually occurs between 6 and 36 h after teliospore deposition in the sugarcane surface

(SUNDAR et al., 2012). It is reported that the rates and patterns of colonization of *S. scitamineum* differ in resistant and susceptible sugarcane tissues (CARVALHO et al. 2016) and the use of resistant varieties is the more effective approach to control smut in sugarcane.

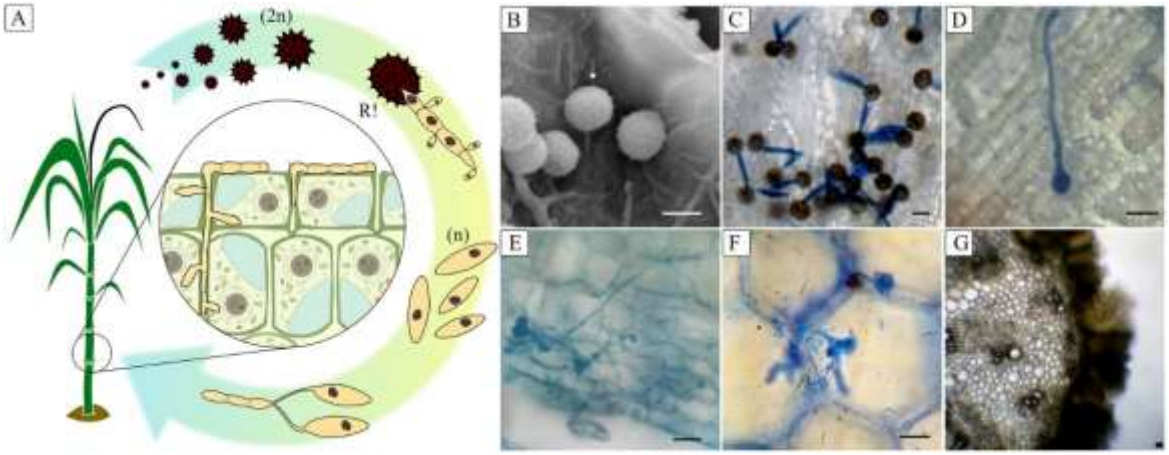

Figure 1 – A - Developmental stages in the *S. scitamineum* life cycle: diploid teliospores (2n); haploid yeast-like sporidia (n) after meiosis (R!); mitosis (E!); dikaryotic mycelium (n+n) after anastomosis. B - Scanning electron microscopy (SEM) image of spores adhered to sugarcane bud surface. C - Germination of spores on bud scale epidermis and tube-like promycelium formation visualized at 6 hai (hours after inoculation); light microscopy and image of tube-like promycelium stained with lactophenol-cotton. D - Light microscopy image of *S. scitamineum* intracellular growth on parenchyma cells of white whip portion; stained with lactophenol-cotton blue. E - SEM image of appressorium-like visualized on bud scale epidermis at 24 hai; arrow show appressorium. F - Light microscopy image of *S. scitamineum* growth on parenchyma cells of bud tissue observed at 120 hai stained with lactophenol-cotton blue. G - Light microscopy image of black whip portion showing the mature spore liberation. Scale bar = 5 µM (TANIGUTI et al., 2015)

Generally, the defense system in plants, besides containing physical barriers such as cuticle and cell wall, has molecular mechanisms that can be activated upon recognition by specific receptors for pathogen-associated molecular patterns (PAMPS) during interaction (LAO et al., 2008; MITTLER et al., 2011; O, BRIEN et al., 2012). Following recognition, another important factor for the defense system is the rapid ROS production (oxidative burst), which occurs at the beginning of the process of plant-pathogen interaction (TORRES et al., 2010). ROS, comprised by the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and *singlet* oxygen ($^1O_2$), play a dual role in plants regarding the defense activation against pathogen attacks, which function as key regulator and toxic compounds for many biological processes (CAO et al., 2012).

During plant-pathogen interaction, ROS can act as local toxins, as well as strengthen the host cell wall by promoting the formation of crosslinks with structural proteins – by participating in the synthesis of physical barriers such as lignification, suberization and

formation of papillae near the infection site (MITTLER et al., 2002). Moreover, another important aspect is the H_2O_2 participation as secondary messenger. This molecule is the most stable reactive species and it is promptly transported through the membrane. Thus, it can modulate the expression of resistance genes and proteins associated with pathogenicity and participate in the signalization network of hormones, such as ethylene, jasmonate and salicylic acid (TORRES et al., 2006).

However, due to the ROS toxicity to the own plant system, there are several antioxidant enzymes and compounds that can effectively scavenge these molecules (APEL; HIRT, 2004). Therefore, alterations in activity of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and compounds such as glutathione and ascorbate, contribute to host resistance against pathogens (MITTLER, 2002; GRATÃO et al., 2005). Major ROS-scavenging mechanisms of plants include SOD, CAT and APX. SOD catalyzes the dismutation of $O_2^{-\bullet}$ into H_2O_2, whereas CAT and APX play crucial roles in the detoxification process of H_2O_2 (Mittler, 2002). The balance between SOD, CAT and APX activities in plant cells is important for determining the steady-state level of these ROS (O'Brien et al., 2012). The antioxidant enzyme thioredoxin (Trx) regulates the function of target proteins through oxidoreductase activity. This enzyme can couples with Trx-dependent peroxidases (Prx) to scavenge H_2O_2 (SEVILLA et al., 2015). In plant-pathogen interactions, Trx are required to catalyse the conversion of the salicylic acid-induced nonexpressor of PR genes 1 (*NPR1*) into a monomer and to activate defence responses (TADA et al., 2008). Furthermore, GSTs enzymes can detoxify pathogen toxins and reduce lipid hydroperoxides, being recognized as important plant defences (LIEBERHERR et al., 2003) (Figure 2).

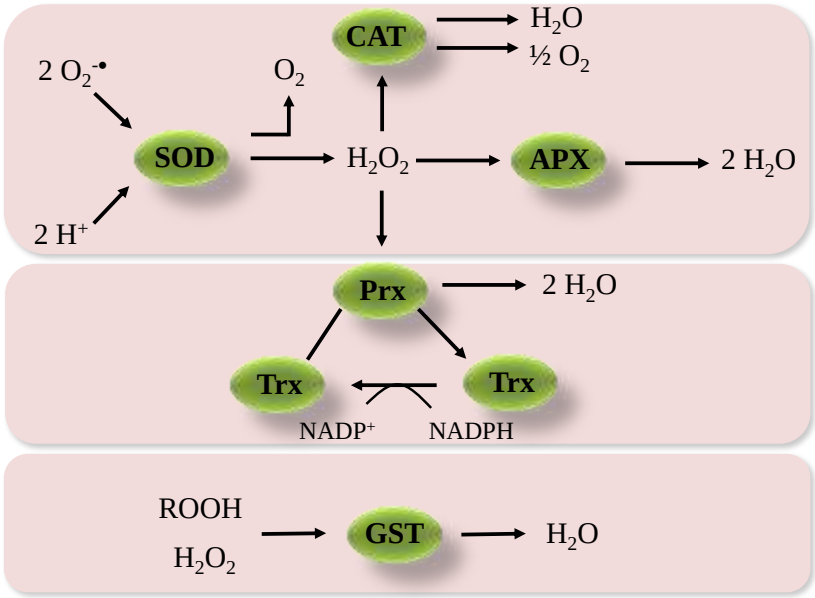

Figure 2 – Some examples of mechanisms for reactive oxygen species-scavenging in plants. Antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (Prx), thioredoxin (Trx) and glutathione S-transferase (GST)

Many efforts have been developed to understand the biochemical and molecular mechanisms in the sugarcane and *S. scitamineum* interaction. For example, this fungus leads to a premature transcriptional reprogramming of the shoot meristem functions continuing until the emergence of the whip (SHACKER et al., 2016). Moreover, the consequences associated with whip emission are modulation of typical meristematic functions toward reproductive organ differentiation requiring strong changes in carbon partitioning and energy production (SHACKER et al., 2016). Also, some studies suggested that effectors as chorismate mutase from *S. scitaminuem* might channelize chorismate to the phenylpropanoid pathway, thus reducing its availability for salicylic acid (SA) biosynthesis in infected sugarcane cells (TANIGUTI et al., 2015; BARNABÁS et al., 2016). Additionally, resistant sugarcane genotypes may present chemical barriers, such as the presence of phenylpropanoids, flavonoids (LLOYD AND NAIDOO, 1983, FONTANIELLA et al., 2002; MILLANES et al., 2005; DE ARMAS et al., 2007), free and conjugated glycoproteins and polyamines increased in sugarcane buds (LEGAZ, et al., 1998; MILLANES et al., 2008). Yet, in sugarcane, genes associated with defense were differentially expressed earlier in the smut resistant variety in comparison to the smut susceptible one (QUE et al., 2014). Oxidative burst and antioxidant system were also listed among the mechanisms involved in the sugarcane-smut interaction (LAO, et al., 2008; SU et al., 2014). However, little information is still available depicting the

oxidative burst and ROS metabolism, as well as the *S. scitamineum* development in smut resistant and susceptible sugarcane.

Similarly, plant pathogens have an efficient antioxidant system in order to overcome the oxidative burst and infect the host (CHAI et al., 2009; HELLER; TUDZYNSKI, 2011). *S. scitamineum* modulates transcription of genes (SOD and CAT) related to surviving against ROS and other toxic metabolites produced by the plant (TANIGUTI et al., 2015). Additionally, in smut fungi a novel effector called protein essential during penetration 1 (Pep1) has been identified and characterized (HEMETSBERGER et al., 2015). In *Ustilago maydis* the Pep1 effector protects fungal hyphae from the oxidative burst and ROS driven by class III peroxidases, which are major components of the plant immune response (HEMETSBERGER et al., 2012). Class III peroxidases in the cell wall can be a source of apoplastic H_2O_2 in the presence of a reductant released from responding cells, contributing for oxidative burst in host (TORRES et al. 2006).

Although there were substantial increment in studies approaching the sugarcane-*S. scitamineum* interaction for the last years, at the present moment, there are few studies comparing ROS and antioxidant enzymes in susceptible and resistant sugarcane genotypes upon the inoculation with *S. scitamineum*. Therefore, the general objective of the present work was to obtain a detailed view of ROS production and the involvement of genes and proteins associated to ROS metabolism in the sugarcane resistance to *S. scitamineum*. To achieve the objective, this work was divided into two steps, which are presented here as two independent chapters.

The objective of the first step (chapter 2) was to identify at which time point occurs the increase of ROS production (oxidative burst), the antioxidant enzymes activities responses, gene expression and protein inductions or repressions associated with the ROS metabolism in smut susceptible (IAC66-6) and smut resistant (SP80-3280) sugarcane genotypes. The novelty of this chapter was to relate the *S. scitamineum* development to the ROS metabolism in early infection of the fungus, using microscopic analysis and biochemical and molecular tools.

In the second step (chapter 3) the main goal was to assess the relationships among antioxidant enzymes and sensitivity of *S. scitamineum* to exogenous H_2O_2, and obtain comprehensive information about the impact of exogenous H_2O_2 on the different SOD and CAT isoenzymes of *S. scitaminuem* through quantification of gene expression analysis and enzyme activities.

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2 FUNCTIONAL ANALYSIS OF OXIDATIVE BURST IN SUGARCANE SMUT-RESISTANT AND -SUSCEPTIBLE GENOTYPES

Abstract

Sporisorium scitamineum is the causal agent of sugarcane smut disease. In this study, we characterized sugarcane reactive oxygen species (ROS) metabolism in response to the pathogen in smut-resistant and -susceptible genotypes. *S. scitamineum* teliospore germination and appressorium formation coincided with H_2O_2 accumulation in resistant plants. The superoxide dismutase (SOD) activity was not responsive in any of the genotypes; however, a higher number of isoenzymes was detected in resistant plants. Additionally, related to resistance were lipid peroxidation, a decrease in catalase (CAT) and an increase in glutathione S-transferase (GST) activities and an earlier transcript accumulation of ROS marker genes (*CAT3*, *CATA*, *CATB*, *GST31*, *GSTt3* and *peroxidase 5-like*). Furthermore, based on proteomic data, we suggested that the source of the increased hydrogen peroxide (H_2O_2) may be due to a protein of the class III peroxidase, which was inhibited in the susceptible genotype. H_2O_2 is sensed and transduced potentially through overlapping systems related to ascorbate-glutathione and thioredoxin to influence signalling pathways, as revealed by the presence of thioredoxin h-type, ascorbate peroxidase and guanine nucleotide-binding proteins in the infected resistant plants. Altogether, our data depicted the balance of the oxidative burst and antioxidant enzyme activity in the outcome of this interaction.

Keywords: *Sporisorium scitamineum*; Antioxidant enzymes; Reactive oxygen species; Phytopathogen; Hydrogen peroxide; Biotic stress

2.1 Introduction

Plants have developed an efficient defence system against pathogens (MOLINA; KAHMANN, 2007), and an early response is one of the strategies acquired for plants' survival. One of the initial defence reactions in plants after pathogen recognition is the increased production of reactive oxygen species (ROS) (TORRES; JONES; DANGL, 2006; O'BRIEN et al., 2012; DEL RÍO, 2015). This ROS burst, mostly consisting of superoxide anion and hydrogen peroxide (H_2O_2) at the site of invasion, is regarded as a core component of the early plant immune response (APOSTOL; HEINSTEIN; LOW, 1989) is regarded as a core component of the early plant immune response (DAUDI et al., 2012; DOEHLEMANN; HEMETSBERGER, 2013). In plant cells, ROS are produced via plasma membrane-localized NADPH oxidase, cell wall peroxidases (class III peroxidases) (O'BRIEN et al., 2012; TORRES, 2010) and pathways such as photosynthesis, photorespiration and respiration (MITTLER, 2002; GRATÃO et al., 2005; GRATÃO et al., 2015). In plants, the ROS increase during the infection process by pathogens and, for instance, high concentrations of H_2O_2 may contribute to the strengthening of host cell walls via-the crosslinking of glycoproteins, lipid

peroxidation (membrane damage), pathogen growth inhibition, induction of gene expression, and acting as a signalling molecule (MITTLER et al., 2011; LEHMANN et al., 2015). Additionally, ROS in incompatible interactions culminate in localized cell death, called the hypersensitive response (HR), which may increase the host resistance to biotrophic pathogens (BARNA et al., 2012).

However, due to ROS toxicity, antioxidant compounds and enzymes work in conjunction to maintain the steady-state level in plant cells (APEL; HIRT, 2004; GRATÃO et al., 2005). Among these enzymes are superoxide dismutase (SOD, EC 1.15.1.1), catalase, (CAT, EC 1.11.1.6), glutathione S-transferase (GST, EC 2.5.1.18) and others (GHELFI et al., 2011; SHARMA et al., 2012; PETERS et al., 2014). Moreover, it is suggested that plant hormones, such as salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA), can influence ROS and antioxidant enzyme activation in this process (MITTLER et al., 2011; MONTEIRO et al., 2011; BARNA et al., 2012; ALCÂNTARA et al., 2015).

The biotrophic fungus *Sporisorium scitamineum* is the causal agent of sugarcane smut, one of the most important diseases of this crop. The teliospore germination of *S. scitamineum* occurs in the bud and internode surface of sugarcane, following the appressorium formation on the inner scales of the young buds. The fungus entrance in the bud meristem occurs between 6 and 36 h after the teliospore deposition (ALEXANDER; RAMAKRISHNAN, 1980; SUNDAR et al., 2012). It has been reported that the colonization pattern of *S. scitamineum* differs between resistant and susceptible sugarcane genotypes (SINGH; SOMAI; PILLAY, 2004; CARVALHO et al., 2016). In susceptible infected plants, the hyphae are progressively built up within sugarcane tissues, culminating in the formation of a whip-like structure in the primary meristem, compromising culms quality and productivity (SUNDAR et al., 2012; DALVI et al., 2012).

The use of resistant varieties is one of the most effective, safe, economical and environmentally sound approaches to control smut in sugarcane. Some studies have demonstrated that resistance to *S. scitamineum* is associated with chemical barriers, such as the presence of phenylpropanoids, flavonoids (LLOYD; NAIDOO, 1983; FONTANIELLA et al., 2002; MILLANES et al., 2005; DE ARMAS; LEGAZ; VICENTE, 2007), free and conjugated glycoproteins and polyamines increased in sugarcane buds (LEGAZ et al., 1998; PIÑON et al., 1999; MILLANES et al., 2008). Glycoproteins prevent the correct arrangement of microtubules and cause nuclear fragmentation defects, contributing to germinative failure of teliospores (SÁNCHEZ-ELORDI et al. 2016). Likewise, resistance may be associated with the presence of trichomes, as well as the quantity of scales present in buds (GLÓRIA et al.,

1994). Furthermore, resistant plants infected with *S. scitamineum* present an increase of phenylalanine ammonia lyase enzyme activity (SANTIAGO et al., 2008; SANTIAGO et al., 2012) and cell wall lignification (DE ARMAS et al., 2007) and chitinase (SU et al., 2015). It has been reported in sugarcane that the gene nonexpressor of pathogenesis-related 1 (*NPR1*) was upregulated (RT-qPCR analysis) in response to SA and *S. scitamineum* (CHEN et al., 2012). This gene plays a pivotal role in systemic acquired resistance in plants (CAO et al., 1997; MOU; FAN; DONG, 2003). Additionally, regarding the oxidative burst, the *poxN* gene for peroxidase (*ScSs36*) was found to be weakly induced in smut-susceptible plants at 24 hours post-inoculation (hpi), whereas it was upregulated in the resistant plants at 72 hpi (LAO et al., 2008). Additionally, the catalase gene (*ScCAT1*) was associated with *S. scitamineum* because CAT activity in smut-resistant plants was higher than that in susceptible plants (SU et al., 2014). Recently, Su et al. (2016) revealed that antioxidant enzymes (SOD, CAT, APX and peroxidase) are useful biochemical indicators of smut resistance. Nevertheless, at present, there are few studies comparing both ROS production and antioxidant enzymes in susceptible and resistant sugarcane genotypes upon the inoculation with *S. scitamineum*. The present results are expected to provide a better understanding of the sugarcane resistance mechanisms against *S. scitamineum* and shall help the management of selection strategies aiming at the development of resistant cultivars.

2.2 Development

2.2.1 Material and methods

2.2.1.1 Biological materials

Two sugarcane genotypes were used to investigate the stress response after inoculation with *Sporisorium scitamineum* (= *Ustilago scitaminea*): 1) the clone IAC66-6, smut susceptible genotype**,** and 2) the cultivar SP80-3280, smut resistant genotype (Figure 2.1A). The plants were maintained at the experimental field of the Departamento de Genética, ESALQ, USP. The IAC66-6 clone and public domain variety SP80-3280 were kindly provided by the Centro de Cana, Instituto Agronômico, IAC/APTA. *S. scitamineum* SSC39 teliospores were obtained from a diseased plant of the RB925345 intermediate-resistant variety and were maintained for subsequent experiments in the Genomics Laboratory (ESALQ, USP) (TANIGUTI et al., 2015).

No special permits were necessary for the teliospores or genotypes used in the experiments because this project was developed in collaboration with IAC researchers. This work does not involve endangered or protected species.

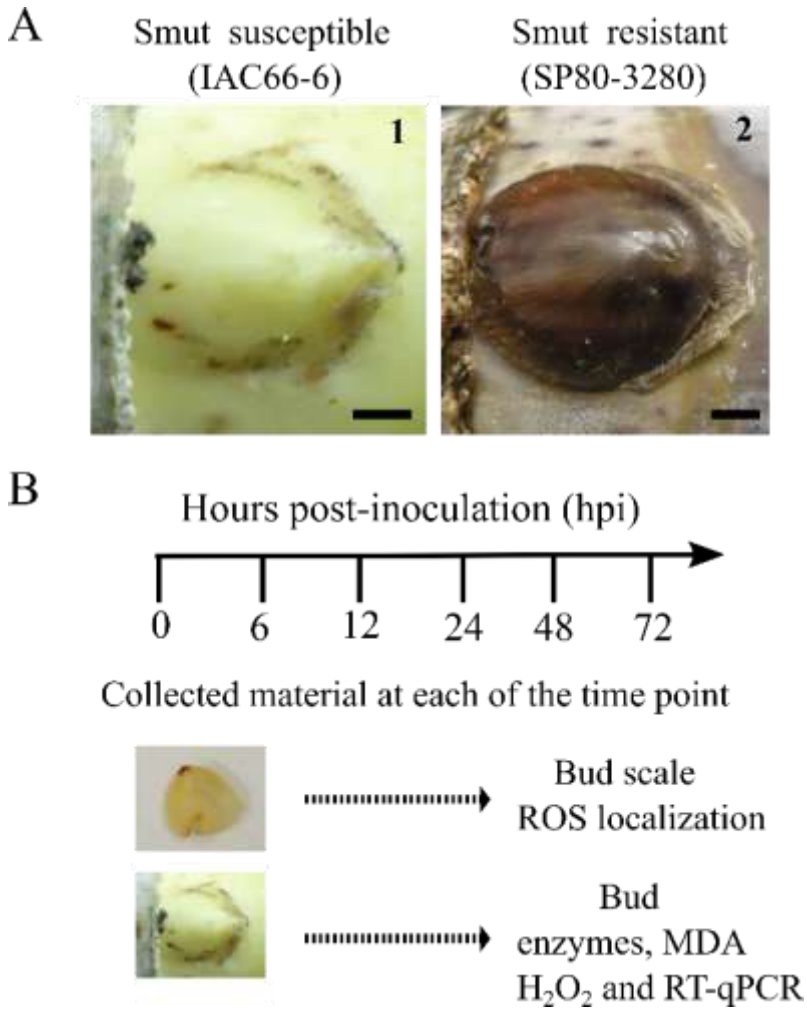

Figure 2.1 - A - Sugarcane bud collected from 10 months old plants from genotypes: 1) IAC66-6 (smut susceptible genotype) and 2) SP80-3280 (smut resistant genotype). B - Time course experiment and tissues in which different analyses were performed for each assay. Bar = 0.5 cm

2.2.1.2 Inoculation procedure and time course collection data

Single bud sets of 10-month-old healthy plants of both genotypes (Figure 2.1A) were surface disinfected and incubated for 16 h at 28°C according to CARVALHO et al. (2016). These bud sets were drop inoculated with 20 µL of a suspension containing 5 x 10^6 teliospores mL^{-1} in 0.1% (v/v) Tween-20, whereas controls were inoculated with 20 µL of sterile saline solution (0.85%) in 0.1% (v/v) Tween-20 (mock-inoculated). All of the inoculated bud sets were maintained in vermiculite at 28 °C under conditions of 12 h light/12 h dark and 85% relative humidity. (Figure 2.2). Twenty buds of each genotype were collected

at each of the time point of 6, 12, 24, 48 and 72 hours post-inoculation (hpi) and were maintained at -80 °C until further experiments (Figure 2.1B).

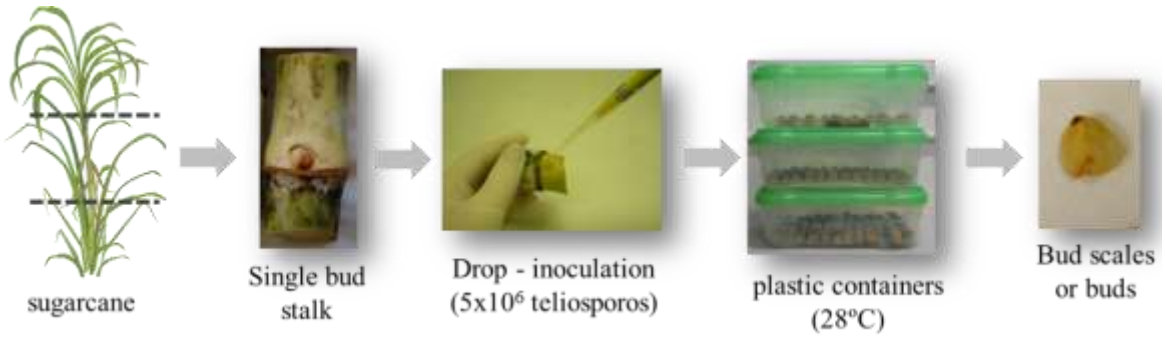

Figure 2.2 – Workflow for inoculation procedure

2.2.1.3 Microscopy analysis of fungal structures in plant tissues

Infected bud scales collected at 6, 12, 24, 48 and 72 hpi were detached from buds and were used to detect fungal structures stained with lactophenol-cotton blue (10 g phenol, 10 mL glycerol, 10 mL lactic acid, 0.02 g cotton blue, 10 mL deionized water) (TUITE, 1969). Light microscopy analyses were conducted in an Optika B-350 microscope (Optikam B5 digital camera) for all time points. The experiment was performed with three biological replicates. The percentage of spore germination was obtained at 6 and 12 hpi (100 spores were counted for each replicate) (JAMES, 1973). The percentage of appressorium was quantified observing at least 100 germinated spores for each replicate (APOGA et al., 2004). Statistical analysis was performed as described in the Experimental design and statistical analysis section.

2.2.1.4 ROS localization of sugarcane-*Sporisorium scitamineum* interaction

Changes in reactive oxygen species (ROS) production in sugarcane bud tissues as a result of *S. scitamineum* infection were assessed. To detect superoxide ions, scales were excised from inoculated and mock-inoculated buds and vacuum infiltrated for 1 h in 0.1% (w/v) NBT (nitroblue tetrazolium) solution in 50 mM potassium phosphate buffer (pH 6.5) (HÜCKELHOVEN et al., 2000). Bud scales were then placed into a 0.15% trichloroacetic acid (TCA) in ethanol and chloroform (4:1; v/v) solution to make tissues clear in appearance (FREITAS et al., 2012). After 72 h, they were mantained in the dark in 50% glycerol solution until light microscopy analysis (Optika B-350; Optikam B5 digital camera). Histochemical detection of H_2O_2 was performed according to HÜCKELHOVEN et al. (2000), with modifications. A similar protocol was followed to detect superoxide, but NBT was used

instead with a 1% (w/v) DAB (3,3' diaminobenzidine) solution in 50 mM potassium phosphate buffer (pH 3.8, adjusted with HCl).

2.2.1.5 Biochemical analysis

For all of the biochemical assays described below, twenty sugarcane buds (mock inoculated and inoculated) of each genotype were collected at 6, 12, 24, 48 and 72 hpi. The sugarcane buds were maintained in liquid nitrogen during sampling and were subsequently stored at -80°C until further analysis.

2.2.1.5.1 Hydrogen peroxide concentration

The content of H_2O_2 was determined as described by ALEXIEVA et al. 2001. Sugarcane buds (100 mg) 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 supernatant was added to 200 μL of 100 mM potassium phosphate buffer (pH 7.0) and 800 μL of 1 M potassium iodide (KI). The absorbance was read at 390 nm (Perkin Elmer Lambda 40). The H_2O_2 content for all samples was determined using a known H_2O_2 concentration curve as a standard. The result was expressed in μmol g^{-1} fresh weight.

2.2.1.5.2 Lipid peroxidation assay

Membrane damage was determined by estimating the content of thiobarbituric acid reactive substance (TBARS) following the method of HEATH; PACKER (1968). A total of 100 mg of powdered sugarcane buds was homogenized in 1 mL of 0.1% (w/v) TCA solution and centrifuged at 12,000 *g* for 10 min at 4°C. Next, 250 μL of the supernatant from the 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 centrifuged for 5 min at 12,000 *g*. Malondialdehyde (MDA) was monitored by absorbance measurements at 535 and 600 nm in a Perkin Elmer Lambda 40 spectrophotometer, and the concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. The result was expressed in nmol g^{-1} fresh weight.

2.2.1.5.3 Antioxidant enzyme extraction and activity assays

One gram of fine sugarcane buds powder were homogenized (2:1, 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 4ºC, and the supernatants were stored in separate aliquots at -80 ºC prior to enzymatic analysis. The concentration of protein was determined using bovine serum albumin as the standard (BRADFORD, 1976).

SOD activity staining

SOD activity staining was carried out as described by BEAUCHAMP; FRIDOVICH (1971) and optimized by AZEVEDO et al. (1998). The non-denaturing 12% PAGE gels were loaded with 30 µg of biological extract protein, and electrophoresis was carried out with a constant current until migration was completed. After non-denaturing PAGE separation, 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. One unit of bovine liver SOD (Sigma) was used as a positive control of activity. After 30 min, the gels were rinsed with distilled deionized water and then were illuminated in water until the development of achromatic bands of SOD activity on a purple-stained gel. SOD isoenzyme characterization was performed as described by Giannopolitis and Ries (1977) and as modified by Azevedo et al. (1998). SOD isoenzymes were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide and 5 mM hydrogen peroxide.

CAT total activity determination

CAT activity was assayed as described previously by GRATÃO et al. (2012) at 25ºC in a reaction mixture of 1 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 µL of 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 the changes in absorbance at 240 nm. CAT activity is expressed as µmol min^{-1} mg^{-1} protein.

GST total activity determination

The methodologies described by MOZER; TIEMEIER; JAWORSKI, (1983) and LABROU et al. (2005) were used to determined GST activity. The activity was assayed spectrophotometrically at 30°C in a mixture containing 900 µL of 100 mM potassium phosphate buffer (pH 6.5), 25 µL of 40 mM 1-chloro-2,4-dinitrobenzene (CDNB), 50 µL of 1 mM GSH and 25 µL of enzyme extract. The reaction mixture was followed by monitoring the increase in absorbance at 340 nm over 3 min. GST activity was expressed as µmol min^{-1} mg^{-1} protein.

2.2.1.6 Protein preparation

In view of the cytological and biochemical changes induced during the infection process, we sought to analyse proteins associated with an oxidative burst. The time point selected for protein extraction corresponded to the increase in H_2O_2 and lipid peroxidation concentration (72 hpi) detected in the resistant genotype. Proteins were extracted from sugarcane buds according to the protocol described by HURKMAN; TANAKA (1986) with modifications. Buds samples (50 mg) were finely powdered in liquid nitrogen and homogenized in 800 µL of extraction buffer (1% PVPP, 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl pH 7.5, 500 mM EDTA, 1 mM (PMSF), 2% b-mercaptoethanol). Phenol (800 µL) was added, and the mixture was homogenized for 30 min at 4°C and finally centrifuged at 10,000 g for 30 min. The upper phenol phase was removed and re-extracted two times with extraction buffer as above. Proteins were precipitated from the final phenol phase with two volumes of saturated ammonium acetate in methanol overnight at 4°C and were pelleted by centrifugation at 10,000 *g* for 30 min. The protein pellets were solubilized in lysis buffer (7 M urea and 2 M thiourea). The concentration of protein was determined using bovine serum albumin as the standard (BRADFORD, 1976).

2.2.1.7 Mass spectrometry MS/MS and data analysis

For protein analysis, an aliquot of 4.5 µL of proteins resulting from peptide digestion was separated in C18 (100 mm 6100 mm) RP-nano UPLC (nanoAcquity, Waters) coupled with a Q-Tof Premier mass spectrometer (Waters) with a nanoelectrospray source at a flow rate of 0.6 µL/min. The gradient was 2%–90% acetonitrile in 0.1% formic acid over 45 min. The nanoelectrospray voltage was set to 3.5 kV, a cone voltage of 30 V and the source temperature was set to 100°C. The instrument was operated in the ‘top three’ mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on an exclusion list for 60 s and for the analysis of endogenous cleavage peptides, a real-time exclusion was used. The spectra were acquired using software MassLynx, v.4.1 and the raw data files were converted to a peak list format (mgf) without summing the scans by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science Ldt.) and searched against (9,747 sequences, 3,345,870 residues) using Mascot engine v.2.3.01 (Matrix Science Ltd.), with carbamidomethylation as fixed modifications, oxidation of methionine as variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions. The Scaffold software was used to calculate the normalized spectral counts and to validate peptide and protein

identifications (NESVIZHSKII et al., 2003), considering the scoring parameters (95% of peptide confidence level identification, 99% peptide probability and at least 2 unique peptides) to obtain a false discovery rate (FDR) of less than 1% for proteins and peptides.

2.2.1.8 RNA extraction and gene expression analysis

The time points selected for gene expression analysis corresponded to appressorium formation (24 hpi) of *S. scitamineum* in the susceptible genotype and the increase in H_2O_2 and lipid peroxidation concentration (72 hpi) in the resistant genotype. The genes analysed were selected from previous work based on differential expression (analysed by RNAseq) in plants infected with *S. scitamineum* (Appendix A) (SHACKER et al., 2016). Furthermore, other genes coding for proteins identified as present or absent during the proteomic assay were selected for gene expression analysis. RNA extraction was performed using Trizol® (Sigma) and the Direct-zol™ RNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions. Total RNA was treated with DNAse (Sigma), and RNA quality was verified in agarose gel. The primers were manually designed and the quality verified using Gene Runner (http://www.generunner.net/) and Beacon Designer™ Free Edition (http://www.premierbiosoft.com) softwares (Table 2.1). To confirm the absence of genomic DNA contamination, PCR assays were performed with samples. All RT-qPCRs 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 50 ng of RNA, 6.5 μL of GoTaq® qPCRMaster 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 was used for three biological replicates and two technical replicates. The 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. Primer specificity was confirmed obtaining the dissociation curve for every reaction (Appendix B). Sugarcane housekeeping genes encoding for polyubiquitin (PAPINI-TERZI et al., 2005) and 14:3:3 (ROCHA et al., 2007) were used to normalize expression signals. PCR efficiencies and Ct values were obtained using the LinReg PCR program (RAMAKERS et al., 2003). The relative changes in the gene expression ratios were calculated using REST software (PFAFFL et al., 2004). Control samples (mock-inoculated plants) were used as calibrators.

Table 2.1 - Primers used in RT-qPCR experiments

Name	gene ID		Sequence
Catalase (CAT3)	comp189288_c1_seq1	F	GATCCCACCAAGTTCCGTCC
		R	CTTCTCGATCAGGTGGTAGTCC
Catalase (CATA)	comp189288_c0_seq1	F	GTAGTAGTCCACCTCCTCATCCC
		R	CTACTACTCCGACGACAAGATGC
Superoxide dismutase (SOD)	comp186491_c0_seq1	F	CTGGCGAGCAACCTACAATGG
		R	GTTGTTGGGAGAGCATTTGTGG
Catalase (CATB)	comp191235_c0_seq1	F	ATATAACCACCACCAGTCATCAGC
		R	AAGATTGACAAGGAAGAAAGCAGG
Peroxidase (P5)	comp127311_c0_seq1	F	CACAACGAACCAGGCTATGC
		R	GTCAAGATGGGCACTGTCGG
Glutathione S-tranferase (GST31)	comp201441_c0_seq1	F	AGAATCTACAATGACAGGCACC
		R	TATCTTTCTTGTGTGGTGTCGG
Glutathione S-tranferase (GSTt3)	comp179663_c0_seq1	F	TTCGGAACCTTCGCCTTGTC
		R	TCAGCCAGGGGAAGCACTAC
Peroxidase III class	evm.model.scga7_uti_cns_0172034.2	F	CATCGAGCTGACCCTGAAGAAG
		R	TTGATCATGCTGGTGCCGAAC
guanine nucleotide-binding protein subunit beta-like protein a	Sb09g027690.1|PACid:1981757	F	GAAGGACGGTGTTACTCTGCTG
		R	GATCTGGATGTCGGGCTTGAG
thioredoxin h-type	evm.model.scga7_unitig_341686.1	F	CCAAGAAGAACCCCAGCGTG
		R	CACCCTGTCCTTCACGTCGG
Polyubiquitin	Papini-Terzi et al., 2005	F	CCCTCTGGTGTACCTCCATTTG
		R	GGTGGCCGGCTTGGA
14-3-3 protein	Rocha et al., 2007	F	TTTGTTTCGGTTTCAAGTCGATAA
		R	TTCACACTTGGAGTGAAGCAGAT

2.2.1.9 Experimental design and statistical analysis

The experiment was performed in a completely randomized design, and each treatment was conducted on three biological replicates. The significance of the observed differences was verified using Student's *t* test (P <0.05). All statistical analyses were carried out using R software (URL http://www.r-project.org).

2.2.1.10 DNA extraction of *S. scitamineum* and infected buds

The DNA extraction of *S. scitamineum* (SSC39A) was performed using Wizard Genomic DNA purification kit (Promega) from cells grown to exponential phase in Yeast Medium (YM) consisting of 3 g yeast extract, 3 g malt extract, 5 g soybean peptone and 10 g glucose per L of distilled water at 28 °C, and shaken at 180 rpm in an aerobic environment. The DNA extraction of plants was carried out using CTAB method with modifications (POREBSKI; BAILEY; BAUM, 1997). In the extraction of plants DNA, smut susceptible and smut resistant sugarcane buds (50 mg) at 6, 12, 24, 48 and 72 hpi were blended in a 1.5 mL-tube with CTAB-buffer (total volume of 1 mL consisting of: 10 mM Tris, 20 mM EDTA, 0.02 M CTAB, 0.8 M NaCl), pH was set to 8.0 with NaOH. Mercaptoethanol (8 µL) was added to the mixture. After an incubation period of 60 min at 65 °C, during which the content of the tubes was mixed every 15 min, chloroform-isoamyl alcohol (500 µL, 24:1) was added. The samples were centrifuged for 10 min at 8,000 g at 25 ºC. A portion of the upper phase

was transferred to a 1.5-mL tube containing isopropanol (350 µL), mixed, and centrifuged for 10 min at 10,000 g at 25 °C. The pellet was washed with 70% (v/v) ethanol, dried and dissolved in sterile ultrapure water (50 µL). The concentration of DNA was measured spectrophotometrically (NanoDrop® 1000 – Thermo Scientific) and DNA quality was assessed in 1% agarose gels using 1Kb Plus ladder (Invitrogen).

2.2.1.11 qPCR conditions and standard curve for fungal quantification *in planta*

Primers selection and the standard curve for fungal quantification is presented in the Appendix C. The set of primers named SSC-C targeted the IGS region (*Intergenic Spacer*) of *S. scitamineum* genome (TANIGUTI et al., 2015) was used in this study (F 5' CGGCTATTGTCGCACATCTC 3' and R 5' CCAAACGCAGGTCACAGTCT 3'). Reactions were composed of 6.5 µL of *2X LuminoCt SYBR Green qPCR ReadyMix*, 0.2 µM of each primer (F and R), 5 µL of each DNA dilution, 0.5 µl BSA (10 mg/mL), and water to a final volume of 12.5 µL. Standard curve was generated by plotting the threshold cycles (Ct) versus the logarithmic values of known quantities of target DNA (Appendix C). Fungal DNA amount was assessed in infected buds at 6, 12, 24, 48 and 72 hpi, and calculated as pathogen DNA amount in 100 ng of plant DNA. Tukey's test ($P \leq 0.05$) was used to compare the DNA quantity of *S. scitamineum* in the smut susceptible and resistant genotypes infected with the pathogen.

2.2.2 Results

2.2.2.1 *S. scitamineum* development delayed in sugarcane tissues of resistant plants

To understand the initial sugarcane reaction to *S. scitamineum*, we sought to investigate the infection process of the fungus in smut-susceptible and -resistant plants. Sugarcane bud scales were used to analyse the production of ROS in a time-course experiment, at 6, 12, 24, 48 and 72 hpi, monitored by light microscopy. The results revealed that the fungus presented well established infection stages and differentiated structures in both of the analysed genotypes (susceptible and resistant). These modifications comprised filament formation upon recognition of the host surface and the development of infection structures such as the appressorium. At 6 hpi, 81% of teliospores were germinated in the susceptible genotype (Figure 2.3A), forming promycelium (Figure 2.4-1 and 2.4-7); however, in the

resistant genotype SP80-3280, teliospore germination had initiated only in 36% of teliospores (Figure 2.3A), reaching a maximum rate at 12 hpi (53%) (Figure 2.3A).

Appressorium formation was quantified by observing lactophenol-cotton blue- stained fungal filaments on bud scale surfaces. In the susceptible genotypes 71% of the hyphal tips developed appressorium (Figure 2.3B, 2.4-3) at 24 hpi, whereas in the resistant genotype we observed 43% of the tips developing appressorium later at 48 hpi (Figure 2.3B, 2.6-9). At 72 hpi, the formation of an extensive network of filaments in both genotypes was observed (Figure 2.4-5 and 2.4-10).

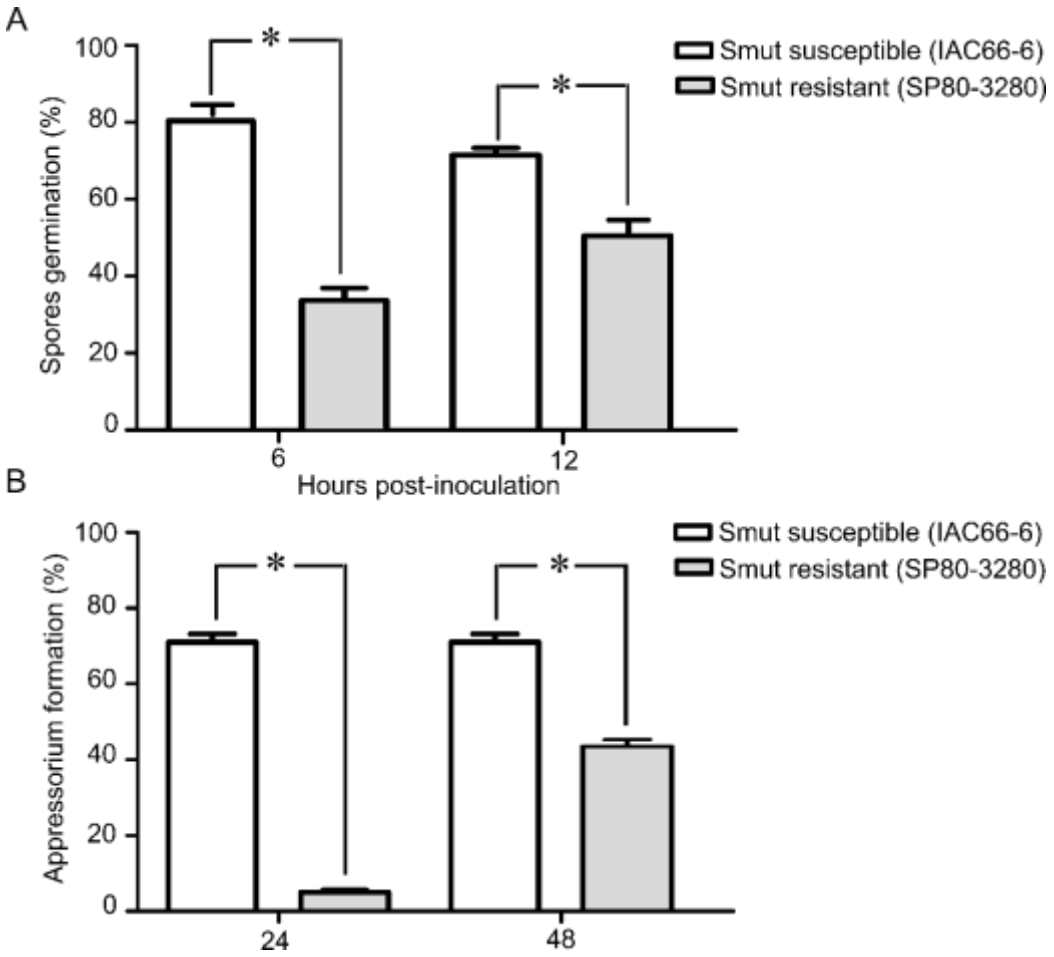

Figure 2.3 - Teliospore germination and appressorium formation of *S. scitamineum* on sugarcane bud scale surface in smut-susceptible and -resistant genotypes. A - Teliospore germination (%) of *S. scitamineum* on sugarcane bud scale surface in smut-susceptible and -resistant genotypes. B - Appressorium formation (%) of *S. scitamineum* on sugarcane bud scale surface from smut susceptible and resistant genotypes. Bars represent the standard deviations of three independent biological replicates. * represents statistically significant differences ($P < 0.05$) between the smut-susceptible and -resistant genotypes

2.2.2.2 Sugarcane-resistant plants over produce ROS earlier after *S. scitamineum* inoculation

The production of superoxide anion and H_2O_2 in infected bud scales was determined by the *in situ* oxidation of nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB), respectively. The results revealed that the main ROS compound produced is H_2O_2, and the response became more evident at 72 hpi for both genotypes (Figure 2.6-5 and 2.6-10). This is the time when the network of filaments is well established for both genotypes. However, for resistant plants, H_2O_2 accumulation was initiated earlier at 6 hpi along with teliospore germination (Figure 2.6-6). Furthermore, accumulation of H_2O_2 was also observed in the plant epidermal cells in direct contact with the appressorium at 48 hpi (Figure 2.6-9). Interestingly, as the colonization progressed, the fungal hyphae started to produce internal vesicular bodies, probably containing H_2O_2 (Figure 2.6-8 and 2.6-3). These vesicles were evident in hyphal growth at 12 and 24 hpi in the susceptible genotypes and at 24 hpi in the resistant one. However, at the other time points analysed for the genotypes there was not accumulation of H_2O_2 in fungal vesicles (Figure 2.6). The microscopic analysis of sugarcane bud scale (mock inoculated) in a time course from 6 to 72 hpi are in Figure 2.7. Bud scales controls were also stained with DAB to detect the possible production of H_2O_2.

The production of superoxide anion was also evaluated in sugarcane tissues infected with *S. scitamineum*. The results showed that the plant cells of the resistant genotype produced superoxide at 6 and 12 hpi (Figure 2.4-6 and Figure 2.4-7). At these time points, the presence of superoxide anion was restricted to the surrounding areas of the promycelium hyphal tips. Superoxide production was not observed in any other time point analyzed of the resistant genotype as well as in any observed time of the susceptible genotype using the proposed technique. The microscopic analysis of sugarcane bud scale (mock inoculated) in a time course from 6 to 72 hpi are in Figure 2.5. Bud scales control were also stained with NBT to detect the production of superoxide anion.

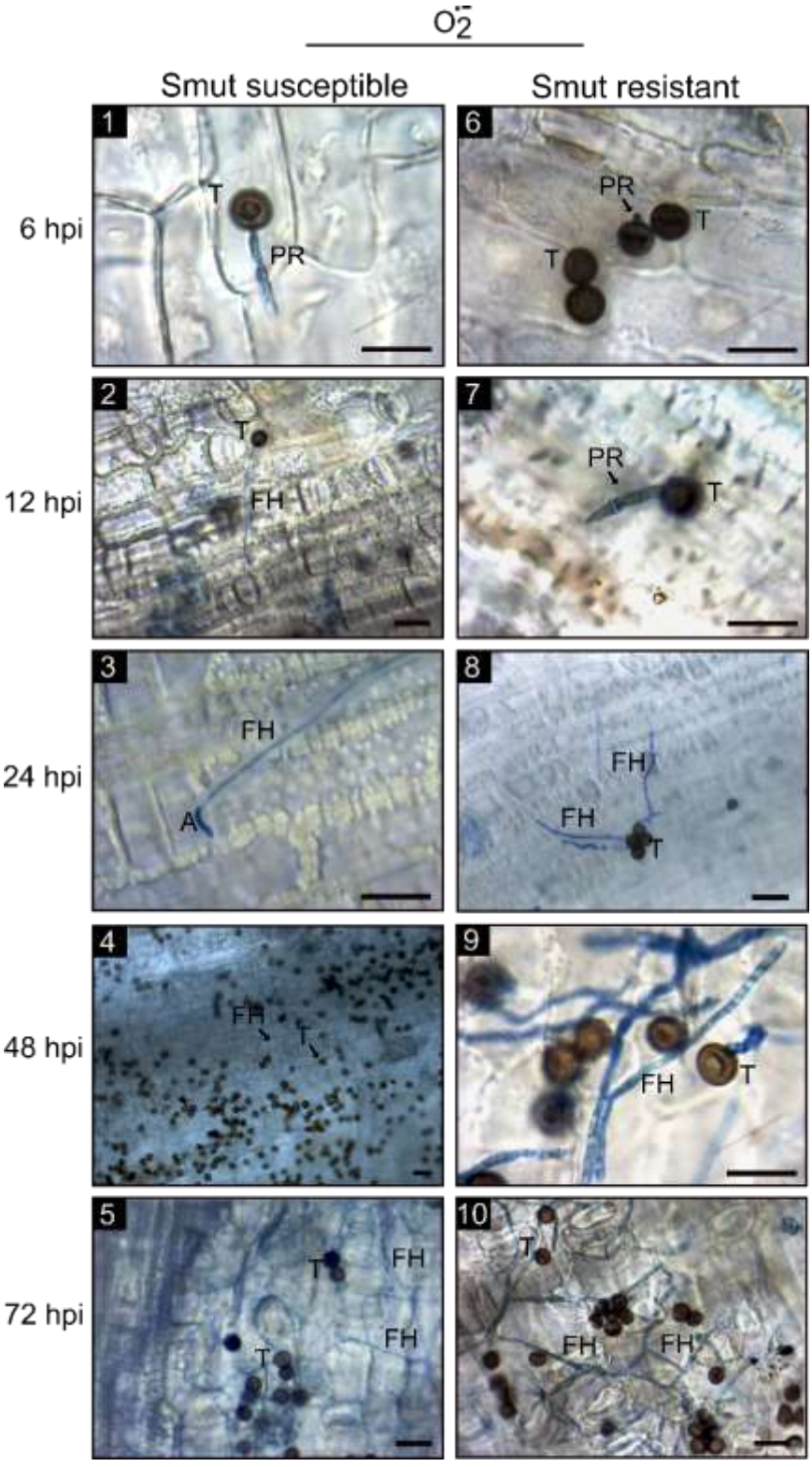

Figure 2.4 - Microscopic analysis of the *S. scitamineum* infection sites on inoculated sugarcane bud scale and ROS produced on sugarcane bud scales during *S. scitamineum* infection in a time course from 6 to 72 hpi. Inoculated bud scales were stained with nitroblue tetrazolium (NBT) to detect the production of superoxide anion (formation of dark blue formazans) in sugarcane smut-susceptible (numbers of 1-5) and -resistant plants (numbers of 6-10). T, teliospore; PR, promycelium; FH, fungal hyphae; A, appressorium; V, vesicular bodies. Bar = 100 μm

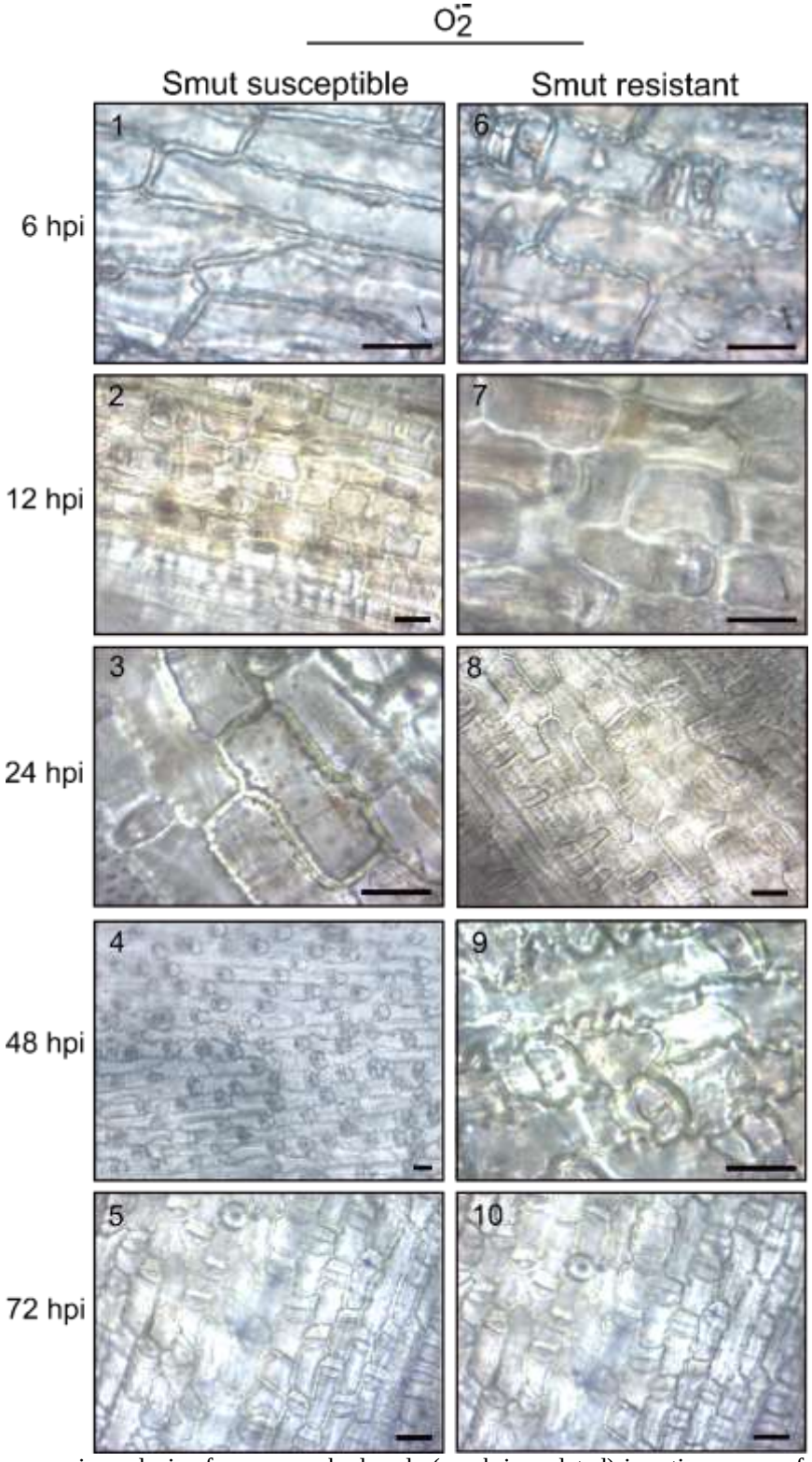

Figure 2.5 - Microscopic analysis of sugarcane bud scale (mock inoculated) in a time course from 6 to 72 hpi. Bud scales controls were stained with nitroblue tetrazolium (NBT) to detect the production of superoxide anion (formation of dark blue formazans) in sugarcane smut-susceptible (numbers of 1-5) and -resistant plants (numbers of 6-10). Bar = 100 μm

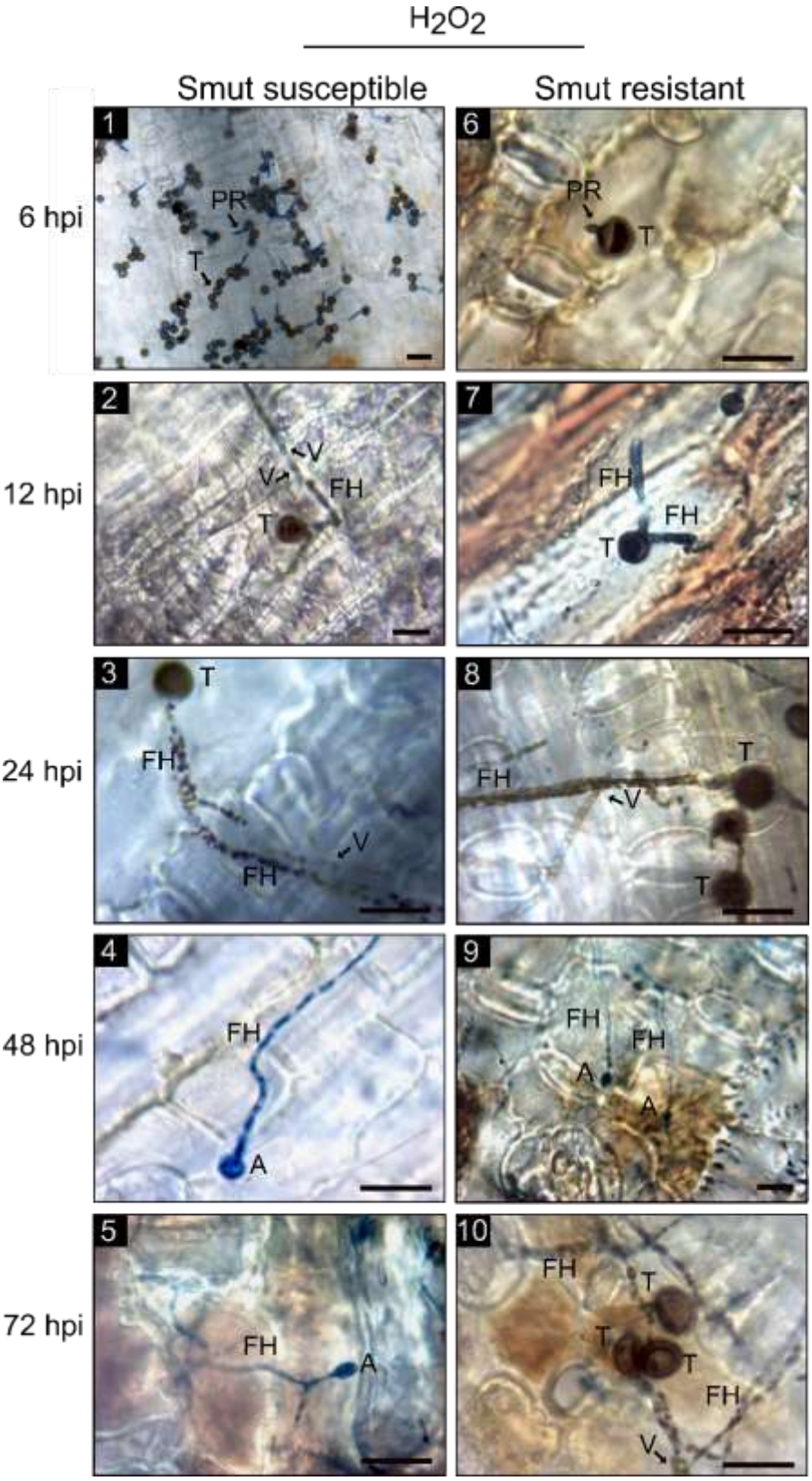

Figure 2.6 - Microscopic analysis of *S. scitamineum* infection sites on sugarcane bud scale inoculated and ROS produced on sugarcane bud scale during *S. scitamineum* infection in a time course from 6 to 72 hpi. Inoculated buds scale were stained with 3,3"-diaminobenzidine (DAB) to detect the production of H_2O_2 (DAB polymerization, brown) in sugarcane smut susceptible (numbers of 1-5) and resistant (numbers of 6-10). T, teliospore; PR, promycelium; FH, fungal hyphae; A, appressorium; V, vesicular bodies. Bar = 100 μm

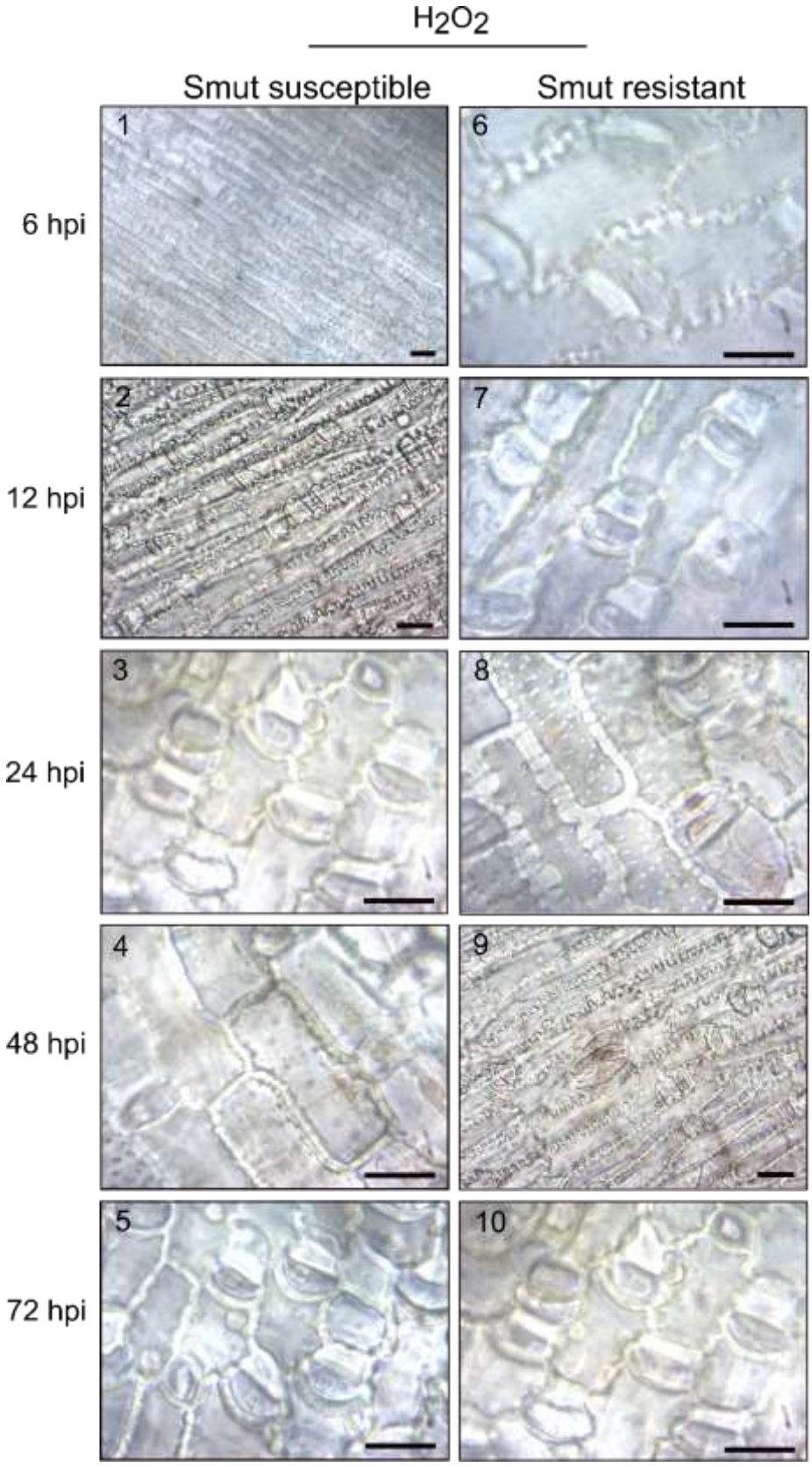

Figure 2.7 - Microscopic analysis of sugarcane bud scale (mock inoculated) in a time course from 6 to 72 hpi. Buds scales controls were stained with 3,3'-diaminobenzidine (DAB) to detect the production of H_2O_2 (DAB polymerization, brown) in sugarcane smut susceptible (numbers of 1-5) and resistant (numbers of 6-10). Bar = 100 µm

Biochemical assays allowed the quantification of H_2O_2 produced in both inoculated and non-inoculated buds of the two genotypes. The accumulation of H_2O_2 at 6, 48 and 72 hpi in smut-resistant plants was higher (23, 22 and 70%, respectively) than that in mock controls (Figure 2.8A), however, for the other time points analysed, changes in the H_2O_2 content were not observed. Likewise, smut-susceptible buds did not exhibit the accumulation of H_2O_2 at all time points analysed (Figure 2.8A).

2.2.2.3 *S. scitamineum* infection induces lipid peroxidation in resistant sugarcane

We examined the extension of oxidative damage in sugarcane buds challenged with *S. scitamineum* by determining the content of lipid peroxidation (MDA- malondialdehyde). The results showed that the MDA content in inoculated plants of the susceptible genotype was not altered significantly throughout the experiment (Figure 2.8B). Although a similar result was observed for the resistant genotype, an increase of 41% in the MDA content was detected at 72 hpi (Figure 2.8B). There was a major intrinsic difference in MDA content between the smut-susceptible and -resistant genotypes regardless inoculation, with the susceptible genotype exhibiting a much higher lipid peroxidation rate (about 70%) during the entire course of the experiment (Figure 2.8B).

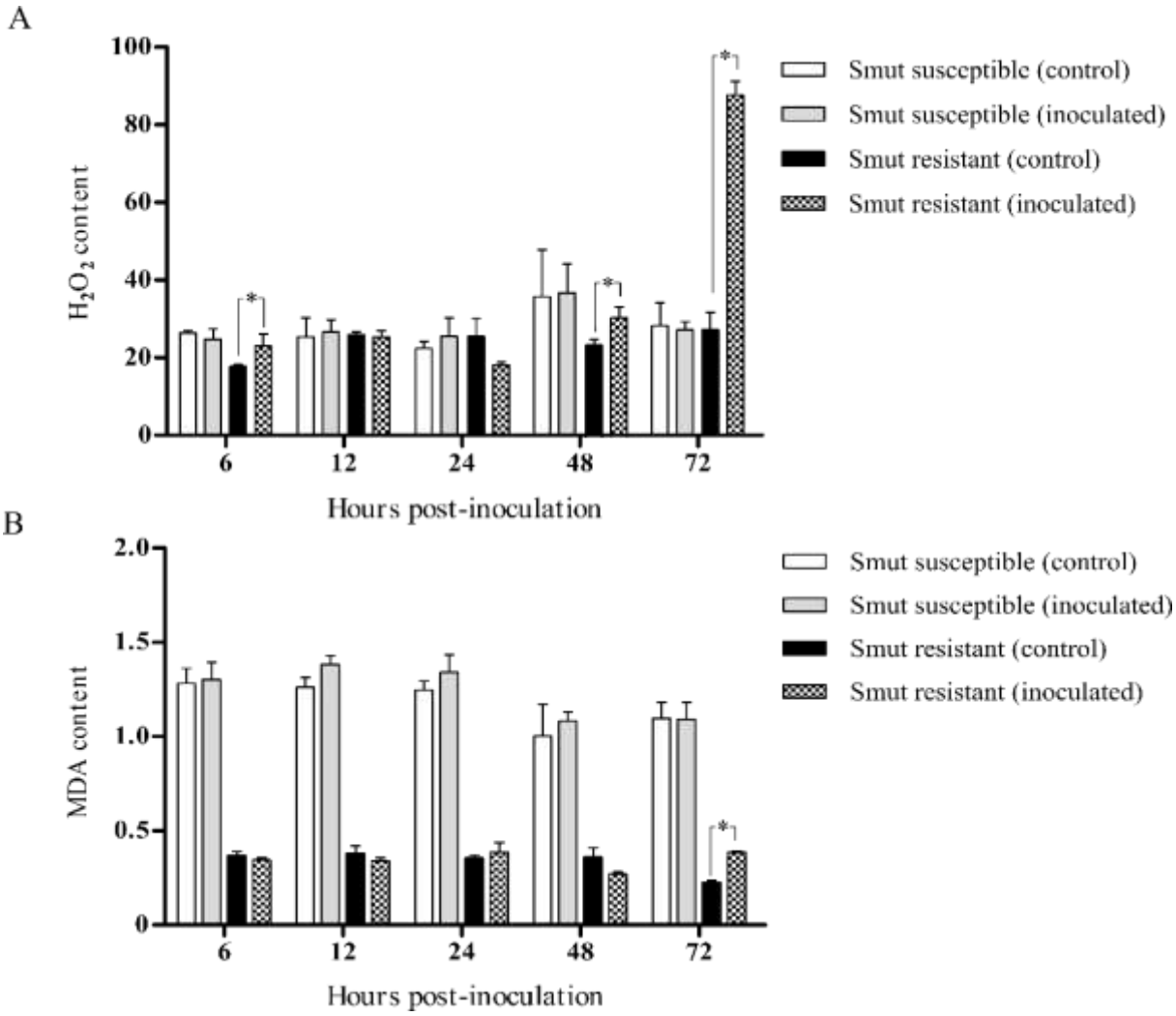

Figure 2.8 – Detection of H_2O_2 and lipid peroxidation caused by *S. scitamineum* in sugarcane buds. A - Effects of *S. scitamineum* infection on the H_2O_2 content (µmol g^{-1} fresh weight) (quantitative results) and B - Effects of this fungus on the malondialdehyde (MDA) content (nmol g^{-1} fresh weight) in susceptible and resistant sugarcane genotypes over the time course from 6 hpi to 72 hpi. Values represent the means from three independent biological replicates ±SD. * represents statistically significant differences ($P < 0.05$) between control buds (mock inoculated) and inoculated buds

2.2.2.4 *S. scitamineum* alters the activities of antioxidant enzymes in sugarcane

The overall enzyme activity patterns related to ROS scavenging (SOD, CAT and GST) were distinct in the resistant and susceptible genotypes. SOD activity was determined by non-denaturing PAGE staining for isoenzyme identification (Figure 2.9A and 2.9B). The results revealed the existence of five isoenzymes characterized as Mn/SODs (SOD I, II and III) and Cu-Zn/SODs (SOD IV and V) in the smut-susceptible genotype (Figure 2.9A), and 10 isoenzymes identified as Mn/SODs (SOD I, II, III and IV) and Cu-Zn/SODs (V, VI, VII, VIII, IX and X) in the smut-resistant genotype (Figure 2.9B). However, SOD activity did not exhibit any major visible changes or specific alterations in the expression of the distinct isoenzymes in any of the genotypes in response to the inoculation with *S. scitamineum*. Furthermore, the five isoenzymes present in the susceptible genotype were also present in the

resistant one (susceptible SOD II, III, IV and V corresponding to resistant SOD IV, V, VI and X), three of them (IAC66-6 II, III, IV; SP8032-80 IV, V and VI) accounting for most of the SOD activity.

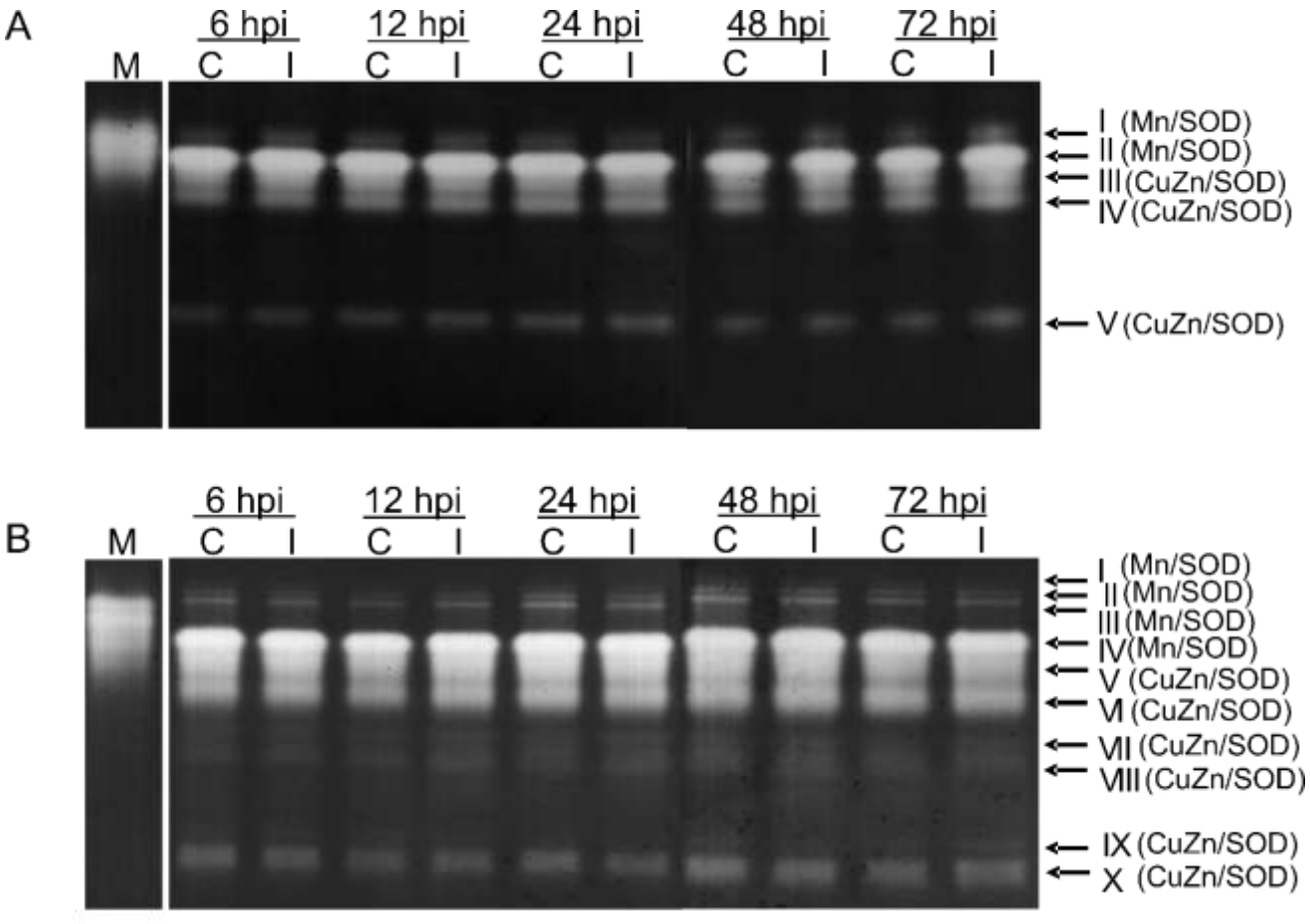

Figure 2.9 – Effects of *S. scitamineum* infection on the activity of superoxide dismutase (SOD). A - Activity staining for SOD following non-denaturing PAGE from smut-susceptible genotype over the time course from 6 hpi to 72 hpi. B - Activity staining for SOD following non-denaturing PAGE from the smut-resistant genotype over the time course from 6 hpi to 72 hpi. The first lane is a bovine SOD standard, and arrows indicate sequentially numbered SOD bands for smut-susceptible (I–V) and smut-resistant (I–X) plants. The letters C and I represent control and inoculated plants, respectively.

CAT total activity was not altered in the susceptible genotype throughout the experiment (Figure 2.10A). By contrast, for the resistant genotype a decrease in CAT activity was observed at 12 hpi (67%) and at 72 hpi (275% decrease) (Figure 2.10A). CAT activity was consistently higher, especially at 6 and 12 hpi, in the susceptible genotype than in the resistant genotype independent of the inoculation (Figure 2.10A).

Total GST activity exhibited contrasting results between the two genotypes. The susceptible genotype exhibited an early decrease (42% at 12 hpi) (Figure 2.10B); however, the resistant genotype increased the activity even earlier (37% at 6 hpi), reaching its maximum value at 12 hpi (70% increase). These changes co-occurred with the teliospore

initial germination and maximum germination rates, respectively (Fig. 2.4-6, 2.6-6). However, GST activity in the resistant genotype decreased 30% and 24% at 48 and 72 hpi, respectively (Figure 2.10B).

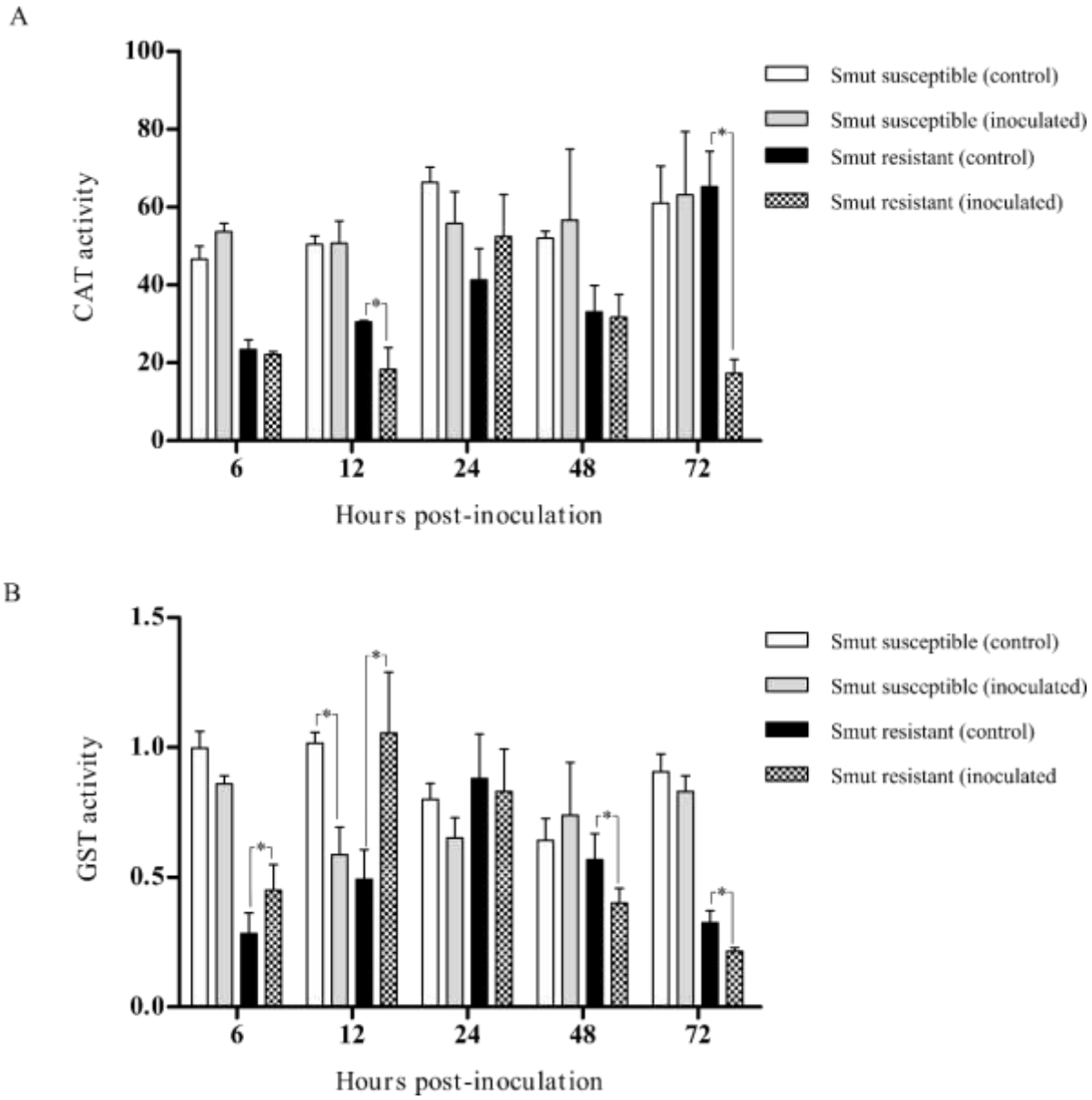

Figure 2.10 - Effects of *S. scitamineum* infection on the total specific activity of catalase (CAT) and glutathione S-transferase (GST) in susceptible and resistant sugarcane genotypes over the time course from 6 hpi to 72 hpi. A - Total specific activity of CAT (μmol min^{-1} mg^{-1} protein). B - Total specific activity of GST (units min^{-1} mg^{-1} protein). Values represent the means from three independent biological replicates ±SD. * represents statistically significant differences ($P < 0.05$) between control (mock inoculated) buds and inoculated buds

2.2.2.5 Sugarcane proteins associated with oxidative burst are induced or repressed in response to *S. scitamineum* infection

Based on the results of protein identification and functional categorization (SETTA et al. 2014; BARNABÁS et al., 2016), four proteins associated with oxidative burst were detected from a set containing 38 proteins (4 proteins from susceptible genotype and 34 proteins from resistant genotype) that were particularly present or absent between the two genotypes (Figure 2.11). The complete list of present or absent proteins consistently detected in replicates is presented in Appendix D. Among these proteins, a cationic peroxidase spc4-like (peroxidase III class) was repressed in the susceptible genotype (Table 2.2). Three proteins were induced in response to infection in the resistant genotype: ascorbate peroxidase, thioredoxin h-type and guanine nucleotide-binding protein subunit beta-like (Table 2.2).

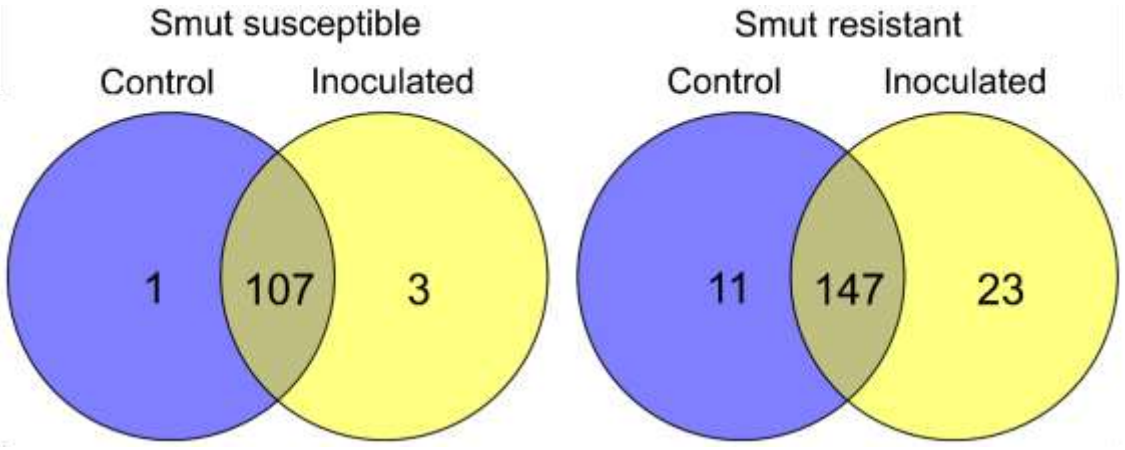

Figure 2.11 - Venn diagram of induced or repressed proteins in susceptible and resistant sugarcane genotypes inoculated with *S. scitamineum* (72 hpi)

Table 2.2: Induced or repressed proteins related to ROS metabolism at 72 hpi

Treatment[a]	Protein Description[b]	Protein threshold[c]	% sequence coverage[d]					
			C1	**C2**	**C3**	**Inoc1**	**Inoc2**	**Inoc3**
Smut resistant (inoculated)	ascorbate peroxidase	0.9%	0	0	0	10.00%	10.00%	0
Smut resistant (inoculated)	guanine nucleotide-binding protein subunit beta-like protein a	0.9%	0	0	0	18.09%	15.09%	
Smut resistant (inoculated)	thioredoxin h-type	1%	0	0	0	25.4%	25.4%	31.5%
Smut susceptible (mock inoculated)	Class III peroxidase	1%	14.5%	14.5%	9.44%	0	0	0

[a] Corresponds to treatment that protein is present.

[b] Protein description based on UniProt Knowledgebase.

[c] Protein threshold corresponds to false discovery rate (FDR).

[d] % sequence coverage represents the percentage of all the amino acids in the protein sequence that were covered by identified peptides detected in the sample. C1, C2 and C3 represent control 1, 2 and 3, respectively. Inoc1, Inoc2 and Inoc3 represent inoculated 1, 2 and 3, respectively.

2.2.2.6 Gene expression analysis

The ROS-related marker genes were selected based on previously obtained RNAseq data (SCHACKER et al., 2016) and based on protein sequences induced and repressed by the fungus identified in this work. A total of 10 genes were analysed in two time points: 24 hpi and 72 hpi (Figure 2.8). These two time points were chosen because 24 hpi coincided with appressorium formation in the susceptible genotype, and 72 hpi coincided with the increase in H_2O_2 and lipid peroxidation concentration in the resistant genotype. At 24 hpi (Figure 2.12A), the genes encoding *SOD* and the two *GST*s exhibited a similar regulation pattern in both genotypes, in which *SOD* and *GST*s were downregulated. *GDP* and *PRX4* were significantly upregulated only in the resistant genotype. By contrast, the *CAT* genes along with the genes coding for thioredoxin and *POX5* peroxidases showed opposite regulation. They were all upregulated in the resistant genotype but were downregulated in the susceptible one. At 72 hpi (Figure 2.12B), *SOD* remained downregulated for both genotypes, and *PRX4* remained upregulated for the resistant genotype. However, the *CAT* genes and peroxidases *POX5* and *TRX h* had their expression regulation inverted. They were all downregulated in the resistant genotype, but were upregulated in the susceptible one. *GDP* was significantly downregulated at 72 hpi in the susceptible genotype.

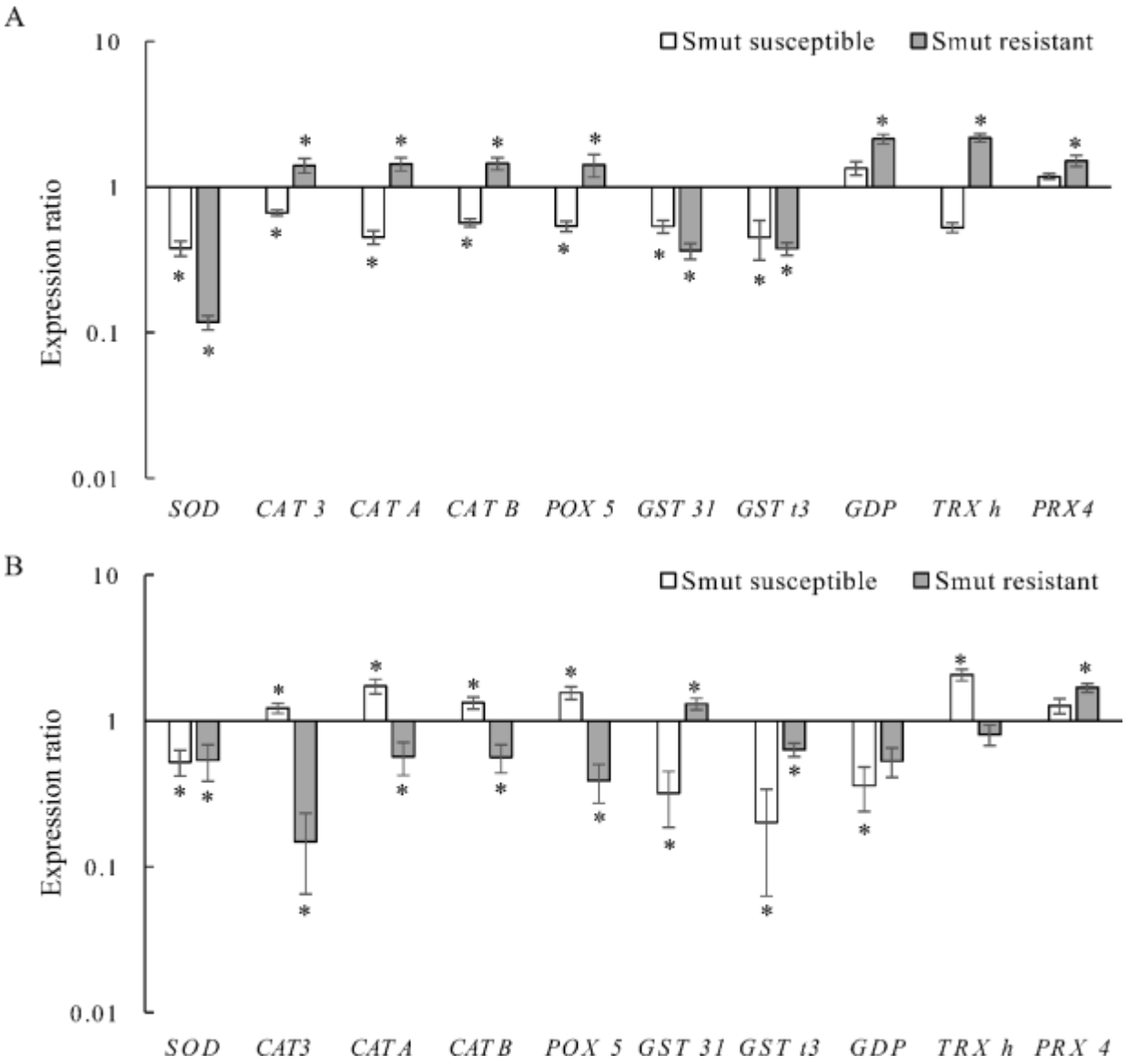

Figure 2.12 - Expression profiling of superoxide dismutase (SOD-*comp186491_c0_seq1*), catalase 3(CAT3-*comp189288_c1_seq1*), catalase A (CATA-*comp189288_c0_seq1*), catalase B (CATB-*comp191235_c0_seq1*), peroxidase 5-like (POX5-*comp127311_c0_seq1*), glutathione S-transferase 31 (GST31-*comp179663_c0_seq1*), glutathione S-transferase t3 (GST t3-comp198747_c0_seq1), guanine nucleotide-binding protein a (GDP-*Sb09g027690.1|PACid:1981757*), thioredoxin h like (TRX h-*evm.model.scga7_unitig_341686.1*) and peroxidase III class (PRX4-*evm.model.scga7_uti_cns_0172034.2*) genes associated with the antioxidant system in smut-susceptible and -resistant genotypes by RT-qPCR analysis. A - Gene expression at 24 hpi. B - Gene expression at 72 hpi. Statistical analysis was performed using REST® software. * represents genes differentially expressed by RT-qPCR ($P < 0.05$)

2.2.2.7 Fungal quantities within plant tissues confirmed the reduction of fungal growth in resistant plants

In order to detect the dynamic of *S. scitamineum* growth in infected sugarcane, we used the qPCR protocol developed in this study to quantify the fungus in infected buds. The results revealed that the pathogen could be detected within the first 6 hpi in smut susceptible buds (Figure 2.13A). In addition, starting from 24 hpi (48 and 72 hpi) the DNA quantity of *S.*

scitamineum markedly increased in the susceptible variety (Figure 2.13A). On the other hand, this dynamic of *S. scitamineum* growth was opposite in the resistant variety. At 12 hpi, the DNA quantity of *S. scitamineum* was similar to the observed at 24 hpi. However, in the other tested times (48 and 72 hpi) the fungus DNA presented smaller quantity in comparison to the observed at 6, 12 and 24 hpi (Figure 2.13B).

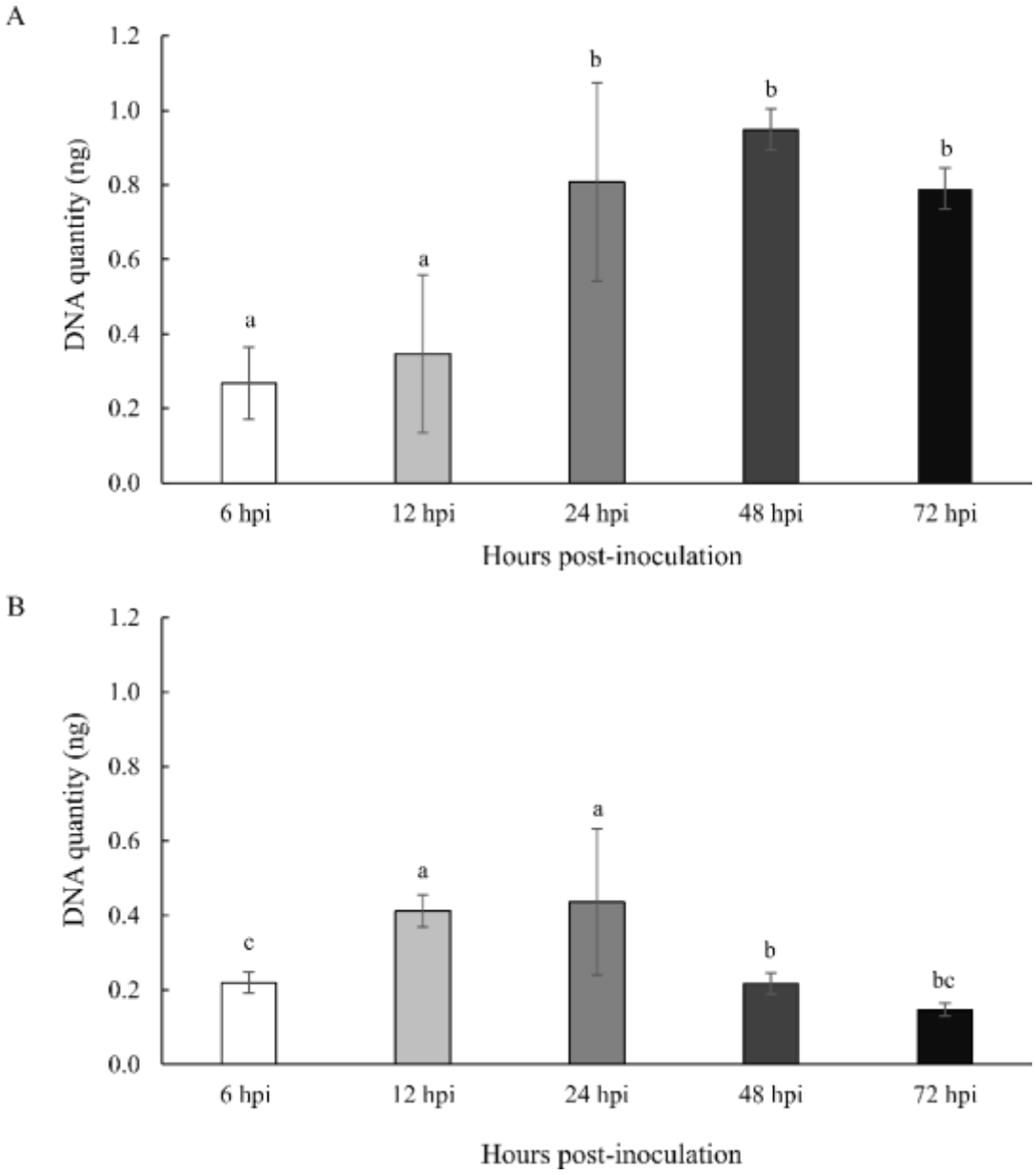

Figure 2.13 - DNA quantity (ng) of *S. scitamineum* assessed by qPCR (present in 100 ng of DNA extracted from infected plants) using SSC-C primer pair. A - Infected smut susceptible sugarcane at 6, 12, 24, 48 and 74 hpi. B -Infected smut resistant sugarcane at 6, 12, 24, 48 and 74 hpi. Values of DNA quantity represent the means from three biological replicates. Means with different letters are significantly different ($P \leq 0.05$) by one-way analysis of variance (ANOVA) and Tukey's test

2.2.3 Discussion

To characterize the sugarcane responses in the early stages of interaction, we performed histochemical and biochemical studies and assessed the gene expression profile and protein identification associated with the antioxidant system of sugarcane genotypes susceptible and resistant to smut. Similar to other smut species, *S. scitamineum* is a biotrophic fungus that during the early stage of infection penetrates plant tissues colonizing the primary meristem (ALEXANDER; RAMAKRISHNAN, 1980; SUNDAR et al., 2012). The sugarcane mechanisms of resistance to smut is complex and involves several mechanisms (LLOYD AND PILLAY, 1980; LEGAZ et al., 1998; PIÑON et al., 1999; FONTANIELLA et al., 2002; MILLANES et al., 2005; LEGAZ et al., 2005; DE ARMAS et al., 2007; SANTIAGO et al., 2012) including the oxidative burst (LAO et al., 2008; SU et al., 2014). To depict the events related to ROS, we used a resistant SP80-3280 genotype, which is considered highly resistant to smut and is largely cultivated in Brazil, and the IAC66-6 genotype, which is highly susceptible to smut and is maintained only for research purposes (CARVALHO et al, 2016).

Buds of these two genotypes were clearly distinguished by the format, hardness and pigmentation (Figure 2.1), suggesting that some of the above mentioned mechanisms of resistance should be present in these plants. Our data revealed that teliospore germination was delayed in the resistant genotype, however, it still proceeded reaching its maximum at 12 hpi. It is noteworthy that fungal colonization was achieved for both genotypes using the inoculation method that did not injure sugarcane tissues. Following the germination results, appressorium formation was also delayed in resistant plants, but not completely impaired. The study of CARVALHO et al (2016) showed that plants of the SP80-3280 are colonized by the fungus at least until 180 days after inoculation (dai). Other resistant genotypes also allow fungal colonization but not whip emission (WALLER, 1970; LLOYD; PILLAY, 1980; MILLANES et al., 2005). The fungal quantification using qPCR confirmed the genotype influenced fungal growth. The susceptible genotype was more conducive to the pathogen proliferation than the resistant one. The results corroborated the data of spore germination and appressorium formation, which were delayed in the resistant genotype.

ROS production causes a direct toxic effect to the pathogen (TORRES, 2010) along with localized injuries to the plant cell membrane, which delays or impairs fungal colonization (HAMMOND-KOSACK; JONES, 1996; MAY et al., 1996). Although some previous studies (LAO et al., 2008; SONG et al.; 2013; SU et al., 2014) have revealed the relevance of the oxidative burst to counteract fungal colonization, we were able to relate each fungal developmental stage to the variation of the plant responses. Histochemical detection of

superoxide anion and H_2O_2 accumulation in sugarcane smut infected was carried out in this work. Our results revealed that *S. scitamineum* markedly induced H_2O_2 accumulation at 6, 48 and 72 hpi in inoculated buds of the resistant genotype. These three time points were related to the three phases of fungal development: phase I, coinciding with initial spore germination at 6 hpi (beginning of promycelium formation); phase II: proceeding at 48 hpi, at the moment of the accumulation of H_2O_2 surrounding the appressorium and; the third phase at 72 hpi, when an extensive net of fungal hyphae was observed and DAB staining revealed H_2O_2 spread over the epidermal cells of the bud surface (H_2O_2 increased by 70 % compared with the control). This triphasic type of H_2O_2 accumulation in incompatible interactions has been described for other monocot involving interactions such as those of *Blumeria graminis* f. sp. *hordei*-infected barley (HÜCKELHOVEN; KOGEL, 2003) and *Septoria tritici*-infected wheat (SHETTY et al., 2003). Other pathosystems seem to rely on two phases of a ROS-associated response to pathogens (LAMB; DIXON, 1997; TORRES et al., 2006; TORRES, 2010). These differences presumably are determined by the host genotype, as well as by the pathogen infection process (SHETTY et al., 2007). H_2O_2 is also a signaling compound and the response observed by the resistant genotype may indicate the key role of this ROS in signaling the stress response, which does not occur in the susceptible genotype. In case the role of the ROS in the plant-pathogen interactions, the timing and concentration are also important in site action, signaling and downstream responses (HÜCKELHOVEN; KOGEL, 2003).

Similarly to ROS production, lipid peroxidation is a biochemical marker of oxidative stress (GRATÃO et al., 2015) and it is among the plant defense responses induced by pathogenic fungi in early stages of infection (HEATH, 2000). It has been proposed that lipid peroxidation is a key process for membrane alteration in plants (KEPPLER, 1986; LAMB; MAY et al., 1996; DIXON, 1997; MONTILLET et al., 2005). In many cases, this response is efficient against biotrophic pathogens that depend on living cells to survive (KOECK; HARDHAM; DODDS, 2011). In our results, the smut-resistant genotype showed increased levels of MDA content at 72 hpi, a finding that was not detected for the susceptible genotype. Interestingly, the reduced GST antioxidant enzyme activity and particularly the CAT activity, probably contributed to the increased levels of H_2O_2, leading consequently to this increased lipid peroxidation. CAT is referred as a key H_2O_2-scavenging enzyme in plants (GRATÃO et al., 2005) and is generally localized in the peroxisomes where most of the cellular H_2O_2 is produced as well as several signalling molecules derived from β-oxidation, including salicylic acid (SA) (DEL-RÍO; LÓPEZ-HUERTAS 2016). For instance, CAT activity may be inhibited by the action of salicylic acid (SA) (TAKAHASHI et al., 1997; MITTLER et al., 1999) during

HR, raising H_2O_2 levels and inducing the expression of defense genes in response to pathogens (MITTLER et al., 1999; KLESSIG et al., 2000). In addition, inhibition of CAT can convert SA into a free radical, which can also initiate lipid peroxidation (DURNER; KLESSIG, 1996). It was also described that SA signalling is the major pathway activated by biotrophic pathogens (GLAZEBROOK, 2005) associated with the early defence response of sugarcane to *S. scitamineum* (CHEN et al., 2012). These results lead us to propose a key role of H_2O_2 in the sugarcane early defence response to smut, probably in association with SA. In addition, the MDA data showed that the constitutive antioxidant system between the two sugarcane genotypes are notably different. In all treatments, the smut resistant exhibited 70% lower MDA content that of the in smut-susceptible genotype independent of conditions applied.

The ROS-scavenging systems play an important role in managing ROS generated in the plant-pathogen interaction (TORRES et al., 2006). SOD catalyses the dismutation of superoxide anion to H_2O_2 and represents the first line of defence against ROS (GIANNOPOLITIS; RIES, 1977; MITTLER, 2002; DE GARA; DE PINTO; TOMMASI, 2003; GRATÃO et al., 2015). In the present study, SOD activity did not exhibit any major alterations that could be due to the inoculation in either the resistant or susceptible genotype. This is true when no major alterations in the band intensity or specific induction or repression of isoenzymes were observed between the control and inoculated plants. Conversely, the two genotypes clearly exhibited a different number of isoenzymes, explained by the genetic background, but not the infection with *S. scitamineum* Moreover, the isoenzyme pattern observed for the SP80-3280-resistant genotype agrees with the previous report by FORNAZIER et al. (2002) who used the same genotype to study cadmium-induced stress in sugarcane. These authors also identified a large number of SOD isoenzymes with the same ones also accounting for the majority of the SOD activity observed. All SOD isoenzymes were classified by FORNAZIER et al. (2002) as Cu-Zn/SOD. Because SOD dismutates the superoxide ion into H_2O_2, the results suggest that, at least under the conditions tested, the increased production in superoxide observed in plants cells of the resistant genotype at 6 and 12 hpi was not high enough to increase SOD activity, in other words, the base SOD activity in the resistant genotype appears to be high enough to deal with an increment in superoxide production in response to the inoculation. These results also clearly indicate that the changes in H_2O_2 observed cannot be explained by changes in SOD activity.

The increase in CAT activity in the buds of resistant sugarcane plants (Yacheng 05–179) infected with *S. scitamineum* in the early stages of interaction (6 and 24 hpi) was

previously detected, whereas the susceptible genotype (Liucheng 03–182) did not alter the activity levels during the analysis (SU et al., 2014). According to the authors, there is a positive correlation between the CAT activity and the sugarcane resistance to smut. Our data corroborate with this previous results regarding the susceptible genotype. However, CAT activity decreased at 12 and 72 hpi in the smut resistant genotype used here suggesting that resistant genotypes may respond differently than previously suggested to *S. scitamineum* infection. Our work revealed that particularly at 72 hpi, the infected buds presented increased H_2O_2 content and lipid peroxidation, and decreased CAT activity.

Likewise, GSTs are responsible for antioxidant activity, reducing damage caused by pathogens through the removal of lipid hydroperoxides produced by the peroxidation of membranes (LIEBERHERR et al., 2003; DEAN; GOODWIN; HSIANG, 2005; GHELFI et al., 2011). In this study, GST activity was increased in the infected resistant genotype at 6 and 12 hpi probably contributing to the inhibition of lipid peroxidation. We conclude that this increased GST activity may be associated with smut resistance because we did not detect alterations in the susceptible genotypes, except at 12 hpi, in which GST activity was decreased. In other pathosystems, the GST increased activity has also been associated with pathogen resistance (DEBONA et al., 2012; FORTUNATO et al., 2015).

To characterize sugarcane responses to *S. scitamineum* regarding ROS, we analysed susceptible and resistant sugarcane plants at 72 hpi at the protein level. Thirty-nine proteins were detected as induced or inhibited in sugarcane in response to *S. scitamineum* infection, and four of these were oxidative stress-related. In this first proteomic attempt, we did not use a quantitative approach; instead, proteins were identified as particularly present in only one of the two genotypes tested upon infection. The protein cationic peroxidase spc4-like (*evm.model.scga7_uti_cns_0172034.2*) (classified as class III peroxidase) was inhibited in susceptible buds. Additionally, this same protein-encoding gene tested via RT-qPCR was upregulated in the smut-resistant genotype at 24 and 72 hpi; however, in the smut-susceptible genotype, its expression did not change throughout the time points analysed. We speculate that *S. scitamineum* caused the inhibition of this peroxidase potentially through the action of effectors. The pathosystem involving maize and the smut fungus *Ustilago maydis*, expressed the PEP1 effector that inhibits this same peroxidase (identity 86%) in susceptible plants, leading to blockade of the oxidative burst and suppression of the early immune responses of maize (HEMETSBERGER et al., 2012). T The gene encoding an orthologue of PEP1 is present in the *S. scitamineum* genome (QUE et al., 2014; HEMETSBERGER et al., 2015). We also identified that the infected sugarcane resistant genotype presented proteins such as

thioredoxin h-type (evm.model.scga7_unitig_341686.1), guanine nucleotide-binding protein subunit beta-like protein a (*Sb09g027690.1|PACid:1981757*- classified as G-protein) and ascorbate peroxidase (*Sb02g044060.1|PACid:1959988*), which were not detected in the susceptible genotypes, potentially involved in the resistance process. Thioredoxins can regulate the redox status of proteins through thiol-disulphide exchange reactions (SEVILLA et al., 2015). In plant-pathogen interactions, thioredoxins are required to catalyse the conversion of the SA-induced nonexpressor of PR genes 1 (*NPR1*) into a monomer and to activate defence responses (TADA et al., 2008). Previous data have shown that sugarcane plants infected with *S. scitamineum* upregulated *NPR1* (CHEN, et al., 2012). Also associated with defence signalling in plants are G-proteins (NITTA et al., 2015), in which the activation of these proteins (Gβ) occurs in response to pathogen elicitors leading to ROS increase (LIU et al., 2013; TORRES et al., 2013) and the synthesis of pathogenesis-related (PR) proteins (BEFFA et al., 1995).

We further investigated the expression of genes at 24 and 72 hpi that were identified here by histo-biochemical and proteomic data regarding the processes of oxidative burst and antioxidative defence. These two points were relevant and markedly different considering the two genotypes analysed. The transcription profiles of marker genes of the antioxidant system followed the pattern of ROS management discussed here between sugarcane-susceptible and -resistant genotypes. The response of the smut-resistant genotype was earlier (at 24 hpi), showing high transcript accumulation after infection for several of the analysed genes; however, in the smut-susceptible genotype, these same genes were downregulated. Conversely, these same genes were upregulated in the susceptible genotype later at 72 hpi. Previous RNAseq data from sugarcane cultivars resistant (Yacheng 05-179) and susceptible ("ROC"22) to *S. scitamineum* revealed that the genes associated with resistance in the resistant cultivar were earlier expressed (24-48 hpi) than those detected for smut susceptible cultivar (28-120 hpi) (QUE et al., 2014). Other regulatory processes may be involved because the expression of genes encoding the antioxidant enzymes did not necessarily coincide with the enzyme response. Moreover, other antioxidant enzymes may be involved in the stress response and must be considered in future studies. In particular, we suggest that special attention should be given to apoplastic class III peroxidases and the enzymes of the ascorbate-glutathione cycle.

In conclusion, we described the sugarcane response regarding the oxidative burst induced by *S. scitamineum* using a combination of molecular and biochemical tools to identify candidate genes that reveal relevant information regarding resistance to smut. At the

initial step of smut infection, ROS production and antioxidant enzymes have different outcomes between the susceptible and resistant sugarcane genotypes. Our results demonstrated that fungal developmental stages within sugarcane tissues (smut resistant) impose an earlier oxidative burst response by reducing the activity of antioxidant enzymes and significantly increasing H_2O_2 accumulation, resulting in severe lipid peroxidation (Figure 2.14A and 2.14B). This timely response in the sugarcane-resistant genotype is stronger at 72 hpi. The fungal hyphae upon exposure to the plant response accumulated H_2O_2 in vesicles throughout its extension. This is an effect that needs to be further explored. It is also clear that the different enzymes analysed may respond differently depending on the period tested, inoculation and genotype. However, regardless of the genotypes fungal colonization is achieved for both genotype at 72 hpi.

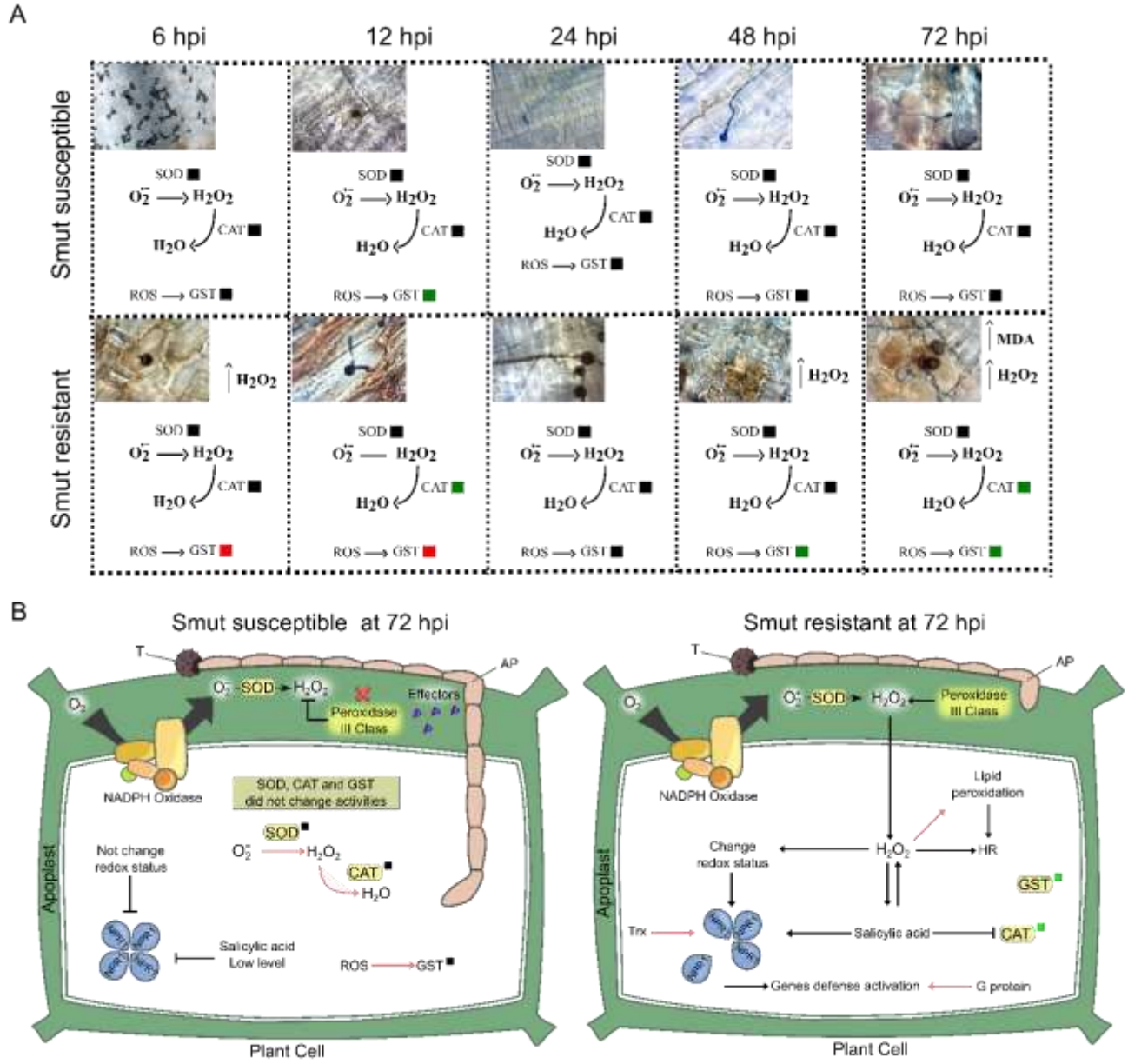

Figure 2.14 – General view of ROS metabolism of sugarcane buds inoculated with *S. scitamineum*. A - Summary of the antioxidant system, oxidative stress markers and infection process of *S. scitamineum* on sugarcane bud scales in a time-course experiment (6, 12, 24, 48 and 72 hpi) based on the data obtained in the present study. SOD (superoxide dismutase); CAT (catalase); GST (glutathione S-transferase); MDA (lipid peroxidation) e H_2O_2 (hydrogen peroxide). Green squares indicate decreases in enzymatic activity; red squares indicate increases in enzymatic activity; black squares indicate no alterations. B - Overview of the mechanisms associated with ROS and antioxidant enzymes of susceptible and resistant sugarcane inoculated with *S. scitaminum* at 72 hpi. Red arrows represent results from this study. Green squares indicate decreases in enzymatic activity; black squares indicate no alterations. Symbol indicates "x" repression (in only smut-susceptible plants). T represents teliospore, and Ap represents appressorium. All changes are relative to the mock control

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3 *Sporisorium scitamineum*, THE CAUSAL AGENT OF SUGARCANE SMUT DEALING WITH OXIDATIVE STRESS IMPOSED BY HYDROGEN PEROXIDE

Abstract

Increase of reactive oxygen species (ROS) following plant-pathogen interactions can trigger plant defence responses and directly damage pathogens. Thus, it is essential for pathogens to scavenge host-derived ROS to establish a pathogenic relationship. However, the mechanisms protecting pathogens to oxidative stress remain unclear. In this study, biotrophic fungus *Sporisorium scitamineum* was exposed to exogenous hydrogen peroxide (H_2O_2) for 30 and 180 minutes. The results revealed that the fungus is resistant to H_2O_2-induced oxidative stress and, in which lipid peroxidation was not observed at 2 mM of exogenous exposure to H_2O_2. Three superoxide dismutase (*SOD*) and two catalase (*Kat*) genes were identified in the *S. scitamineum* genome that encode a cytoplasmic *KatG*, the mitochondrial *SOD2* and *SOD3* and, extracellular *SOD1* and *KatE*. The resistance to exogenous H_2O_2 was associated with an increased activity of CAT and SOD as well as an adaptive response to the stress-agent. Also, the SOD1 isoenzyme seemed to act against the external superoxide, while SOD2, SOD3 and KatE were more associated to intracellular requirements of the fungus. Altogether, the data suggest that *S. scitamineum* have an efficient antioxidant system and responds to the ROS generated endogenously during metabolism and counteracts exogenous H_2O_2.

Keywords: Oxidative stress response; ROS; Antioxidant enzymes; Phytopathogen fungi

3.1 Introduction

The biotrophic and basidiomycete fungus *Sporisorium scitamineum* (= *Ustilago scitaminea* Sydow; P. Sydow) is the causal agent of sugarcane smut disease responsible for considerable losses to the crop worldwide (PIEPENBRING; STOLL; OBERWINKLER, 2002; SUNDAR et al., 2012). The life cycle of *S. scitamineum* can be described in three distinct phases: haploid yeast-like cells, dikaryotic hyphae and diploid teliospores. The fusion of two haploid sporidia belonging to opposite mating-types results in the infective dikaryotic hyphae, which is necessary to infect the host cells (SINGH; SOMAI; PILLAY, 2004; TANIGUTI et al., 2015). Fungal colonization induces the development of a whip-like structure composed of host and fungal cells that harbors sporogenesis producing billions of teliospores as a result of karyogamy and hyphal fragmentation (Trione, 1990). (TRIONE, 1990). Under favorable environmental conditions, teliospores germinate leading to meiosis and production of sporidial cells, which can fuse to produce the dikaryotic infective hyphae over again. Sporidia can be propagated as yeast-like haploid cells in lab conditions (BANUETT; HERSKOWITZ, 1996).

The interaction between plant pathogens and their hosts is very complex and resulting of a long-standing battle, in which the pathogen attacks to invade and multiply and the plant

recognizes and protects itself from the infection (FONES; PRESTON, 2012). Reactive Oxygen Species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are constantly being produced during normal aerobic metabolism, but also plays a major role in plant pathogen-interaction in response to the pathogen recognition (HELLER; TUDZYNSKI, 2011). In plant defenses, ROS act not only as toxins, able to directly kill or slow the pathogen growth, but also as part of a signaling cascade that may activate hypersensitive response, cell wall modifications and changes in gene expression (TORRES; JONES; DANGL, 2006).

The overcome of host-derived ROS is essential for fungal virulence and relies on the assumption that biotrophic/hemibitrophic fungi depend on the survival of strong oxidative burst, either by suppressing the basal defense reaction or by scavenging the ROS built up at early stages of infection (HELLER; TUDZYNSKI, 2011; KOMALAPRIYA et al., 2015). Additionally, the regulation of cellular levels of ROS is critical for developmental differentiation of many pathogenic fungi (GESSLER; AVERYANOV; BELOZERSKAYA, 2007), since excess ROS leads to oxidative stress, causing directly damage lipids, proteins and nucleic acids (HALLIWELL, 2006; LUSHCHAK, 2011). The mechanisms evolved to surpass ROS are generally divided into enzymatic and non-ezymatic systems (MITTLER, 2002; GRATÃO et al., 2005). Non-enzymatic defense consists of the synthesis of small soluble molecules that are oxidized by ROS, like glutathione (GSH). Enzymatic system includes superoxide dismutase (SOD), peroxidases such as glutathione peroxidase (GPx) and catalase (CAT) (APEL; HIRT, 2004).

SODs work as scavengers of superoxide anion ($O_2^{\cdot-\bullet}$) to form O_2 and H_2O_2 and have a protective function during oxidative stress (FRIDOVICH, 1995). SODs are metalloenzymes found in prokaryotes and eukaryotes organisms and have several isoenzymes depending on the metal cofactor(s) that interact with the active site: manganese co-factored (MnSOD), iron co-factored (FeSOD) copper-zinc co-factored (Cu-ZnSOD) and nickel co-factored (NiSOD) (ZELKO; MARIANI; FOLZ, 2002; MILLER, 2004). These different types of SOD are distributed in different cellular compartments (mitochondria, peroxisomes, cytoplasm and others) (GESSLER; AVERYANOV; BELOZERSKAYA, 2007). In fungi, for example, CuZnSOD cytoplasmic have been extensively studied in the yeast *Saccharomyces cerevisiae* where this isoenzyme accounts for 90 % of the total SOD activity of the cell. In addition, in the human pathogenic *Candida albicans* (GLEASON et al., 2014) and in phytopathogens such as *Fusarium graminearum* (YAO et al., 2016) and *Sclerotinia sclerotiorum* (VELUCHAMY et al., 2012) different types SODs are required for virulence and protect against the oxidative burst of host innate immune.

CATs are metalloenzymes that protect cells from oxidative damage originated from exposure to H_2O_2 (HANSBERG; SALAS-LIZANA; DOMÍNGUEZ, 2012). As example of SODs, some CATs in fungal pathogens have proven to be important in the maintenance of cell integrity and virulence during host invasion (SKAMNIOTI et al., 2007). For instance, in *C. albicans*, exogenous H_2O_2 detoxification is firstly catalysed by CAT, which is induced by transcriptional activation of the single catalase gene (*CAT1*) (SMITH et al., 2004) and after H_2O_2 detoxification is mediated by the glutathione and thioredoxin systems, respectively (KOMALAPRIYA et al., 2015). In general, fungi have monofunctional heme catalase and bifunctional heme catalase-peroxidase (ZAMOCKY; FURTMÜLLER; OBINGER, 2008; ZAMOCKY et al., 2012), which are localized in different cellular compartments (HANSBERG; SALAS-LIZANA; DOMÍNGUEZ, 2012).

Therefore, for understanding of the effect of ROS is important when defining the components of the fungal antioxidant response and to determinate the biochemical and molecular changes required for adaption to external or internal stimuli. In order to investigate how *S. scitamineum* cope with external ROS, we considered the effects of exogenous H_2O_2 on the antioxidant system of haploid sporidia cells of *S. scitamineum in vitro.*

3.2 Development

3.2.1 Material and Methods

3.2.1.1 *S. scitamineum* strain and growth conditions

The *S. scitamineum* SSC39A and SSC39B haploid cells of compatible mating types were isolated from teliospores recovered from a whip emerged in a susceptible sugarcane genotype as previously described by TANIGUTI et al. (2015). The SSC39A cells used in this study were maintained in liquid Yeast Medium (YM) (composed of 3 g of yeast extract, 3 g of malt extract, 5 g of soybean peptone and 10 g glucose per L of distilled water) shaken at 180 rpm in an orbital shaker controlled at 28 °C.

3.2.1.2 Sensitivity curve of *S. scitamineum* to hydrogen peroxide

S. scitamineum haploid cells were grown to early-exponential phase (6 hours) in YM medium at 28°C and shaken at 180 rpm in an orbital shaker. At this point, hydrogen peroxide was added at 0, 2, 4, 6, 8, 10, 20 and 30 mM final concentrations and the growth was monitored by optical density (OD_{600}) every 2 h until growth reached the end-exponential

phase (12 h). The experiment was maintained in the dark. The measurements were performed in triplicate and the results were scored as mean and standard deviation (SD).

3.2.1.3 Hydrogen peroxide effects on *S. scitamineum* haploid cells

S. scitamineum haploid cells were grown at 28°C, 180 rpm until reaching early-exponential phase (6 hours - OD_{600} = 0.8) in YM medium added of hydrogen peroxide 2 mM. The experiment was composed of the following treatments: 1) time 0, the cells were analysed immediately after 6h of growth; 2) control, growth was maintained for an additional 30 min 3) H_2O_2 – 30, the peroxide (2mM) was added after 6h of growth and maintained for an additional 30 min; 4) control – 180 min, after 6 h, growth was maintained for an additional 180 min; and 5) H_2O_2 – 180 min, after 6 h, cells were exposed to 2 mM of H_2O_2 and growth was maintained for an additional 180 min. The experiment was performed with three biological replicates (Figure 3.1).

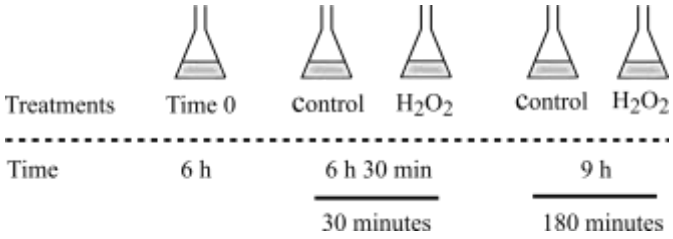

Figure 3.1 - Illustration of the experimental design used in this study. Treatments and time correspond to the addition of H_2O_2 and points analyzed, respectively

3.2.1.4 Sequence analyses of SOD and CAT isoenzymes

In order to identify the SODs and CATs isoenzymes encoded by the *S. scitamineum* genome, similarity searches were used (BLASTp analysis with default parameters using the non-redundant protein sequence database). For all the identified genes, the encoded protein sequences were analysed for domain conservation by InterProScan (ZDOBNOV et al., 2001) and subcellular location using CELLO v. 2.5 (YU et al., 2006). Additionally, the molecular weights of the SODs and CATs 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 conduct dendogram (protein sequences from fungi of different phylum - Ascomycota, Basidiomycota, glomeromycota, Zygomycota, Chytridiomycota and Microsporidia) (Table 1) (MIR et al., 2015). The protein sequences were aligned using ClustalW software with default parameters. Dendograms were constructed using the neighbor-joining method in the MEGA 6.0 software (TAMURA et al., 2013).

Table 3.1 - Superoxide dismutase and catalases genes from 11 selected fungal genomes. SOD and CAT are the copy number of genes encoding superoxide dismutases and catalases in the genomes, respectively

Phylum	Species	SOD	CAT	Characteristic of fungus
Basidiomycota	*Ustilago maydis*	3	1	maize smut
Basidiomycota	*Ustilago hordei*	3	2	barley smut
Basidiomycota	*Sporisorium reilianum*	3	2	maize and barley smut
Basidiomycota	*Sporisorium scitamineum*	3	2	sugarcane smut
Ascomycota	*Magnaporthe oryzae* 70-15	6	2	rice blast fungus
Ascomycota	*Candida albicans*	6	1	disease in human
Ascomycota	*Saccharomyces cerevisiae*	2	2	saprophytic
Chytridiomycota	*Batrachochytrium dendrobatidis* JAM81	9	1	disease in amphibians
Zygomycota	*Rhizopus arrhizus* var. *delemar*	6	2	disease in human
Glomeromycota	*Rhizophagus irregularis* DAOM	4	1	arbuscular mycorrhizal
Microsporidia	*Encephalitozoon cuniculi* GB-M1	1	-	disease in human

3.2.1.5 Gene expression levels analysed by RT-qPCR

Total RNA was obtained from *S. scitamineum* of different treatments using the Trizol reagent (Invitrogen) and treated with DNase I (Thermo Scientific). RNA quality was verified after electrophoresis on agarose gels. Primers were manually designed and analysed using Gene Runner (http://www.generunner.net/) and Beacon Designer™ Free Edition (http://www.premierbiosoft.com) softwares (Table 3.2). 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 25 ng of RNA, 6.5 μL of GoTaq® qPCRMaster 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. Primer specificity was confirmed by the analysis of the dissociation curve in every reaction. *S. scitamineum* housekeeping gene encoding for GAPDH (designed in this study) 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., 2002). Control samples were used as calibrators.

Table 3.2: List of primers of *S. scitamineum* used in this study

Gene ID	Primers
g651_chr01_Ss	5’ TGTGCTGAAGGATGCTATTGAC 3’ 3’ CCTTGACGTTCTGGTACTGGAG 5’
g2980_chr07_Ss	5’ CAACCCTAAGAACTCCAAGCTC 3’ 3’ AAGTTGATGACCGACCAGATG 5’
g3299_chr08_Ss	5’ TGATCAGTAACCAGCCAAACC 3’ 3’ GCACTCAACAACAGCTTCTTCC 5’
g4614_chr13_Ss	5’ CTTGAGGTAGAGGTTGGTCCAC 3’ 3’ GTAAGACTCACGGTGCTGGTAAC 5’
g691_chr01_Ss	5’ GGTCAACATCGGTATCAACGG3’ 3’ CTCGAGGTCAATGAAGGGGTC 5’
g1075_crh02_Ss	5’ CCCGTCCCAACTACATCTCG 3’ 3’ GTTGCCAATCGTCTGCTTCC 5’

3.2.1.6 MDA and H_2O_2 content determination of *S. scitamineum*

Malondialdehyde **(**MDA) content was determined by measuring the content of thiobarbituric acid reactive substance (TBARS) according the method described by HEATH; PACKER (1968), which estimates the lipid peroxidation rate. A 100 mg of frozen powdered cells 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 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 after centrifuged for 5 min at 12,000 *g*. MDA content was monitored by absorbance measurements at 535 and 600 nm in a Perkin Elmer Lambda 40 spectrophotometer, and the concentration was 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 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 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 (Perkin Elmer Lambda 40) and the H_2O_2 content was determined using known concentration as a standard curve. The results were expressed in μmol fresh weight g^{-1}

3.2.1.7 Superoxide dismutase and catalase activity

For SOD and CAT activity assays, 400 mg of a fine powder cells were grounded 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 ºC. The concentration of protein was determined using bovine serum albumin as standard (BRADFORD, 1976).

SOD total activity was carried out as described by GIANNOPOLITIS; RIES (1977) by measuring the enzyme 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 controls tubes 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; 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 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 development of achromatic bands of SOD activity on a purple-stained gel.

CAT total activity was assayed as described by GRATÃO et al. (2008) at 25 ^{o}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. (2012). Gels were incubated in 0.003% hydrogen peroxide (H_2O_2) for 10 min and subsequently in a 1% (w/v) ferric chloride (FeCl3) and 1% (w/v) potassium hexacyanoferrate III (K3Fe(CN6) solution for additional 10 min. One unit of bovine liver CAT (Sigma, St. Louis, USA) was used as a positive control.

3.2.1.8 Statistical Analysis

The experiment was 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 ($P<0.05$). All statistical analyses were carried out by using R software (URL http://www.r-project.org).

3.2.2 Results

3.2.2.1 *S. scitamineum* cells are resistant to exogenous H_2O_2

Hydrogen peroxide-induced oxidative stress was characterized by the analysis of cell growth in different concentrations of H_2O_2 exposed to increasing concentrations of H_2O_2 (2, 4, 6, 8, 10, 20 and 30 mM) after growth to the early-exponential phase. The H_2O_2 affected cellular growth in all time points and concentrations evaluated, excepted for 2 mM of H_2O_2 (Figure 3.2A and 3.2B). *S. scitamineum* growth decreased in the concentrations of H_2O_2 4, 6, 8, 10, 20 and 30 mM when compared with the control, whereas doses between 0 and 2 mM H_2O_2 were not statistically significant (Appendix E). This dose was chosen for further experiments because is far higher than what it is usually detected in plants. In addition, the results of the residual fraction of hydrogen peroxide (H_2O_2) in Yeast Medium are in figure 3.3 These results showed that the exogenous H_2O_2 was consumed when fungus grew in medium + H_2O_2 for 30 minutes and the amount of H_2O_2 remained in medium without the presence of *S. scitamineum* until 180 minutes (Figure 3.3).

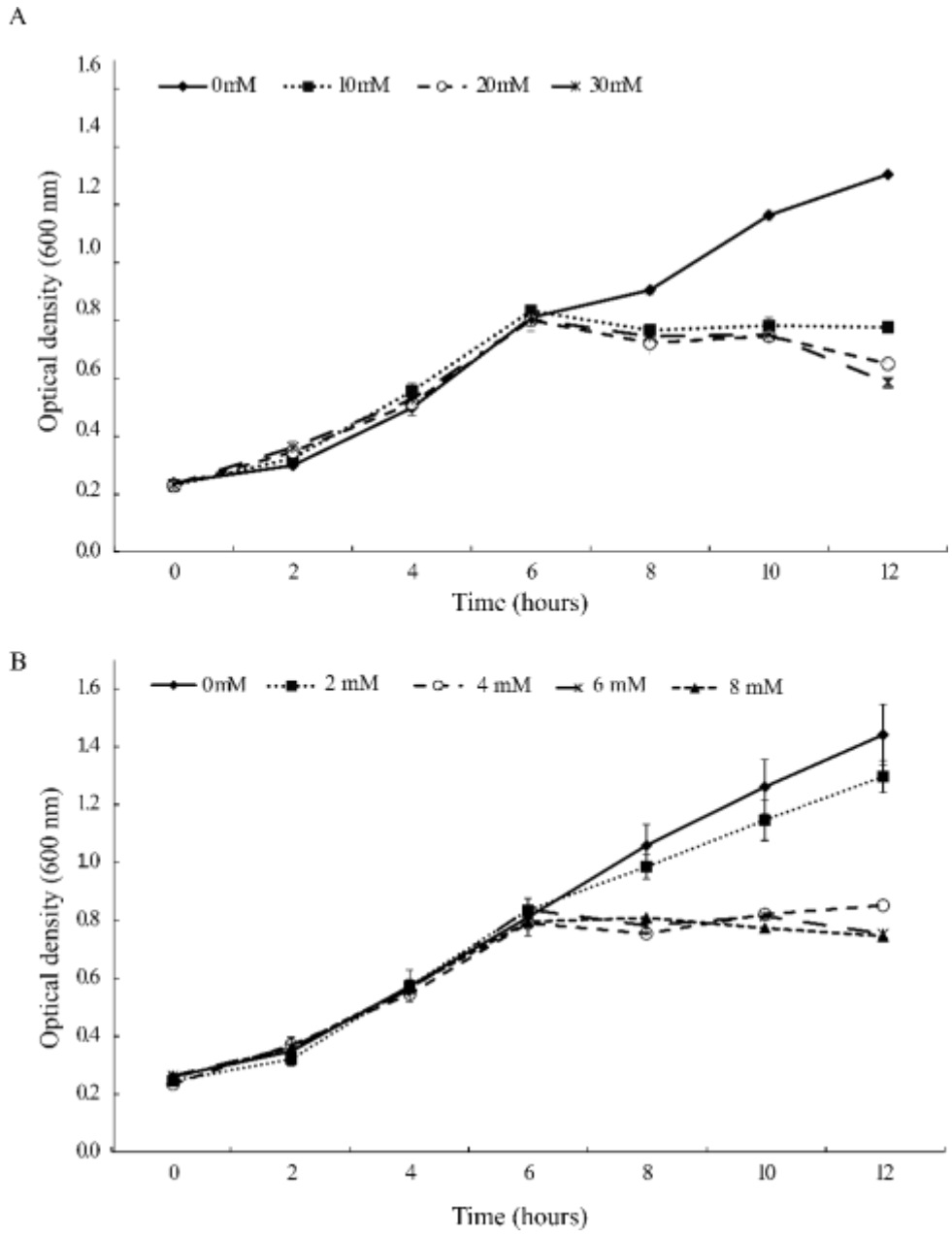

Figure 3.2 - Effect of exogenous hydrogen peroxide (H_2O_2) upon *S.scitamineum* growth in liquid medium. A - Growth curve at 0, 10, 20 and 30 mM H_2O_2 concentration. B - Growth curve at 0, 2, 4, 6 and 8 mM H_2O_2 concentration

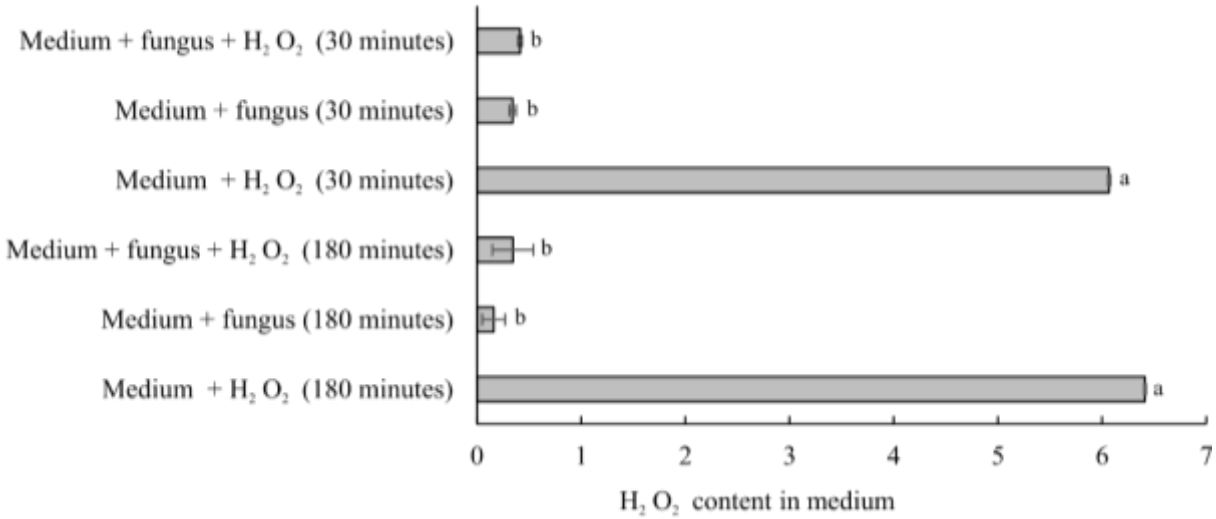

Figure 3.3 - Residual fraction of hydrogen peroxide (H_2O_2) in Yeast Medium. The treatments used were 1) Yest medium + fungus + H_2O_2 (2 mM); 2) Yest medium + fungus, 3) Yest medium + H_2O_2 (2 mM). The treatments were evaluated for 30 and 180 minutes. Values of H_2O_2 content (μmol g^{-1} fr. wt) represent the means from three replicates. Means with different letters are significantly different (P<0.05) by one-way analysis of variance (ANOVA) and Tukey's test

3.2.2.2 *S. scitamineum* cells did not exhibit lipid peroxidation in presence of exogenous H_2O_2

The oxidative damage of the cell membrane was studied by measuring the malondialdehyde (MDA) content in *S. scitamineum* cells following the same treatments described before using 2mM of H_2O_2 (Figure 3.4A). The results revealed that MDA content in the treatment H_2O_2 – 30 minutes decreased significantly (68 %) when compared with its control and it was maintained unaltered considering longer exposure to H_2O_2 (180 minutes) (Figure 3.4A).

The intracellular level of H_2O_2 was similar to the results observed for MDA content (Figure 3.4B). The treatment with H_2O_2 – 30 minutes resulted in a significant decrease of 28% of the intracellular level of H_2O_2 when compared to the control, whereas the content of H_2O_2 was stable after 180 min of exposure (Figure 3.4B).

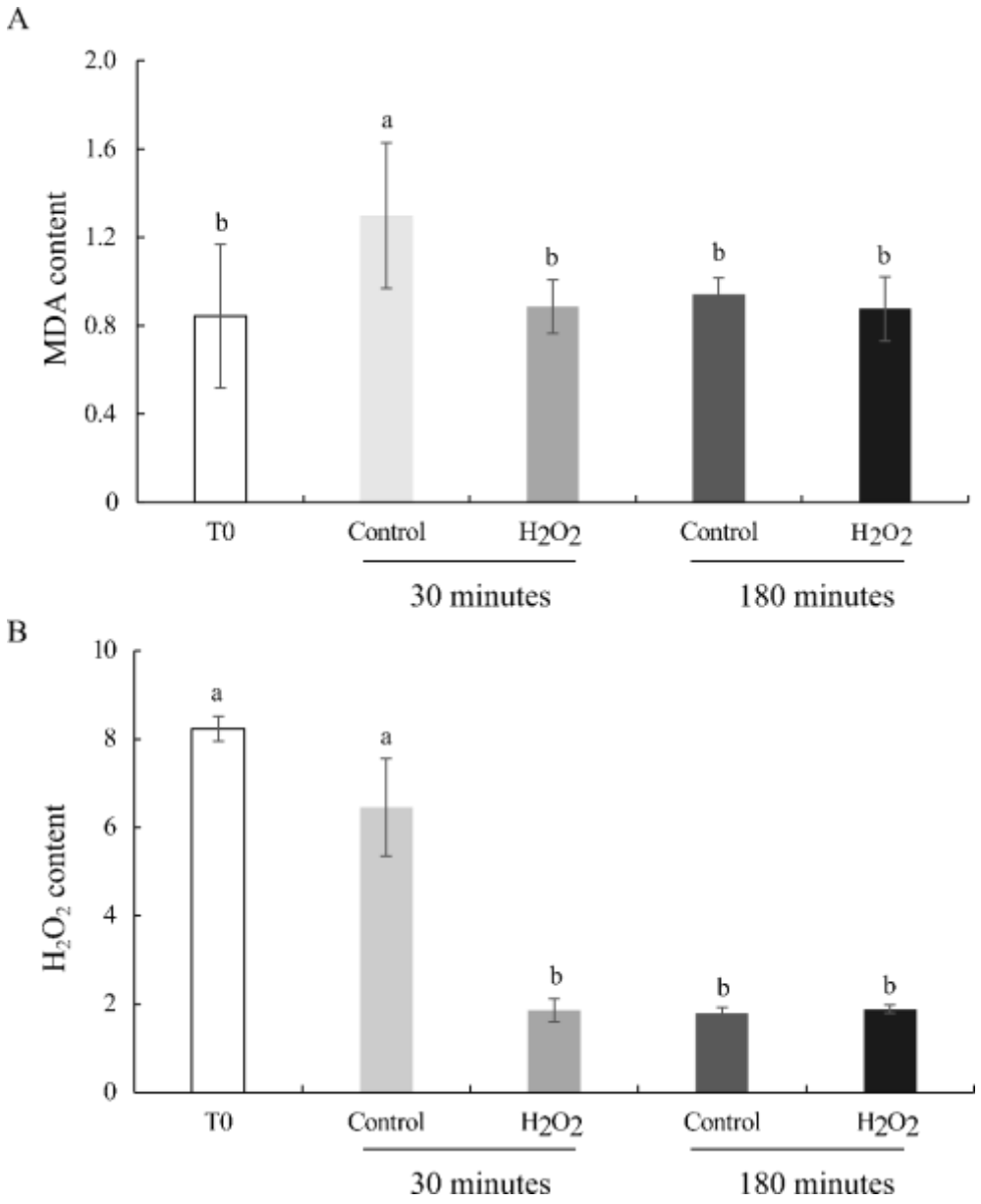

Figure 3.4 – A - Lipid peroxidation (MDA content) of *S. scitamineum* sporidia exposed to exogenous H_2O_2 for 30 and 180 minutes. Values of MDA content (nmol g^{-1} fr. wt) represent the means from three replicates. Means with different letters are significantly different ($P<0.05$) by one-way analysis of variance (ANOVA) and Tukey's test. B - Intracellular hydrogen peroxide content (H_2O_2 content) of *S. scitamineum* sporidia exposed to exogenous H_2O_2 by 30 and 180 minutes. Values of H_2O_2 content (μmol g^{-1} fr. wt) represent the means from three replicates. Means with different letters are significantly different ($P<0.05$) by one-way analysis of variance (ANOVA) and Tukey's test

3.2.2.3 Exogenous H_2O_2 alters activities of antioxidant enzymes in *S. scitamineum* cells

In order to examine in more detail the biochemical basis of the protective response to H_2O_2, we sought to assay the SOD and CAT activities (Figure 3.5 and 3.6). Total SOD specific activity increased 26 % in *S. scitamineum* cells treated with H_2O_2 for 30 minutes, whereas in the treatment for 180 minutes the fungus showed no significant difference compared with its control (Figure 3.5A). SOD activity gel staining revealed the existence of

one isoenzyme (I) in *S. scitamineum* cell in all treatments evaluated in this study (Figure 3.5B).

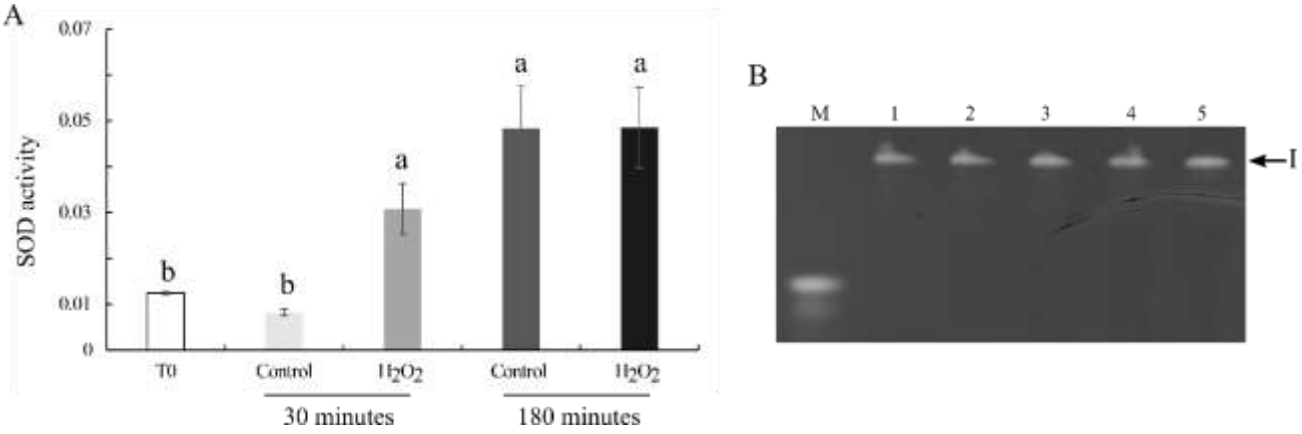

Figure 3.5 – A - Total specific activity of superoxide dismutase (SOD) (units min^{-1} mg^{-1} protein) of *S.scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 minutes. Values of total specific activity SOD represent the means from three replicates. Means with different letters are significantly different ($P<0.05$) by one-way analysis of variance (ANOVA) and Tukey's test. B - Activity staining for SOD following non-denaturing PAGE of *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 minutes. First lane (M) is a bovine SOD standard, lanes 1, 2, 3, 4 and 5, *S. scitamineum* grown in the treatments Time 0 (T0), control (30 minutes), H_2O_2 (30 minutes), control (180 minutes) and H_2O_2 (180 minutes), respectively. Arrows indicate numbered SOD band for *S. scitamineum* cells (I)

Total CAT specific activity exhibited an increase when *S. scitamineum* cells were exposed to H_2O_2 for 30 (85% increase) and 180 (84% increase) minutes (Figure 3.6A). The assessment of CAT activity by non-denaturing PAGE revealed the existence of 2 isoenzymes (I and II) in all treatments assessed (Figure 3.6B). In general, the CAT isoenzymes activities were in accordance with the results obtained for total CAT activity. Moreover, a distinct modulation in CAT activity was observed in *S. scitamineum* subjected to H_2O_2 for 30 and 180 minutes. In these treatments, an increased intensity of bands I and II occurred (Figure 3.6B).

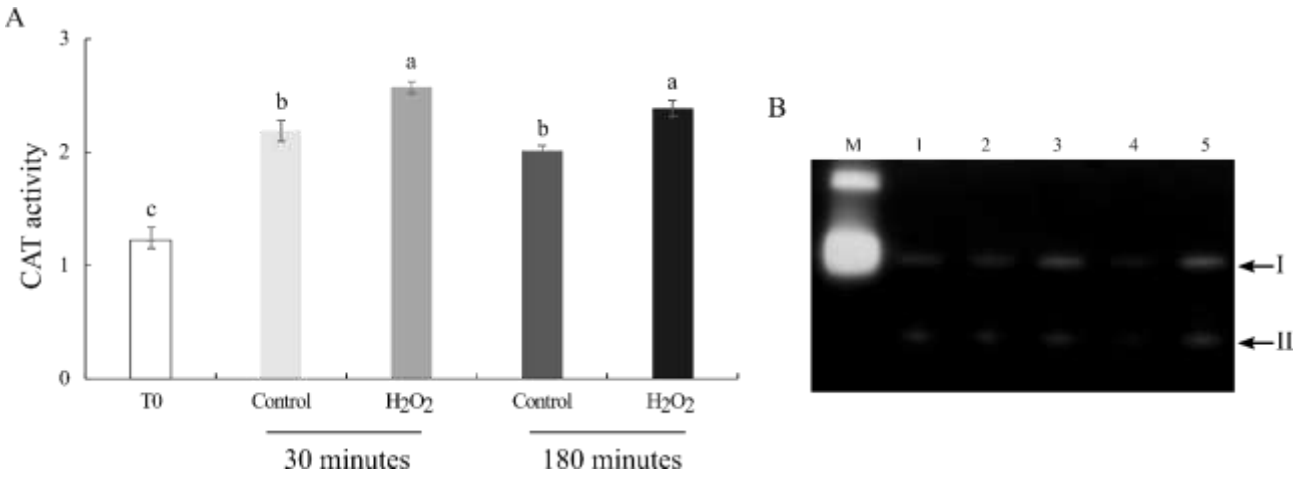

Figure 3.6 - Total specific activity of catalase (CAT) (μmol min^{-1} mg^{-1} protein) of *S.scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 minutes. Values of total specific activity SOD represent the means from three replicates. Means with different letters are significantly different ($P<0.05$) by one-way analysis of variance (ANOVA) and Tukey's test. B. Activity staining for CAT following non-denaturing PAGE of *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 minutes. First lane (M) is a bovine CAT standard, lanes 1, 2, 3, 4 and 5, *S. scitamineum* sporidia grown in the treatments Time 0 (T0), control (30 minutes), H_2O_2 (30 minutes), control (180 minutes) and H_2O_2 (180 minutes), respectively. Arrows indicate numbered CAT band for *S. scitamineum* cells (I and II)

3.2.2.4 *S. scitamineum* genome encodes three SOD and two CAT isoenzymes

Sequence analyses revealed that *S. scitamineum* genome encodes three *SOD* (*g2980_chr07_Ss, g651_chr01_Ss* and *g3299_chr08_Ss*) and two *CAT* (*g1075_crh02_Ss* and *g4614_chr13_Ss*) proteins (Table 3.3). We investigated the evolutionary relationships of *SOD*s and *CAT*s encoded by various fungi. The results revealed that SODs of *S. scitamineum* were closely related to those of other smut fungi (*S. reilianum*, *Ustilago maydis* and *U. hordei*) (Figure 3.7). Reference fungal SOD enzymes, such as those described for *C. albicans* and *S. cerevisiae*, were used to classify *S. scitamineum* SODs isoenzymes (Figure 3.7). All three SODs (g2980_chr07_Ss, g651_chr01_Ss and g3299_chr08_Ss) had the domains described for Mn/Fe proteins (PF: PF00081: alpha N-terminal domain; PF02777: alpha/beta C-terminal domain) (Figure 3.9). Additionally, the protein sequence encoded by *g3299_chr08_Ss* gene had two Mn/Fe C-terminal domain divided by an interval of 49 amino acids (Figure 3.9). In *S. scitamineum*, the protein encoded by *g2980_chr07_Ss* gene is predicted extracellular, whereas g651_chr01_Ss and g3299_chr08_Ss encoded for predicted mitochondrial proteins (Table 3.3). In this study the *g2980_chr07_Ss, g651_chr01_Ss* and *g3299_chr08_Ss* genes were named like *SOD1*, *SOD2* and *SOD3*, respectively.

Table 3.3**:** Superoxide dismutase and catalase genes of *S. scitamineum* cell

Gene ID	Gene name	Protein domain	Subcellular localization	Molecular weight (kDa)	Protein Description
g2980_chr07_Ss	*SOD1*	Mn or Fe SOD	Extracellular	26.6	Superoxide dismutase
g651_chr01_Ss	*SOD2*	Mn or Fe SOD	Mitochondrial	27.9	Superoxide dismutase
g3299_chr08_Ss	*SOD3*	Mn or Fe SOD	Mitochondrial	32.7	Superoxide dismutase
g1075_crh02_Ss	*KatE*	Imune-responsive domain/haem domain	Extracellular	63.8	catalase
g4614_chr13_Ss	*KatG*	Haem domain/ haem domain	Cytoplasmic	82.4	peroxidase/catalase

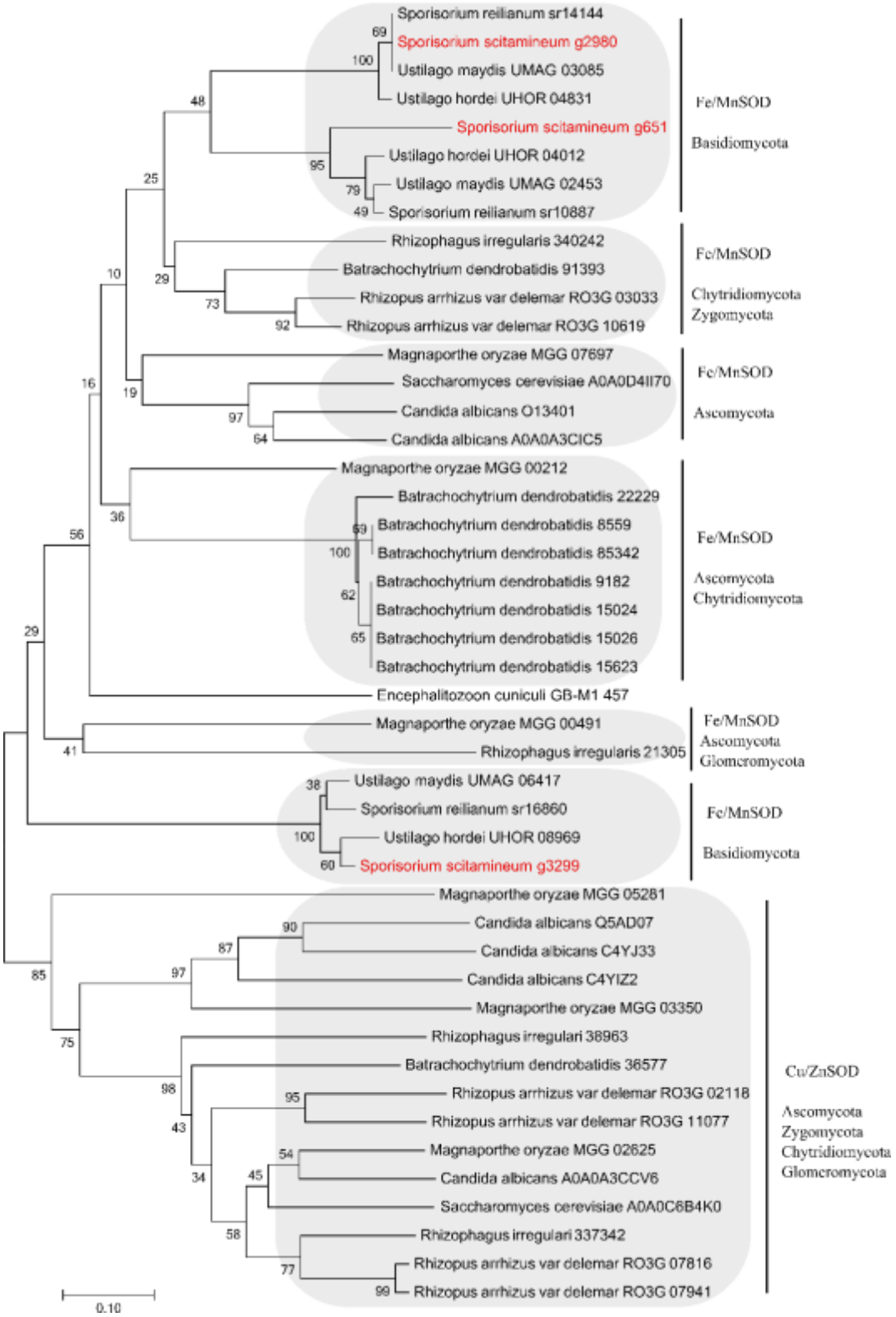

Figure 3.7 - Dendogram of putative superoxide dismutase genes in selected fungi of different phylum (basidiomycota, ascomycota, chytridiomycota, zygomycota, glomeromycota and microsporidia). A neighbor-joining tree was constructed based on the amino acid sequences of representative fungal superoxide dismutase (*SOD*) proteins. Numbers at nodes represent bootstrap confidence values, or percentage of clade occurrence in 2,000 bootstrap replicates. *S. scitamineum SOD* genes and characterized genes from other fungi are depicted in red or black, respectively. Fe/MnSOD are SOD isoenzymes manganese co-factored (MnSOD) or iron co-factored (FeSOD). Cu/ZnSOD are SOD isoenzymes copper-zinc co-factored (Cu-ZnSOD). The scale bar represents the number of amino acid differences per site

CATs were separated in two distinct groups (Figure 3.8). One, composed of mono-functional CATs of pathogenic and non-pathogenic fungi. The CAT encoded by the *g1075_crh02_Ss* gene was closer to that of *U. maydis* UHOR 03771. The second group was composed by bifunctional peroxidase/catalase proteins, also present in *maydis* but *S. scitamineum* enzyme was closer to that of *U.hordei*. (Figure 3.8). Sequence feature analysis revealed that the *CAT* (*g1075_crh02_Ss*) had the haem domain and immune-response domain, whilst the *CAT* (*g4614_chr13_Ss*) had two haem domains (Figure 3.9) (Table 3.3). In this study, the *g1075_crh02_Ss* and *g4614_chr13_Ss* genes were called like *KatE* and *KatG*, respectively.

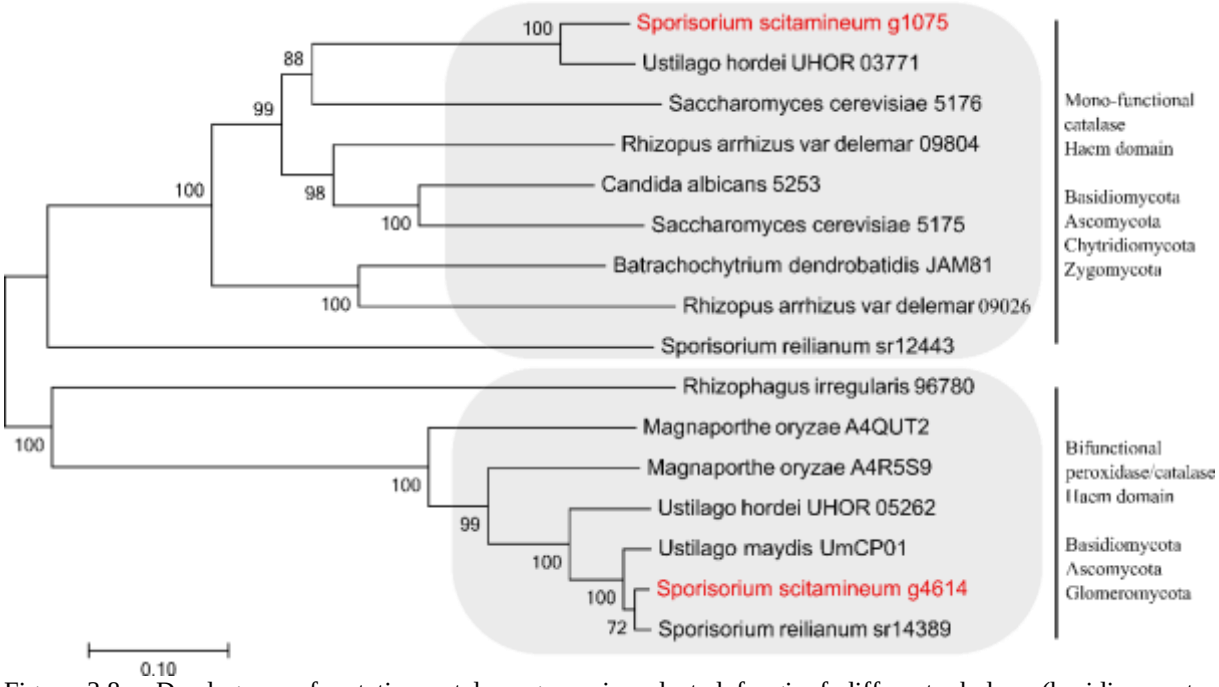

Figure 3.8 - Dendogram of putative catalase genes in selected fungi of different phylum (basidiomycota, ascomycota, chytridiomycota, zygomycota, glomeromycota and microsporidia). A neighbor-joining tree was constructed based on the amino acid sequences of representative fungal catalase (*CAT*) genes. Numbers at nodes represent bootstrap confidence values, or percentage of clade occurrence in 2,000 bootstrap replicates. *S. scitamineum CAT* genes and characterized genes from other fungi are depicted in red or black, respectively. The scale bar represents the number of amino acid differences per site

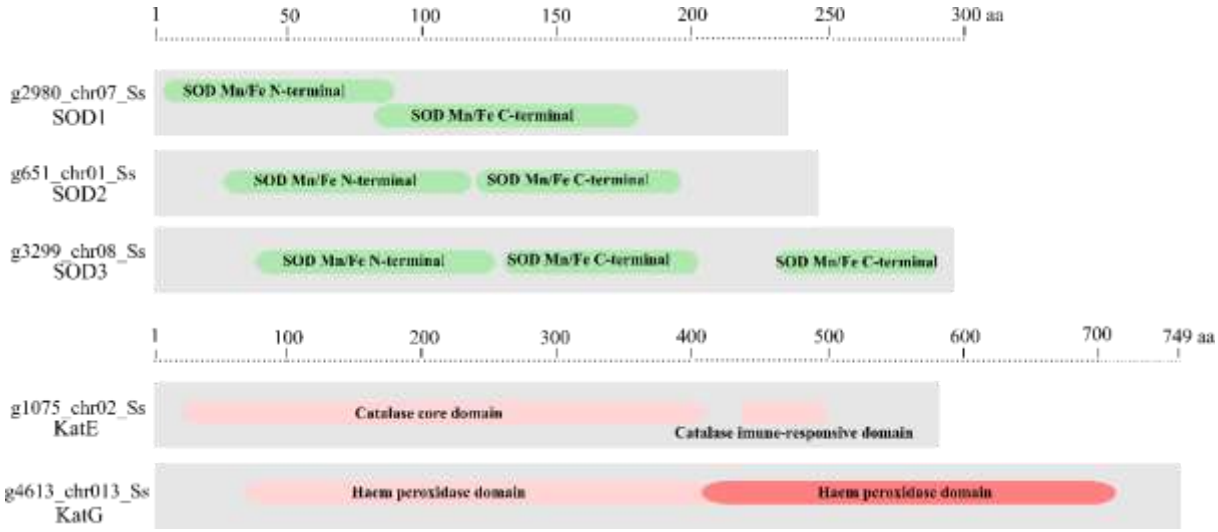

Figure 3.9 - Functional annotation and putative protein domains of identified SODs and CATs in *S. scitamineum* genome

3.2.2.5 *SOD*s and *KatG* genes are differentially expressed in *S. scitamineum* exposed to exogenous H_2O_2

We analyzed gene expression level of each SOD and CAT isoenzymes of *S. scitamineum* exposed to exogenous H_2O_2 for 30 and 180 min. At 30 min the *SOD1*, *SOD2*, *KatG* and *KatE* genes were upregulated, whereas *SOD3* gene did not show significant difference when compared to *S. scitamineum* growth without exogenous H_2O_2 (Figure 3.10). Additionally, *KatG* was the gene with the highest expression level in treatment 30 min. In contrast, when *S. scitamineum* cells were exposed for 180 min to exogenous H_2O_2, all genes tested did not exhibit significant difference in the expression (Figure 3.10).

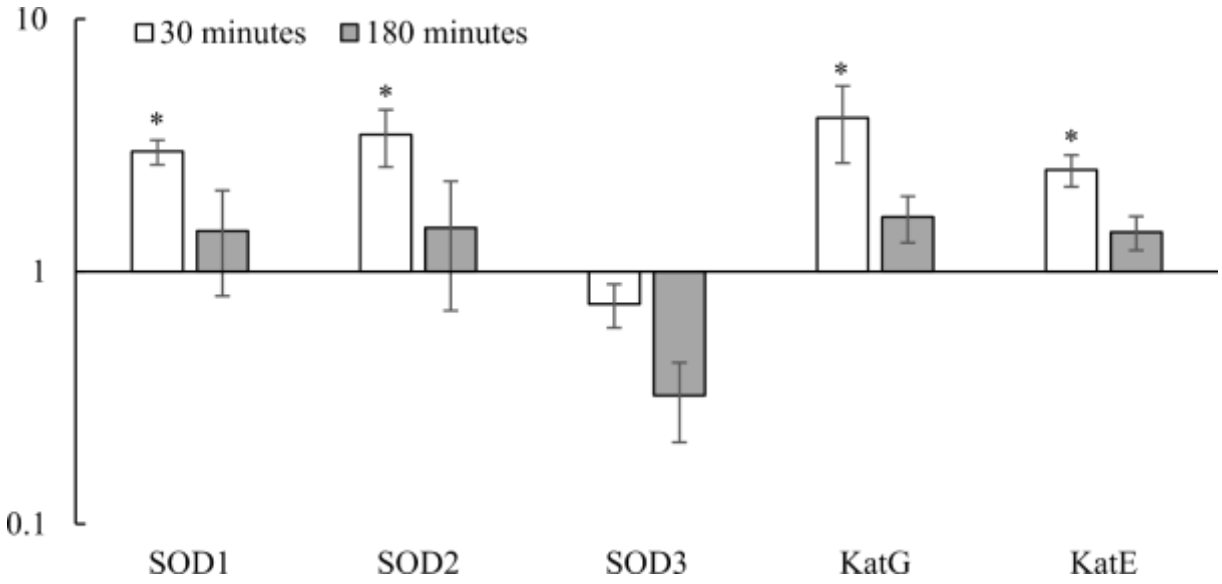

Figure 3.10 - Expression profiling of superoxide dismutase (*SOD1-g2980_chr07_Ss*), (*SOD2-g651_chr01_Ss*), (*SOD3-g3299_chr08_Ss*) and catalase (*KatG-g4614_chr13_Ss*), (KatE- g1075_crh02_Ss) genes of *S. scitamineum* cells exposed to exogenous H_2O_2 for 30 and 180 minutes by RT-qPCR analysis. Statistical analysis was performed in REST® software. * represents genes differentially expressed in RT-qPCR reactions (p-value < 0.05)

3.2.3 Discussion

The success of a pathogen is associated with its survival and strategies to avoid recognition by the host innate immune defense (CHAI et al., 2009). One of the first defenses against pathogen attack is the oxidative burst (TORRES; JONES; DANGL, 2006) and, in response, pathogens rely on an efficient antioxidant system (MOLINA et al., 2007; HELLER; TUDZYNSKI, 2011; VELUCHAMY et al., 2012; MIR et al., 2015; YAO et al., 2016). Additionally, in fungal pathogens the ROS production is also related to intracellular differentiation processes, such as that of the appressoria formation (MARSCHALL; TUDZYNSKI, 2014). Because we have determined that teliospore germination and appressorium formation were related to increased levels of H_2O_2 *in plant* (Chapter 2), it is possible that the reaction of *S. scitamineum* to peroxide shall be related to signaling and not to resistance per se, since the fungus can adapt to its toxic effects. Generally, biotrophic fungi are more sensitive to ROS during pre-infection and infection stages, whereas necrotrophic pathogens use the increment of host ROS production linked to its lifestyle and takes benefits from dead cells for nutritional purposes (BARNA et al., 2012). We observed that *S. scitamineum* cells showed a high *in vitro* resistance to exogenous H_2O_2, which ranged from 4 to 30 mM (sub-lethal doses). These concentrations are much higher than that imposed by the plants in early stages of infection. In stress conditions, the physiological concentrations of H_2O_2 in plant cells may change within the range of 0.1 to 150 μM (PATYKOWSKI; URBANEK, 2003; CHAPARZADEH et al., 2004; FORTUNATO et al., 2015). We have

shown that resistant sugarcane plants (genotype SP80-3280) infected with *S. scitamineum* increased the H_2O_2 concentration from 23 μM to 90 μM (Chapter 2). In addition, this burst contributed to retard the fungal development and colonization, but not impaired them completely. In this study we sought to investigate the influence of antioxidant system regarding SOD and CAT enzymes in survival of *S. scitamineum* to oxidative burst.

The sensitivity growth curve of *S. scitamineum* to peroxide was similar to the control in concentrations up to 2 mM H_2O_2, but survival could be determined in concentrations up to 30 mM. A large range of variation considering resistance to peroxide is seen among biotrophic fungi (LI et al., 2005; LI et al., 2011). For instance, the *Blumeria graminis* f. sp. *tritici* grew in seeds treated with 10 mM H_2O_2 caused the disease in wheat plants (LI, et al., 2011), whereas *Mycosphaerella fijiensis* survived in culture medium in concentrations up to 30 mM H_2O_2 (BELTRÁN-GARCÍA et al., 2009). Our results indicate that *S. scitamineum* is as resistant to peroxide as some necrotrophic fungus that can support an oxidative burst for more than 2 h.

Upon various stressors, cells usually counterbalance an antioxidant system for its protection and the production of ROS, to avoid oxidative damage to their own proteins, nucleic acids and lipids (HALLIWEL, 1991; APEL; HIRT, 2004; GESSLER et al., 2007). The oxidative damage to lipids, results in a variety of lipid hydroperoxides, that can be measured by the presence of malondialdehyde (MDA) (HEATH; PACKER, 1968; JENTZSCH et al., 1996; AYALA et al., 2014). *S. scitamineum* cells did not show lipid peroxidation in the treatments evaluated, suggesting an effective system to surpass the secondary products of the oxidative stress due to peroxide *in vitro*. However, *S. scitamineum in plant* is affected by ROS imposed by resistant plants (Chapter 2). Also, H_2O_2 vesicles are formed within the filaments formed by the fungus during plant colonization (Chapter 2). The ways that peroxide affects *S. scitamineum* growth needs to be further investigated since it was not associated with lipid peroxidation and membrane damage, at least for the concentration used in the *in vitro* assay. Resistance to peroxide, for instance, could be related to the presence of melanized cells in association with laccase activity previously detected in *S. scitamineum* (JACBISON, 2000; BELTRÁN-GARCIA et al., 2014; TANIGUTI et al, 2015).

SOD has pivotal role for stress protection by contributing to hyphal growth and virulence (ANGELOVA et al., 2004; GLEASON, et al., 2013; GLEASON, et al., 2014; YAO et al., 2016). Different H_2O_2 treatments had distinct effects on SOD activity in *S. scitamineum* cells. Total SOD activity was induced in *S. scitamineum* treated with H_2O_2 for 30 minutes, whereas, the cells exposed to the same concentration of H_2O_2 for 180 minutes did not affect

SOD activity. These results indicate that *S. scitaminuem* showed an adaptive response to H_2O_2.

Some studies showed that H_2O_2 gradients were formed across the plasma membrane when *S. cerevisiae* and *C. albicans* were exposed to exogenous H_2O_2 (BRANCO, et al., 2004; KOMALAPRIYA et al., 2015). This exogenous H_2O_2 is presumed to enter the cell via limited diffusion (BIENERT, et al., 2007), and it is degraded by CAT enzyme. In *C. albicans*, CAT plays a critical role immediately after H_2O_2 induced stress and adaptive response to H_2O_2 (JAMIESON, et al., 1996, KOMALAPRIYA et al., 2015). The CAT activity measured in *S. scitamineum* was elevated by H_2O_2 at 30 and 180 minutes. Two isoenzymes were detected in SDS-page gels. The isoenzymes CAT I and II that corresponding to peroxidase/catalase (82.4 kDa) and catalase (63.8 kDa), respectively.

Accordingly, the intracellular content of H_2O_2 decreased when *S. scitamineum* cells were exposed to H_2O_2 for 30 minutes, whereas after 180 minutes, H_2O_2 content entered in a redox homeostasis restoring the initial environment. These results over again supported the earlier statement that an adaptive response is in course such as those seen for other systems (MARSCHALL et al., 2016). In *C. albicans,* for instance, the antioxidant system activation contributed to acquisition of an adaptive response to H_2O_2 (JAMIESON et al., 1996; GONZÁLEZ-PÁRRAGA et al., 2003).

It is known that the maintenance of a redox homeostasis in cells is the result of a combination of enzymes working to deal with the natural stress provoked by the cellular metabolism. Among the SOD and CAT enzymes are the ones recruited to deal also with exogenous stresses. Previously it was shown that *SOD1* and *KatE* genes, both encoding extracellular enzymes of S. *scitamineum* were differentially expressed 5 days after infecting sugarcane (TANIGUTI et al., 2015).

To investigate all the genes of *S. scitamineum* genome involved and their contribution to enzyme activity in this mechanism we studied by means of sequence similarity (BLAST) the complete set of SOD and CAT encoding genes. Reference fungal sequences were used to allow the identification of three SOD and two CAT isoenzymes in *S. scitamineum* genome. The *SOD2* and *SOD3* proteins were predicted to have a mitochondrial targeting sequence, potentially to maintain the mitochondrial integrity, which is also crucial for pathogenicity (BORTFELD et al., 2004). Despite our efforts and due to technical difficulties, we were not able to detect biochemical evidence for the presence of all three SOD isoenzyme in PAGE staining.

Through RT-qPCR analysis, it was determined the expression profile of each SOD and CAT isoenzymes upon peroxide exposure. At the dose of 2 mM H_2O_2, the *SOD1*, *SOD2*, *KatG* and *KatE* genes were upregulated in *S. scitamineum* after 30 min of exposure. As mentioned before, the *SOD1* gene expression was also upregulated in *S. scitamineum in plant* experiment (Taniguti et al., 2015). The results confirmed the involvement of *SOD1* in response to external stress *in vitro* and *in plant* and suggest an additional role for *SOD2* potentially regarding the intracellular effects of the peroxide. It has been established for other pathosystems the relevance of *SOD1* to hyphal growth and virulence (GODON et al., 1998; YAO et al., 2016). The *KatG* and *KatE* genes were also upregulated. In contrast, the *SOD1*, *SOD2*, *SOD3*, *KatG* and *KatE* genes did not show differential expression in *S. scitamineum* after 180 min exposed to H_2O_2.

S. scitamineum still overcomes a second oxidative stress during sporogenesis and whip emission (TANIGUTI et al., 2015). It is possible that this fungus use the redox system as signal to allow the expression of other genes to establish colonization. It has been demonstrated that *Saccharomyces cerevisiae* and *Candida albicans* (dimorphic yeast) show an adaptive oxidant stress response after an initial exposure to a non-lethal dose of H_2O_2, and became more resistant to further severe doses of the same oxidant agent (COLLINSON; DAVIES 1992; JAMIESON et al., 1996; ALVAREZ-PERAL et al., 2002). *C. albicans* has developed antioxidant systems, required for adaptation to ROS exposure from the host throughout evolution (Alonso-Monge et al., 2003; Herrero-de-Dios et al., 2010). This is also true for plant pathogens (MARSCHALL et al., 2016; MENTGES; BORMANN, 2015; MARSCHALL; TUDZYNSKI, 2014) and potentially for *S. scitamineum*, the cell redox state also changes when exposed to stress by means of an adaptative response and signaling to maintain its biotrophic lifestyle.

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4 FINAL CONSIDERATIONS

In the present study, we investigated how sugarcane controls ROS production and ROS scavenging in response to *S. scitamineum* infection. Thus, analysis were carried out in the early stages of the interaction, using genotypes susceptible and resistant to smut. We showed that both *S. scitamineum* teliospores germination and infection structures, such as the appressorium, were delayed during early infection in the smut resistant genotype, which coincided with H_2O_2 accumulation. These results demonstrated that *S. scitamineum* development was highly affected by host–pathogen interaction. Additionally, we observed that the H_2O_2 accumulation at 72 hours post-inoculation (hpi) is associated with lipid peroxidation and repression of catalase (CAT) in smut resistant genotype, indicating a deliberate imbalance of the ROS scavenging system, since a large amount of H_2O_2 might contribute for hypersensitivity response (HR). Moreover, the proteins thioredoxin h-type, ascorbate peroxidase and guanine nucleotide-binding are associated with sugarcane resistance to smut. Furthermore, we observed an increase in the H_2O_2 concentration at 6 (23 μM), 48 (35 μM) and 72 (90 μM) hpi in the resistant genotype infected with *S. scitamineum*. Although *S. scitamineum* is a biotrophic fungus, which is more sensitive to ROS effects during early infection stages, we showed that the H_2O_2 concentration produced by the plant cell is not sufficient to block the pathogen growth. In addition, we showed that the fungus exhibited an adaptive response to 2 mM exogenous H_2O_2, revealing an efficient antioxidant system. Therefore, this work contributed to a better understand of the biology of the interaction between sugarcane and *S. scitamineum*, as well as pinpointing candidates and mechanisms involved in the sugarcane resistance to *S. scitamineum* that can be further analyzed.

APPENDIX

APPENDIX A – List of transcripts in sugarcane at 5 and 200 days post-inoculation (RNAseq analysis) associated with oxidative stress. * indicate transcripts selected for RT-qPCR analysis

Sugarcane ID	Ortholog	Gene annotation	Gene expression	
			5 DAI	200 DAI
comp189288_c1_seq1	A0A067ZYH9	Catalase *	Down	
comp189288_c0_seq1	A0A059PZB0	Catalase *	Down	
comp186491_c0_seq1	A0A0A7DNY4	Superoxide dismutase *	Down	
comp178898_c0_seq1	C5X5K6	Peroxidase 42	Down	
gg_00273	C5Y1Y1	Peroxidase c5y1y1	Down	
gg_13547	B6THP7	Thioredoxin-like 1	Down	
gg_11882	C5Z8S7	Peroxidase	Down	
gg_09892	A0A096S9H8	Thioredoxin reductase	Down	
gg_06628	A0A059PZF8	Peroxidase	Down	
comp175577_c0_seq1	B6TDA9	2-cys peroxiredoxin bas1	Down	
comp195045_c1_seq1	O24595	Glutathione s-transferase t3-like	up	
comp188391_c0_seq1	C5YB25	Cationic peroxidase spc4-like		Down
gg_05291	P84516	Cationic peroxidase spc4		Down
comp191235_c0_seq1	A0A088FFP9	Catalase *		up
comp186041_c0_seq1	C5X0X1	Peroxidase 66		up
comp187834_c0_seq1	C5YHR8	Peroxidase 51-like		up
comp127311_c0_seq1	C5WUE5	Peroxidase 5-like *		up
comp190230_c0_seq1	C5Z8S7	Peroxidase c5z8s7		up
gg_05125	C5X744	Peroxidase k3z2d1		up
gg_09987	C5XD24	Peroxidase k3zv96		up
gg_04909	C5WV85	Peroxidase c5wv85		up
gg_03326	C5XGM1	Peroxidase c5xgm1		up
comp200860_c0_seq1	Q9SM20	Glutathione s-transferase iii		up
comp201441_c0_seq1	Q9FQB7	Glutathione s-transferase gst22 *		up
comp205099_c0_seq1	B6TQI2	Glutathione s-transferase		up
comp179663_c0_seq1	Q9FQA8	Glutathione s-transferase gst 31 *		up
gg_07331	Q9FQB7	Glutathione s-transferase gstu6		up
gg_12826	B6TP19	Thioredoxin-like protein 5		up
comp185514_c0_seq1	M7ZT77	Thioredoxin ttl1-like		up
comp194788_c1_seq1	B6U6B7	Thioredoxin superfamily protein		down
comp200907_c0_seq1	B6TN15	nadh-ubiquinone oxidoreductase 24 kda subunit		up
comp203450_c1_seq1	B4FQ98	nadh-ubiquinone oxidoreductase 51 kda subunit		up
comp187953_c0_seq1	B6T8F0	nadh-ubiquinone oxidoreductase 23 kda subunit		up
comp180089_c0_seq1	B4G1P2	nadh-ubiquinone oxidoreductase 20 kda subunit		up
gg_06476	B6T8F0	nadh-ubiquinone oxidoreductase 23 kda subunit		up

APPENDIX B - Primer specificity was confirmed obtaining the dissociation curve for every reaction (examples)

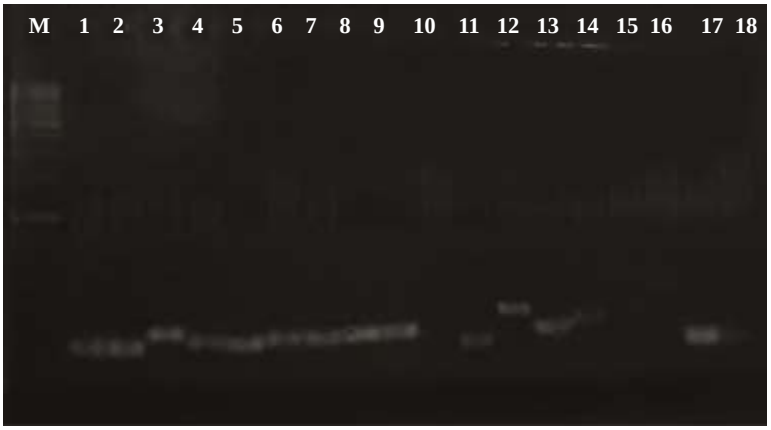

Figure 1 – Qualitative PCR with primers pairs designed for amplification of target genes. M represents molecular marker and number of 1 to 18 represent the gene amplification used in this work, with expept 10, 15, 16

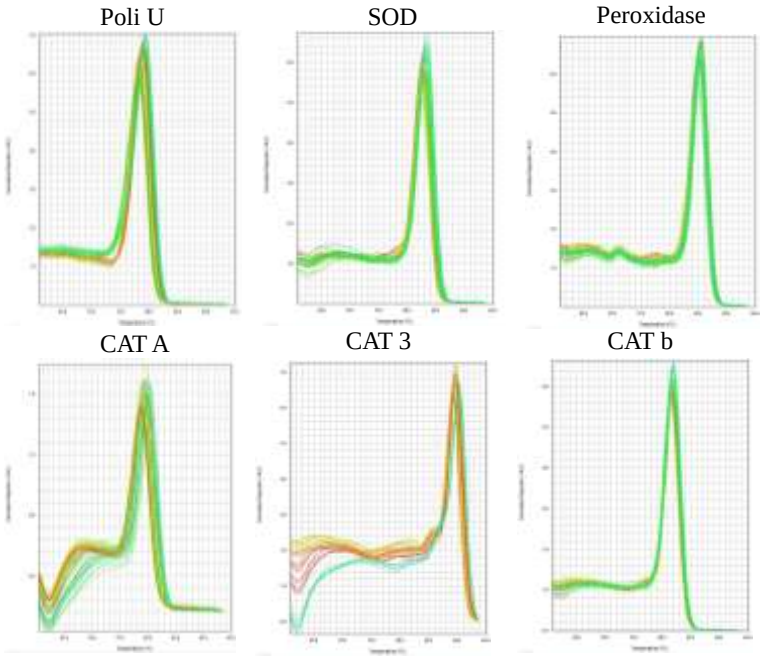

Figure 2 - Dissociation curves generated for the target genes Polyubiquitin (Poli U), superoxide dismutase (SOD), Peroxidase 5, catalase A (CAT A), catalase 3 (CAT 3) e Catalase b (CAT b). These dissociation curves showed good results (examples)

APPENDIX C - Standard curve for *S. scitamineum* quantification

qPCR conditions and standard curve

Six primers pairs were designed to test its performances in qPCR (Table 1). These primers (SSC-A, SSC-B, SSC-C, SSC-D, SSC-E and SSC-F) target the IGS region (*Intergenic Spacer*) of *S. scitamineum* genome (TANIGUTI et al., 2015). Primers were designed using Primer3plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and tested for secondary structures formation using Gene Runner (http://www.generunner.net/) and Beacon Designer™ Free Edition (http://www.premierbiosoft.com) software, respecting the following parameters: size of PCR product 120 to 200 bp, primers size 18 to 24 bp, % GC 40 to 60 and absence of secondary structures.

In order to optimize qPCR reaction conditions, annealing temperature, $MgCl_2$ and primer concentrations were experimentally adjusted. The qPCR was conducted in a 7500 Fast Real-Time PCR System (Applied Biosystems) on the three biological replicates of each treatment, with two technical replicates each. The qPCR assay was carried out using *LuminoCt SYBR® Green qPCR ReadyMix*™ (Sigma-Aldrich) kit. Each reaction was composed by 6.5 µL of *2X LuminoCt SYBR Green qPCR ReadyMix*, 0.2 µM of each primer, 20 ng of *S. scitamineum* (sporidia SSC39A), 0.5 µl BSA (10 mg/mL), and water to a final volume of 12.5 µL. The specificity of the newly designed primer pair was tested using conventional PCR against DNA extracted from the fungal isolates (data not shown). DNA sample of *S. scitamineum*-free sugarcane buds was used as negative control. A standard curve to correlate threshold cycle (Ct) values with different DNA quantities of *S. scitamineum* was established using a ten-fold dilution series of fungal DNA (100 ng/µL) (1:1, 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$), in sterile ultrapure water, using the SSC-C primer.

Pathogen quantification *in planta* using qPCR assay was also performed using the SSC-C primer. Reactions were composed of 6.5 µL of *2X LuminoCt SYBR Green qPCR ReadyMix*, 0.2 µM of each primer (F and R), 5 µL of each DNA dilution, 0.5 µl BSA (10 mg/mL), and water to a final volume of 12.5 µL. Standard curve was generated by plotting the threshold cycles (Ct) versus the logarithmic values of known quantities of target DNA. Fungal DNA amount was assessed in infected buds at 6, 12, 24, 48 and 72 hpi, and calculated as pathogen DNA amount in 100 ng of plant DNA. Tukey's test ($P \leq 0.05$) was used to compare the DNA quantity of *S. scitamineum* in the smut susceptible and resistant genotypes infected with the pathogen.

Table 1 - Primers designed for the quantification of *S. scitamineum* in infected sugarcane buds

Name		Sequence
SCC-A	F	5' GATTGCGAACGAGACTGTGACC 3'
	R	5' GCTACACTTCAAATACGTCGGC 3'
SCC-B	F	5' GCTCAGCCCTATCTTCCACG 3'
	R	5' GACCGCAGCAACTACATTCAC 3'
SSC-C	F	5' CGGCTATTGTCGCACATCTC 3'
	R	5' CCAAACGCAGGTCACAGTCT 3'
SSC-D	F	5' CCCACCGAGAGATGAAACTG 3'
	R	5' CCAAACGCAGGTCACAGTCT 3'
SSC-E	F	5' TCCCACCGAGAGATGAAACT 3'
	R	5' TCACAGTCTCGTTCGCAATC 3'
SSC-F	F	5' GTGATGGGACAGGGTTTGAT 3'
	R	5' AGTCTTTCCTTCCACGAGCA 3'

Results

The SSC-C primer pair (table 1) was selected to detect *S. scitamineum* in planta. This set of primers was tested in other sugarcane pathogens: *Colletotrichum falcatum* (red rot disease), *Thielaviopsis paradoxa* (pineapple disease of sugarcane), *Rhizoctonia* (Root rots) and *Sclerotium rolfsii* (red rot of leaf sheath and sprout rot) by conventional PCR (results not shown) showing a high specificity to smut. Dissociation curves of qPCR using different concentrations of *S. scitamineum* DNA indicated the absence of dimmers, as evidenced by a single peak at the melting temperature of 82.5 ºC (Figure 10A). The standard curve showed a linear correlation between Ct values and fungal DNA, with a correlation coefficient (R^2) = 0.9996 and an amplification efficiency of 1.007%, described by the equation y = –3.3094x + 31.699 (Figure 1).

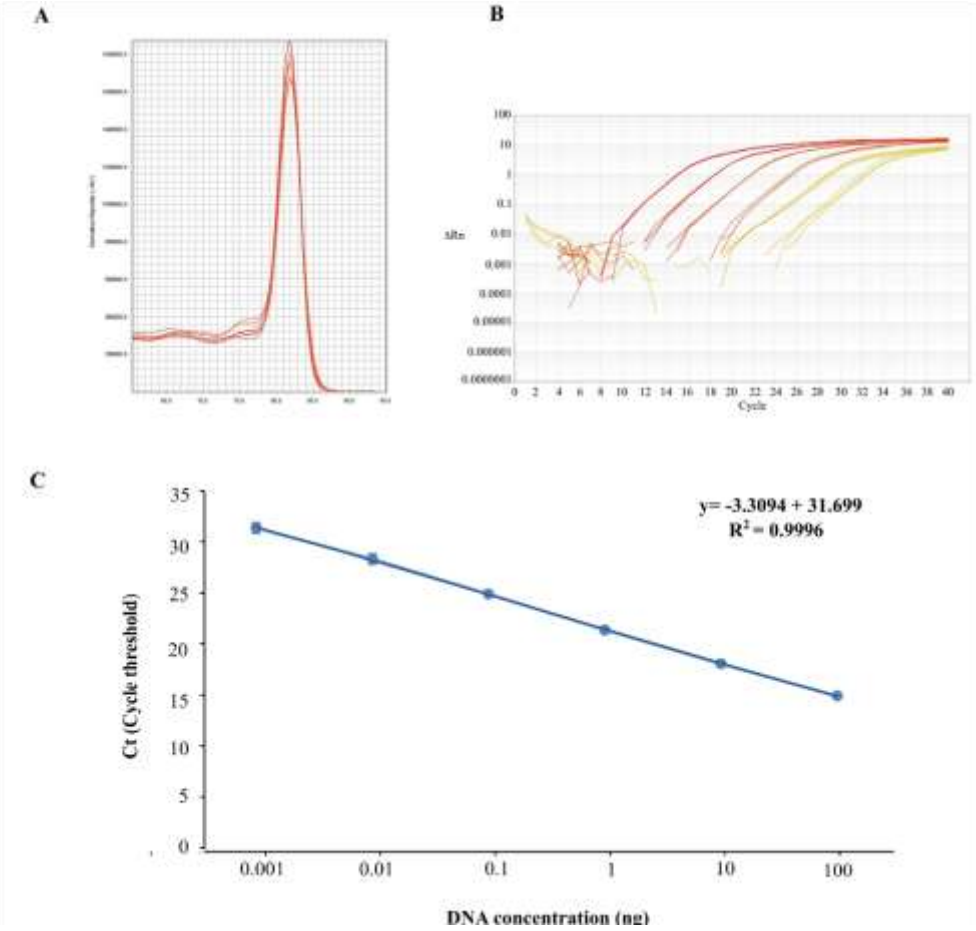

Figure 1 – A- Dissociation curve of SSC-C primers designed for IGS sequence of S. scitamineum. B - The amplification plots show the testing results of a ten-fold serial dilution containing the following amount of *S. scitamineum* DNA: (1:1, 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$ and $1:10^6$). C - Standard regression line of a six-point 10-fold serial dilution of *S. scitamineum* DNA (100 ng/µl) diluted in sterile, ultrapure water. Threshold cycles (Ct) were plotted against the standard curve of known concentrations of genomic DNA

APPENDIX D - List of present or absent proteins of the treatments used in this study

Acession/Treatments	Protein
Smut resistant (mock inoculated)	
Sb03g030370.1|PACid:1962755	tpa: duf1296 domain containing family protein
Sb01g032690.1|PACid:1953025	ef hand family protein
Sb03g000440.1|PACid:1960030	ob-fold nucleic acid binding domain containing protein
Sb01g000380.1|PACid:1949318	ru large subunit-binding protein subunit alpha
Sb01g035740.1|PACid:1953411	60s ribosomal protein l22-2
comp28293_c0_seq1	pvr3-like protein
Sb02g011890.1|PACid:1956732	monodehydroascorbate reductase
Sb01g002150.1|PACid:1949546	glycine-rich rna-binding protein rz1a-like
Sb01g019610.1|PACid:1951682	heterogeneous nuclear ribonucleoprotein 1-like
Sb10g006450.1|PACid:1982937	20 kda chloroplastic-like
comp1838198_c1_seq1	ubiquitin isoform cra_e
Smut resistant (inoculated)	
comp73377_c1_seq1	pvr3-like protein
Sb02g044060.1|PACid:1959988	ascorbate peroxidase
evm.model.scga7_unitig_297107.1	vacuolar atp synthase subunit g
Sb01g041420.1|PACid:1954112	nhp2-like protein 1
Sb09g001020.1|PACid:1979420	maize proteinase inhibitor
comp78817_c0_seq2	40s ribosomal protein s21
Sb03g046730.1|PACid:1964730	ubiquitin-fold modifier 1
SHCRBa_010_J05_R_210_1	acyl- -binding protein
Sb02g033710.1|PACid:1958702	nucleoside diphosphate kinase 1
Sb08g022770.1|PACid:1979242	malate glyoxysomal
comp82541_c0_seq11	sam domain family protein
Sb04g002620.1|PACid:1965179	probable atp synthase 24 kda mitochondrial
comp79484_c0_seq3	stem 28 kda glyco
Sb09g019630.1|PACid:1980782	endo- -beta-d-glucanase-like
evm.model.scga7_unitig_348112.1	eukaryotic translation initiation factor 5a
SHCRBa_028_H13_R_330_1	probable succinyl- ligase
Sb01g017840.1|PACid:1951463	membrane steroid-binding protein 1
Sb03g005550.1|PACid:1960670	histone
Sb09g025900.1|PACid:1981539	heat shock protein 101
Sb09g027690.1|PACid:1981757	guanine nucleotide-binding protein subunit beta-like protein a
Sb01g016810.1|PACid:1951337	auxin-repressed kda protein
Sb03g005190.1|PACid:1960630	t-complex protein 1 subunit beta
evm.model.scga7_unitig_341686.1	thioredoxin h-type
Smut susceptible (mock inoculated)	
evm.model.scga7_uti_cns_0172034.2	peroxidase III class
Smut susceptible (inoculated)	
SHCRBa_010_J05_R_210_1	acyl- -binding protein
comp1838198_c1_seq1	ubiquitin isoform cra_e
Sb08g023050.1|PACid:1979277	acetyl- cytosolic 1-like

APPENDIX E - *S. scitamineum* growth. Mean values with different letters are significantly different ($p < 0.05$)

Time	Treatments	*S. scitamineum* growth	Tukey' test
	4mM	0.234667	a
	2mM	0.246333	a
(0h)	8mM	0.257667	a
	0mM	0.261000	a
	6mM	0.264000	a
	2mM	0.321333	a
	0mM	0.348000	a
(2h)	8mM	0.361000	a
	6mM	0.362000	a
	4mM	0.370000	a
	4mM	0.545667	a
	6mM	0.561000	a
(4h)	8mM	0.568667	a
	0mM	0.572667	a
	2mM	0.573667	a
	4mM	0.790667	a
	8mM	0.796333	a
(6h)	0mM	0.811000	a
	2mM	0.835333	a
	6mM	0.837667	a
	4mM	0.754667	a
	6mM	0.781667	a
(8h)	8mM	0.809333	a
	2mM	0.985000	b
	0mM	1.059333	b
	8mM	0.774000	a
	6mM	0.815000	a
(10h)	4mM	0,821000	a
	2mM	1.146333	b
	0mM	1.261333	b
	8mM	0.746333	a
	6mM	0.754333	a
(12h)	4mM	0.852333	a
	2mM	1.297000	b
	0mM	1.441000	b