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

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Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding
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Piracicaba
2016
To my loves...

Odilon and Dirceia;

Bethânia, Diogo, Danilo, Ana and Moisés.

With love I dedicate this work.
ACKNOWLEDGMENTS

To God for the gift of life and for making me believe that we can make a better world.

To my parents, Dircéa and Odilon, who are examples of life, love, character and faith. For understanding when I couldn’t be there and for teaching me that dreams can come true.

To my sister and brothers, Bethânia, Diogo and Danilo, and my niece Ana for the peace and joy they give me.

To my love Moisés for the patience and companionship.

To Prof. Claudia Barros Monteiro-Vitorello, for the commitment in making me a scientist. I am grateful for the advisement, mentoring, opportunities, patience and friendship. I feel honored and pleased to being a part of your team.

Especially to Prof. Ricardo Antunes de Azevedo for the mentoring, trust and friendship that I will always have with me.

To Prof. Jesús Jorrin-Novó from University of Córdoba - Spain for the advisement and friendship. To Rosa Sanchez and Sabina Zazzu also from University of Córdoba for the friendship and learning.

To Dr. Giselle Carvalho and Dr. Salete A. Gaziola, I would like to thank our friendship during all these years.

To all friends and colleagues that were or still are part of the Genomics Group: Suzane Saito, Leandro de Souza, Natália Teixeira, Nathália de Moraes, Lucas Taniguti, Gustavo Crestana, Pedro Beretta, Síntia Almeida, Daniel Longatto, Jian da Silva, Mariana Marrafon and especially to Juliana Benevenuto and Patrícia Shacker for the rich discussions and friendship.

To Thaís Regiani Cataldi, Felipe Marques, Andressa Bini and Elaine Vidotto for the patience and help.

To family and friends that are part of my life, even those who are far away, and have somehow encouraged me. Especially to my Aunt Christine and friends: Saly Takeshita, Aline Caetano, Daiana Schmidt, Milca Vilhena; Carolina Monteiro, Janice do Livramento, Gisely Ghros, Tânia Niezer, Mônica Franco, Eliene Maia e Gerusa Peters.

To Centro Nacional de Pesquisa em Energia e Materiais (CNPEM) for the opportunity to perform the mass spectrometry analysis in the Brazilian Biosciences National Laboratory (LNBio), and particularly to Bianca Alves Pauletti for the technical support.
To Fundação de Amparo à Pesquisa no Estado de São Paulo (FAPESP), for the financial support to the project entitled “Análise bioquímica e genética das espécies reativas de oxigênio na interação cana-de-açúcar e Sporisorium scitamineum” (Grant number 2013/15014-7).

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (CAPES) for the scholarship.

To “Escola Superior de Agricultura Luiz de Queiroz” and “Departamento de Genética e Melhoramento de Plantas” for the opportunity to carry out my Doctor of Science studies.

To everyone who in some way contributed to the accomplishment of this work.

I sincerely thank you all!
Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.

(Albert Einstein)
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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 biorrófico *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-de-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$_2$O$_2$) 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$_2$O$_2$. No capítulo 3, os resultados demonstraram que *S. scitamineum* é altamente resistente a H$_2$O$_2$ exógeno. O fungo crescendo na concentração de 2 mM de H$_2$O$_2$ 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$_2$O$_2$ exógeno, ele pode adquirir uma resposta adaptativa ao H$_2$O$_2$. 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) 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$_2$O$_2$). 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$_2$O$_2$ accumulation. In chapter 3, the results demonstrated that *S. scitamineum* is highly resistant to exogenous H$_2$O$_2$. At 2 mM exogenous H$_2$O$_2$ 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$_2$O$_2$ concentration it can acquire an adaptive response to H$_2$O$_2$. 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$$^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$) and singlet oxygen (1$^2$O$_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$_2$O$_2$ 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). On the other hand, 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).

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 (TANIGUTI et al., 2015). 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 (TANIGUTI et al., 2015). Also, some studies suggested that effectors as chorismate mutase from _S. scitamineum_ 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; TUDZYNISKI, 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).

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$_2$O$_2$, and obtain comprehensive information about the impact of exogenous H$_2$O$_2$ on the different SOD and CAT isoenzymes of S. scitamineum through quantification of gene expression analysis and enzyme activities.

References


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$_2$O$_2$ accumulation. These results demonstrated that *S. scitamineum* development was highly affected by host–pathogen interaction. Additionally, we observed that the H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$, 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.