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Genetic analysis of ROS modulation in *Sporisorium scitamineum* – energy cane interaction

Joyce Dellavechia Ferreti

Dissertation presented to obtain the degree of Master in Science. Area: Phytopathology

Piracicaba
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1. Espécies reativas de oxigênio 2. Sistema antioxidante 3. Melhoramento genético 4. Genes diferencialmente expressos 5. Doença do carvão I. Título

With all my love and affection,
To all my family,
especially to my beloved mother Silvania Dellavechia.

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"Science and everyday life cannot and should not be separated. Science, for me, gives a partial explanation of life. In so far as it goes, it is based on fact, experience and experiment."

-Rosalind Franklin

SUMMARY

RESUMO

Análise genética da modulação do metabolismo de espécies reativas de oxigênio na interação cana-energia – *Sporisorium scitamineum*

A cana-energia é uma cultura desenvolvida para produção de bioenergia que apresenta grande potencial econômico para o país, diante de sua crescente importância destaca-se estudos sobre aspectos que podem afetar a produção da cultura. Dentre estes aspectos, a doença do carvão causada pelo fungo biotrófico *Sporisorium scitamineum* é uma preocupação no desenvolvimento de novas variedades devido à redução de produtividade ocasionada com a doença. Desta forma, diversos estudos vêm sendo desenvolvidos para melhor entender os mecanismos de defesa do hospedeiro, a fim aprimorar programas de melhoramento genético e controle da doença. Neste trabalho foi realizada uma análise genética sobre os aspectos de defesa do hospedeiro relacionados com o metabolismo de espécies reativas de oxigênio (ROS) de variedades de cana-energia suscetível (Vertix1) e resistente (Vertix2) na interação com *S. scitamineum*. Esta dissertação está apresentada na forma de 3 capítulos, começando com uma revisão no capítulo 1. No capítulo 2, análises a partir do transcriptoma das duas variedades foram realizadas com o objetivo de melhor compreender a modulação genética envolvida no metabolismo de ROS 48 horas após inoculação (hai) com *S. scitamineum*. Foram observados genes diferencialmente expressos (DEGs), relacionados ao metabolismo de ROS, em comum nas duas variedades devido à presença do fungo, porém com padrões de expressão contrastantes. Também foram analisados funcionalmente DEGs específicos para variedades resistentes e suscetíveis. No terceiro capítulo, os resultados obtidos através da análise de expressão gênica dos genes relacionadas ao sistema antioxidante, desencadeado pela resposta de estresse oxidativo na interação em 48 hai e 72 hai, mostraram diferenças significativas apenas para análises em 48 hai. Considerando, nestas variedades de cana-energia, um padrão diferente do já estudado para variedades de cana-de-açúcar convencional na interação com *S. scitamineum*, envolvendo estes mesmos genes de modulação de ROS. Estas informações são relevantes para o desenvolvimento de novas pesquisas relacionadas a estratégias para o melhoramento genético de cana-energia quanto à doença do carvão.

Palavras-chave: Espécies reativas de oxigênio, Sistema antioxidante, Melhoramento genético, Genes diferencialmente expressos, Doença do carvão

ABSTRACT

Genetic analysis of ROS modulation in *Sporisorium scitamineum* – energy cane interaction

Energy cane is a crop developed for bioenergy production and shows a high economic potential for the country. Considering the energy cane increasing relevance, studies on the factors that may impact crop production are particularly important. Among loss production causes, the biotrophic fungus *Sporisorium scitamineum* causal agent of smut disease, is a concern in the development of new resistant varieties given the yield decrease caused by the disease. Therefore, several studies have been developed to improve the understanding of host defense mechanisms to improve genetic breeding programs and disease control. In this study, a genetic analysis was performed for host defense aspects related to reactive oxygen species (ROS) metabolism of susceptible (Vertix1) and resistant (Vertix2) energy cane genotypes in interaction with *S. scitamineum*. This dissertation is submitted in the format of 3 chapters, beginning with a review in chapter 1. In chapter 2, analyses from the two genotypes transcriptome were performed to further understand the genetic modulation involved in ROS metabolism at 48 hours post-inoculation with *S. scitamineum*. Differentially expressed genes (DEGs), related to ROS metabolism, were observed in common in both genotypes due the fungal presence, however showed contrasting expression patterns. Specific DEGs were also functionally analyzed for resistant and susceptible varieties against smut inoculation. In the third chapter, the results obtained through gene expression analysis of genes related to the antioxidant system, triggered by the oxidative stress response in the interaction at 48 hpi and 72 hpi, showed significant differences for *TRX gene* (in susceptible genotype) and *SOD gene* (in resistant genotype) only for analyses at 48 hpi. We observed in energy cane varieties a different pattern than already studied for conventional sugarcane in *S. scitamineum* interaction, involving these same ROS modulation genes. This knowledge is relevant for the new research development related to genetic breeding strategies for energy cane genotypes regarding smut disease resistance.

Keywords: Reactive oxygen species, Antioxidant system, Breeding programs, Differentially expressed genes, Smut diseases

CHAPTER 1: STATE OF THE ART

1. Energy cane

The sugarcane production is the second highest in the world and Brazil is the largest global producer, generating more than 650 million tons in the 2020/21 harvest (CONAB, 2021; LONGATTO et al., 2014). Sugarcane crop is the primary raw material for sugar in the world and essential for ethanol production in Brazil (DE OLIVEIRA et al., 2007). The modern sugarcane genotypes are the result of crosses between individuals of *Saccharum officinarum* (noble cane), accumulating high levels of sucrose in their culms and *Saccharum spontaneum*, an ancestral species, contributing with robustness and general adaptation to stressful conditions (MATSUOKA et al., 2014; SILVEIRA et al., 2016). The higher tolerance to abiotic stresses conditions of *S. spontaneum* along with disease/pest resistance and the high fiber level, vigor and strong post-harvest ratoon growth means that the species has become a valuable genetic resource for sugarcane energy breeding programs (DA SILVA, 2017).

Remarkably, the production of ethanol from sugarcane increased in the mid-1970s with a pursuit for a more sustainable alternative energy source (COOMBS, 1984; MATSUOKA et al., 2014). At that time, Brazil started an ethanol production project, becoming a lead producer of ethanol from sugarcane (COOMBS, 1984; MATSUOKA et al., 2014; NEVES et al., 2011). Therefore, sugarcane became a valuable crop option considering the low production cost and high biomass yield (DIAS et al., 2013; SILVEIRA et al., 2016).

Sugarcane plantations must be adapted to various stresses such as drought, cold, and low nutrient availability to be viable in restrictive environments. In addition, it should not generate competition with food crops and conventional agriculture (CURSI; HOFFMANN; BARBOSA, 2022). Therefore, a successful feedstock for biofuel generation should have features that are significant to biofuels, such as allowing an increase in carbon deposition depth and accumulation, roots capable of capturing water easily, adaptability to contaminated soil areas, and mitigation of greenhouse gas emissions, characteristics possibly found in *S. Spontaneum* germplasm (DA SILVA, 2017; CURSI; HOFFMANN; BARBOSA, 2022).

Traditionally, sugarcane genetic breeding programs have mainly focused on developing cultivars with higher sucrose content for sugar and first-generation ethanol (1G) production. However, given the high potential of this crop for bioenergy production, a new cultivar biotype, called energy cane, has been developed by breeding programs. Genotypes of energy cane are selected for total biomass production rather than focused on sucrose only, and they are used as

feedstock for the production of cellulosic ethanol, also known as second-generation ethanol (2G) (CURSI; HOFFMANN; BARBOSA, 2022). In Brazil, Granbio Investimentos S.A. leads one of the most important energy cane breeding programs globally, with 11 varieties released since 2015[1].

Considering the Planet's climate change rising warnings, the production of renewable energy from energy cane biomass has been perceived as having a high potential of applicability (DA SILVA, 2017). Besides all the important characteristics of *S. spontaneum* for bioenergy production, the high carbohydrate composition found in energy cane is comparable to other lignocellulosic substrates considered as with high potential for second generation bioethanol (2G) production (DA SILVA, 2017; DINIZ et al., 2019).

Tew and Cobill (2008) classified energy cane into two different categories: type I and II. Type I is closer to the conventional ideotype, except for the lower sucrose and the higher fiber contents essential for the energy proposal, whereas type II has higher fiber content than type I, and marginal content of sugar. Both types can be selected for multipurpose use, although primarily used for energy production (SILVEIRA et al., 2016).

Energy cane varieties developed by GranBio focus on twice high cane fiber content in the medium term for Type II varieties and show 20% to 50% lower sugar content in juice than conventional sugarcane. In addition, the energy cane varieties must provide pests and diseases tolerance and higher multiplication rate than sugarcane (CURSI; HOFFMANN; BARBOSA, 2022). Thus, GranBio submits a product concept for the selection of Vertix type1 and Vertix type 2 genotypes (Table 1):

[1] GranBio Investimentos S.A. website information, by http://www.granbio.com.br/

Table 1: Difference in traits between sugarcane and energy cane (type 1 and 2) (adapted from CURSI; HOFFMANN; BARBOSA, 2022).

Trait	Sugarcane	Vertix type 1	Vertix type 2
Productive (X)	X	>1.5 X	>2.0X
Sugars (Kg/t)	150	>100	<100
Fiber (%)	15	18 to 22	>25
Number of cuts	4 to 5	8 to 10	>10
Resistance to pests and diseases	+	++	+++
Industrial use	Sugar and Ethanol	Sugar, Ethanol and Energy	Ethanol 1G, 2G, Biochemicals, Energy and Biomethane

The main criteria for parental breeding for energy cane selection are associated with smut resistance, rhizomes presence, high tillering ability, no pithiness, and flowering absence (CURSI; HOFFMANN; BARBOSA, 2022). Despite the high production potential, the energy cane varieties are moderately susceptible to smut disease caused by the biotrophic fungus *Sporisorium scitamineum* (BISCHOFF et al., 2008). In general, there are no immune sugarcane genotypes to smut colonization. The determination of resistance or susceptibility is given by the number of whips (the main symptom of smut disease) developed in infected plants. Sugarcane genotypes are classified with different resistance or susceptible levels to smut, wherein for resistance, the percentage of whip formation must be less than 12.5%, for susceptibility more than 15%, and intermediaries genotypes are in between these percentage rates (LEMMA et al., 2015).

The high incidence of smut disease in energy cane varieties is one of the three major problems in selection genotypes for this purpose, followed by high flowering rates and low unit stem mass (DINIZ et al., 2019). It is worth noting that the susceptibility can be related to the

features of *S. spontaneum*, which represents a susceptible genotype for smut disease (DA SILVA, 2017; MONTEIRO-VITORELLO et al., 2018; SAKAIGAICHI et al., 2019).

As described earlier, energy cane varieties are built by crossing modern sugarcane varieties with *S. spontaneum.* The modern sugarcane cultivars present a background with major contributions of *S. officinarum* (2n=80) (~90%) and *S. spontaneum* (2n= 40 to 128) (~10%), and a couple of *S. barberi* and *S. sinense* clones - that are derived from *S. officinarum* and *S. spontaneum* (AMALRAJ; BALASUNDARAM, 2006; NAIR, 2012; ALARMELU et al., 2018; THIRUGNANASAMBANDAM; HOANG; HENRY, 2018). Thus, considering the energy cane varieties derived from modern sugarcane and *S. spontaneum* breeding, these varieties must have more than 50% of the characteristics derived from the ancestral species, related to both important features for energy production and susceptibility to smut disease. However, Sakaigaichi et al. (2019) mentioned a great diversity in wild types of *S. spontaneum* considering resistance investigated in accessions collected in Japan. A collection of 30 accessions tested over five years repeatedly revealed a highly resistant genotype collected from the Iriomote Island (Japan) named Iriomote 15. Also, as mentioned by the authors, breeding lines crossed with Glagah Kloet are susceptible to smut disease, which agrees with what we see in Vertix 1 discussed later.

Sugarcane shows one of the most complex genomes due to the elevated polyploidy and aneuploidy degree compared with other crop plants (THIRUGNANASAMBANDAM; HOANG; HENRY, 2018), the number of chromosomes have variations from 100 to 130 (2n) (D'HONT et al., 1996; PIPERIDIS, D'HONT, 2020) and the genome size estimate is 10 Gbp (D'HONT; GLASZMAN, 2001). Advances in genomic tools and next-generation sequencing strategies enable a better understanding of the sugarcane genome, including those of differentiating allele expression (MARGARIDO et al., 2021; THIRUGNANASAMBANDAM; HOANG; HENRY, 2018).

For energy cane, little information is available about gene organization and genome complexity. However, a tetraploid genome of an autopolyploid *S. spontaneum* (AP85-441) facilitated the assembly of 32 pseudo-chromosomes comprising eight homologous groups of 4 members each, bearing 35,525 genes with alleles defined (ZHANG et al., 2018). We used this reference genome in our analysis and, for the first time, collected information on Vertix 1 and 2, both Vertix type 2, transcriptome profiles inoculated with *Sporisorium scitamineum.*

2. Sugarcane smut

The smut fungi belong to the phylum Basidiomycota and cause diseases on various plants, including cereal crops (SINGH *et al*., 2004). Sugarcane smut is caused by the *Sporisorium scitamineum*, which has a long history of spreading worldwide, and becoming a severe disease with up to 60% of sugarcane yield losses (SUNDAR et al., 2012; LONGATTO et al., 2015). The sugarcane smut disease recognizable characteristic sign is the whip structure formed from shoot apical meristem or meristems of lateral buds of infected stalks, where a black spore mass produced resembles soot, reason why the disease is called “smut” (SUNDAR et al., 2012). Smut spores can be carried over long distances by the wind (CROFT; BRAITHWAITE, 2006) and the smut disease is present in nearly all countries producing sugarcane in the world, except for Fiji, an isolated island in Oceania (SUNDAR et al., 2012; TOM et al., 2017; MONTEIRO-VITORELLO et al., 2018). The first disease report came from Natal, South Africa, in 1877, but sugarcane smut is likely to be present in Asia for much longer (CROFT; BRAITHWAITE, 2006). India also has reports of smut, causing severe problems in the susceptible Indian wild genotype (CROFT; BRAITHWAITE, 2006). Around the 1950s, Brazil reported the first case of sugarcane smut in the State of São Paulo (JOKESHI, 2011).

The *S. scitamineum* life cycle is similar to all other smut species involving transitions between three cell types: diploid teliospores (2n) are the resistant cell type and disseminated mainly by wind or rain; the haploid yeast cells (n) are saprophytic; and the dikaryotic mycelia (n+n) which are the plant infective phase (SINGH et al., 2004; LONGATTO et al., 2015). The diploid teliospores germinate by forming a probasidium, where four basidiospores emerge by meiosis. The haploid basidiospores grow by budding and can be cultured on a defined medium (BAKKEREN; KRONSTAD, 1993). When two sporidial cells with compatible mating types fuse, they originate the dikaryotic hyphae able to infect host tissues to proliferate inter and intracellularly (BAKKEREN; KRONSTAD, 1993). Therefore, two haploid yeast-like cells will be sexually compatible (mating-type) only if they have different alleles at two genome loci: *locus a* and *locus b* (ALBERT; SCHENCK, 1996). *Locus "a"* encodes a system required for recognizing and fusing haploid sporidia composed of a membrane receptor and a pheromone. *Locus "b"* encodes two subunits of a heterodimeric transcription factor, bE and bW, that regulates filamentation, dikaryon maintenance, and pathogenicity (ZHU et al., 2019). In summary, two haploid yeast-like cells will be compatible with the pheromone and membrane receptor of the opposite sexual reaction type (ALBERT; SCHENCK, 1996; LONGATTO et al., 2015).

The mating-type loci´s complex structure and function are fundamental to the formation and maintenance of the infectious processes and hence pathogenicity (BAKKEREN; KRONSTAD, 1993). After germination and hyphal anastomosis, fungal development results in the differentiation of an appressorium to penetrate plant tissues. The infective hyphae penetrate through buds at each sugarcane node and shortly reach apical meristem systemically (IZADI; MOOSAWI-JORF, 2007). The hyphae growth progresses for about 1 or 2 months, eventually leading to karyogamy (TRIONE, 1990; LONGATTO et al., 2015), and whip formation containing the diploid teliospores restart the smut cycle. The whips shelter the reproductive structures of *S. scitamineum* with teliospores formed and matured (MONTEIRO-VITORELLO et al., 2018). Finally, the wind releases the teliospores after disrupting a silvery membrane that protects sporogenesis, exposing a mass of black and powdery teliospores (JOKESHI, 1997) (JOKESHI, 1997) (Figure 1).

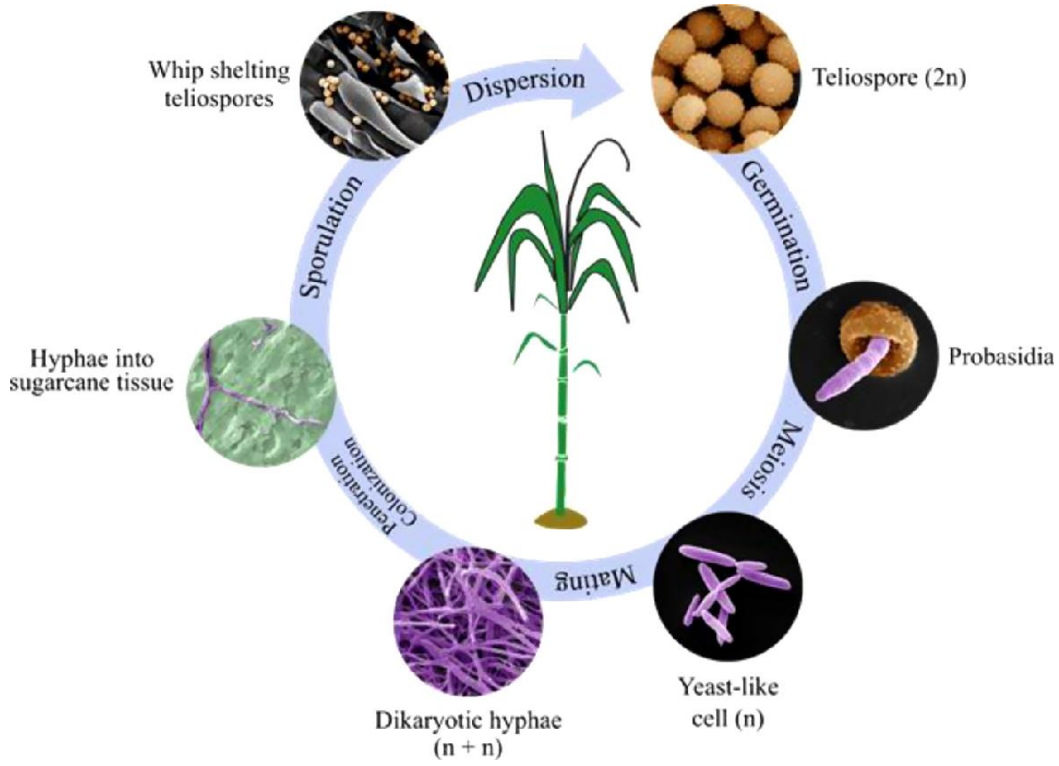

Figure 1. *Sporisorium scitamineum* life-cycle developmental structures in various stages and within host tissues (adapted from MONTEIRO-VITORELLO et al., 2018).

The whips assume various shapes, from short to long, twisted, multiple whips, and their color is black or brown (SUNDAR et al., 2012; MONTEIRO-VITORELLO et al., 2018). The whip corresponding to the fungal *sorus* contains host fibro-vascular tissues, surrounded by parenchymal tissues covering the mass of teliospores (FONTANIELLA et al., 2002). Other

smut disease general symptoms are leaf and stem galls, apical deformity, floral infection, malformed spindle, bud proliferation, and poor cane formation, causing significant cane tonnage and juice quality losses (SUNDAR et al., 2012).

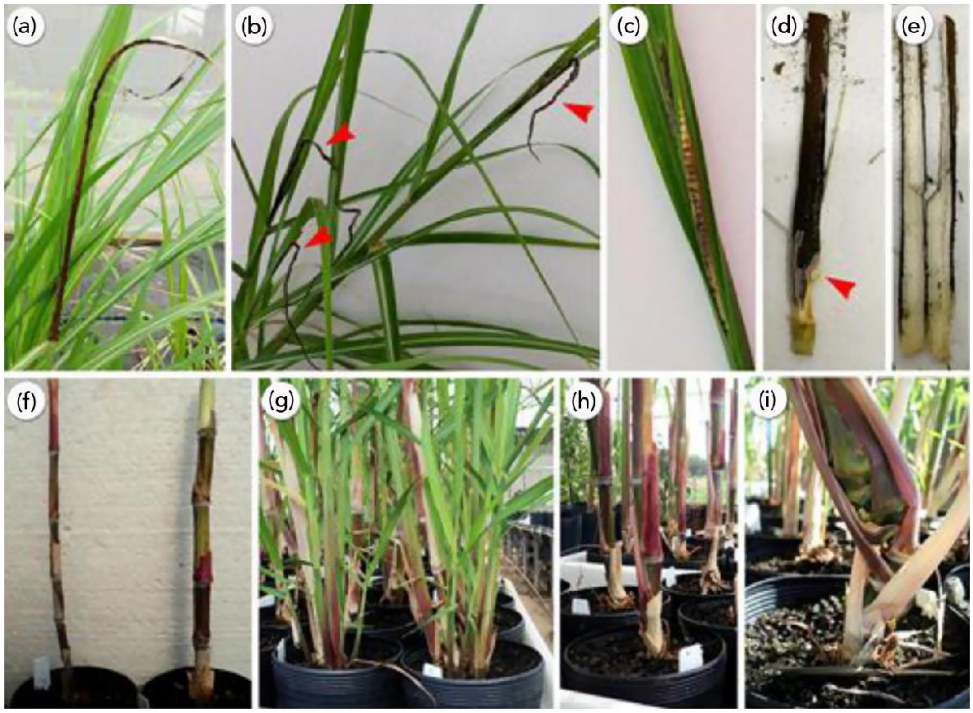

Figure 2. The sugarcane-smut signals and symptoms: (a) Single whip-like structure from sugarcane shoot apical meristem; (b) three whips emerging from sugarcane lateral tillers (red arrows); (c) tumor-like gall developed over a leaf midrib; (d) basal enlargement of a whip (red arrow); (e) longitudinal section of the whip shown in (d); (f) inoculates (left) and control (right) plants showing stalk diameter differences after whip emission (120 days after inoculation); (g) tillering of infected plants; (h) single culm healthy plants; (i) gall formation in the base of sugarcane stalk (adapted from MONTEIRO-VITORELLO et al., 2018).

In some cases, the disease can be asymptomatic, hindering the early diagnosis, being only observed after its development in the field (SUNDAR et al., 2012; LONGATTO et al., 2014). The disease's early diagnosis is essential for right and agile management practices. The PCR assay and microscopy are two common techniques used to detect the smut pathogen in asymptomatic plantlets. (SINGH et al., 2004; MONTEIRO-VITORELLO et al., 2018). Positive detection of the pathogen by conventional PCR is possible three weeks after inoculation using specific primers such as the ITS (*Internal Transcribed Spacer*) region as a target and the bE gene (mating-type). In addition, by light microscopy, the presence of *S. scitamineum* hypha was detected eight weeks after inoculation (SINGH; SOMAI; PILLAY, 2004; LONGATTO et al., 2014).

Another applied molecular technique is a TaqMan real-time qPCR (quantitative real-time PCR), which is employed to detect and quantify *S. scitamineum* in sugarcane inoculation, effective within 12 hours after inoculation using specific primers (bEQ-F/bEQ-R) and a TaqMan probe (bEQ-P), designed based on the bE (b East mating type) gene (YACHUN et al., 2013). The use of qPCR for the bE gene (mating-type) showed higher sensitivity and specificity for smut detection when compared to conventional PCR (SU et al., 2013). In addition, the qPCR on bE gene also provided an improvement in the assessment of smut-resistance of sugarcane genotypes by allowing the quantification of the smut pathogens copy number in asymptomatic infected plants, supporting efficient supervision and management of pathogen-free sugarcane. (YACHUN et al., 2013).

Loop-mediated isothermal amplification (LAMP) also can be used for smut detection. LAMP is an isothermal amplification technology established by Notomi et al. (2000), which is an approach that completes automatic looping, strand displacement and DNA synthesis using two inner (called the forward inner primer – FIP) and two outer primers (called backward inner primer – BIP) and Bst DNA polymerase. The limit of detecting sugarcane infection by smut using LAMP and Pep1 gene is 100 times higher than conventional PCR targeting the bE gene. Also, the LAMP technique shows positive for tested sugarcane buds artificially inoculated with *S. scitamineum* (SU, Y. et al., 2016). The PEP1 gene is a fungal effector in smut diseases with a highly conserved sequence and could inhibit plant peroxidases resulting in plant immunity suppression (HEMETSBERGER et al., 2012; HEMETSBERGER et al., 2015). Studies established that the LAMP method uses the specific Pep1F3/Pep1B3 and Pep1FIP/Pep1BIP primers for *S. scitamineum* in sugarcane and can be used to detect imported or exported sugarcane seeds or seed stems, highlighting a technical support for realizing smut-free sugarcane supervision and management (SU, Y. et al., 2016).

The primary management of smut disease is genetic resistance obtained by breeding methods but rouging of infected plants is also a management alternative (JOKESHI, 2011; SUNDAR et al., 2012). Hot water treatments effectively control the smut fungi residing in the buds and the seedling fungicides application can extend protection (SUNDAR et al., 2012; JOKESHI, 2011). The use of pre-sprouted seedlings with a phytosanitary certificate and seedlings from micropropagation methods are the alternatives to prevent smut on sugarcane cultivation (MONTEIRO-VITORELLO et al., 2018). Integrated disease management strategy is the viable option, but the selection for resistant varieties is still the most effective (SUNDAR et al., 2012; JOKESHI, 2011; MONTEIRO-VITORELLO *et al.*, 2018). For the efficient

development of breeding programs, it is necessary to study the pathogen biology and genetic mechanisms involved in the complex host-pathogen interaction (LONGATTO et al., 2014).

3. Sugarcane-smut defensive response

Sugarcane resistance to *S. scitamineum* may be derived from one or a combination of physiological, biochemical and morphological factors. Sugarcane genotypes are evaluated for resistance through artificial bud inoculation, and the percentage of plants developing characteristic disease symptoms or signals is considered a susceptibility measure for the disease (LEMMA et al., 2015; PETERS et al., 2020). In general, sugarcane defense mechanisms during interaction with smut can be divided between pre-formed and post-formed. The physical mechanisms of resistance are mainly related to the bud morphology in sugarcane since they are the main entry points of the pathogen and can confer increased protection for the host (LONGATTO et al., 2014). Among these pre-formed mechanisms, the number of trichomes on scales protecting buds is highlighted (GLÓRIA et al., 1994; O-HECHAVARRÍA et al., 2011). Among the pre-formed biochemical mechanisms, several studies have already described the role of flavonoids and phenylpropanoids present in the inner scales of the buds, also contributing to the decrease of *S. scitamineum* spore germination (LLOYD; NAIDOO 1983; FONTANIELLA et al. 2002; MILLANES et al. 2005, de ARMAS et al., 2007).

The biochemical defense mechanisms of sugarcane in this pathosystem have been studied with more emphasis. They are produced naturally by the host or as a response to the presence and penetration of the pathogen. Among the biochemical defense mechanisms is highlighted the reactive oxygen species (ROS) metabolism, which involves oxidative stress, antioxidant enzymes, synthesized flavonoids, changes in the concentration of phenolic compounds, glycosylated substances, tissue lignification, salicylic acid accumulation, and polyamides (RODRIGUEZ et al., 2001; SU et al., 2016).

In the interaction sugarcane - *S. scitamineum*, there occurred changes in the expression of genes associated with ROS as a response to the fungal infection by the host. Changes were also associated with ethylene and auxin response pathways, besides other pathways associated with tissue lignification, all related to host resistance (LAO et al., 2008; MENOSSI et al., 2008; SCHAKER et al., 2016; PETERS et al., 2017). Furthermore, the production of chitinase and β-1,3-glucanase represent known responses of sugarcane varieties to fungal infection, acting to target the pathogen's cell wall (BLANCH et al., 2007).

The pathways of signaling and host response can be triggered from the recognition of PAMPS (Pathogen Associated Molecular Patterns), typically conserved molecules that characterize a range of microorganisms and lead to pathogen triggered immunity (PTI - PAMP triggered immunity). PTI confers resistance to most non-adapted pathogens and is known as "non-host resistance". In addition to PTI, plants also feature effector-triggered immunity (ETI). This perception involves intracellular receptors that recognize the effectors secreted by the pathogen, either directly or indirectly (JONES; DANGL, 2006). Candidates for effectors and their function in the host are being investigated (TEIXEIRA-SILVA et al., 2020; LING et al, 2022; MAIA et al., 2022) and will be important in assisting the understanding of this interaction. The significance of this response produced by both mechanisms, PTI and ETI, is to generate matching reactions associated with speed, persistence, and strength of signaling, rather than presenting qualitative differences.

3.1 Reactive oxygen species (ROS)

Plants have a complex antioxidant protection system as a defense mechanism against free radicals, which are formed continuously by regular cell metabolism and during various pathological events. In other terms, they are collectively called reactive oxygen species (ROS), the free radicals produced naturally by organisms as a fundamental part of aerobic life and cellular metabolism or from biological dysfunction, such as pathological events (BARREIROS; DAVID; DAVID, 2006; SIES; JONES, 2020). However, when ROS occurs in excess, it can cause the oxidation of biological molecules. Therefore, the imbalance between oxidative challenge and antioxidant defense capability of the organism is called oxidative stress (MACHADO et al., 2009).

ROS, traditionally a by-product of metabolic processes, is primarily produced in peroxisomes and in the electron transport chain in the chloroplast and mitochondria. The different ROS types are superoxide radical (O_2*), hydrogen peroxide (H_2O_2), hydroxyl radical (OH*), singlet oxygen ($^1O_2$) and tripled oxygen ($^3O_2$). A common characteristic of the different types of ROS is to cause damage to proteins, DNA, RNA, carbohydrates, and lipids due to their reactivity, even on different levels (APEL et al., 2004; WASZCZAK; CARMODY; KANGASJÄRVI, 2018). Also, ROS have an antimicrobial effect, play a role in cell wall stiffening and are important as local and systemic signaling molecules that activate the antimicrobial defenses against plant pathogens (ASZCZAK; CARMODY; KANGASJ¨ARVI, 2018).

During plant-pathogen interaction, the toxic and signaling properties of the ROS act against pathogenic invasion as one of the first cellular responses, and rapidly accumulates after pathogen recognition (O'BRIEN et al., 2012; TORRES, 2010). ROS production is typically apoplastic and has two phases after pathogen inoculation. The first phase is non-specific, presents low amplitude, and may occur minutes after contact with the pathogen. The second phase, usually related to the production of H_2O_2, occurs hours after the pathogenic attack, has high amplitude and is generally associated with the defense responses and plant resistance to diseases (TORRES et al., 2006).

ROS production in the apoplast results from the specific activation of NADPH oxidase and Peroxidase (Prxs III) and is associated with signaling in response to stress (BOLWELL et al., 1995; KIMURA et al., 2017). The NADPH oxidase complex contains an enzymatic subunit, which transfers electrons to the molecular oxygen generating O_2* (SAGI; FLUHR, 2006). Due to their reducing activity, the Prxs III of the cell wall produces H_2O_2 in response to pathogen recognition (TORRES et al., 2006). In addition, the oxidative burst from the apoplast induces the production of chloroplastic ROS from guard cells, contributing to ROS production during the hypersensitivity response in defense against pathogens (MIIGNOLET-SPRUVT et al., 2016).

ROS is also produced in other cell compartments (mitochondria, chloroplasts, peroxisomes, and endoplasmic reticulum) during the interaction, contributing to the plant defense (TORRES et al., 2006). With ROS production, the cells near the site of infection have enzymatic and non-enzymatic mechanisms for detoxification and signal modulation to avoid oxidative damage. Several enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and other peroxidases are involved in this antioxidative system, and there are several isoforms of these enzymes located in multiple cell compartments (DE GARA; DE PINTO; TOMMASI, 2003; QUAN et al., 2008; SHARMA et al., 2012).

The SOD enzyme is part of a complex that catalyzes the formation of H_2O_2 from the O_2* radical, crucial to the antioxidant defense mechanism, and comprises the first line of defense against ROS in cells (SCANSALIOS, 2005). The CAT is widely distributed and is considered a central component of detoxification pathways that prevent the formation of radical OH^-. CAT catalyzes the conversion of two H_2O_2 molecules into water and O_2* by transferring two electrons. Like CAT, ascorbate peroxidase (APX) also has an affinity for H_2O_2 acting in the detoxification together with donors of electrons, such as phenolic, alkaloid, and auxin compounds (ZENG et al., 2010; ZIPOR; OREN-SHAMIR, 2013). Glutathione S-transferase

(GST) and CAT can reduce glutathione and H_2O_2 to water and oxidized glutathione (GSSG) (BLONDET et al., 2006). Another critical antioxidant enzyme involved in the process is the thioredoxin (trx), which can connect with Trx-dependent peroxidases to eliminate H_2O_2, by the activity of oxidoreductase (PETERS, 2016) (Figure 3).

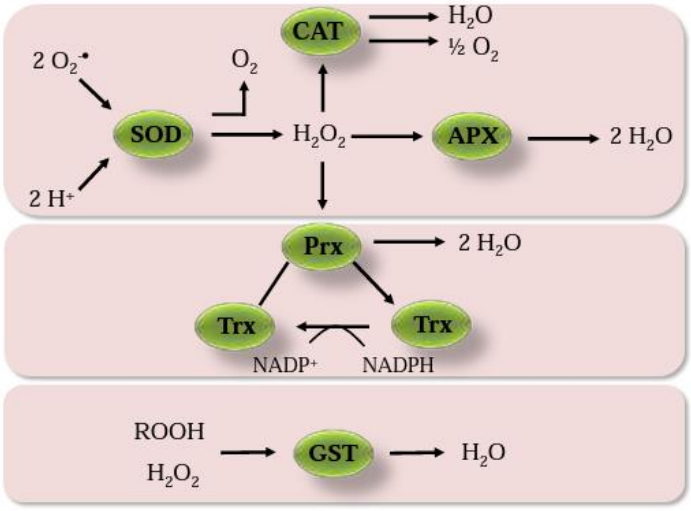

Figure 3. Mechanisms for reactive oxygen species-scavenging examples in plants. Antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (Prx), thioredoxin (Trx) and glutathione S-transferase (GST) (adapted from PETERS, 2016).

Studies with *Colletotrichum falcatum* infecting sugarcane plants showed that the resistant cultivar CoS8436 exhibited elevated activities of SOD, CAT, and PRX as compared to the susceptible cultivar CoJ64 (ASTHIR et al., 2009). In wheat, the overexpression of Prxs (TaPrx103), secreted at the invasion site, showed evidence of association with resistance against powdery mildew (SCHWEIZER, 2008). Also, Su et al. (2016) reported evidence, through *Principal Component Analysis* (PCA) for nine sugarcane varieties tested for *S. scitamineum* interaction, that SOD, GPX, Prx, and CAT contributed to about 43% of smut resistance.

Peters et al. (2017), in a study about the smut-sugarcane interaction, concluded that the high level of H_2O_2 observed in resistant genotypes in response to smut is related to the signaling and triggering of the plant defense responses. In addition, resistant plants have a larger number of ROS and antioxidants enzymes isoforms when compared to susceptible ones (PETERS et al., 2017) (Figure 4).

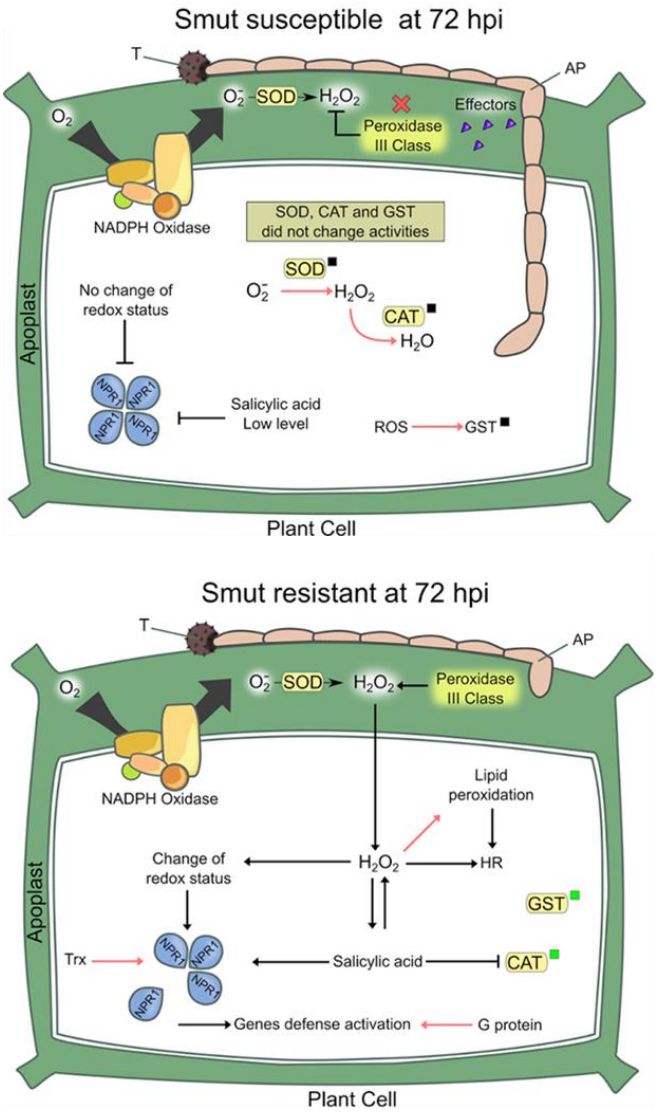

Figure 4. Overview of mechanisms associated with ROS and antioxidant enzymes in susceptible and resistant sugarcane inoculated with *S. scitaminum* at 72 hpi. *Red arrows* represent results from their study and *green squares* indicate decreases in enzymatic activity; *black squares* indicate no alterations. *Symbol* indicates "x" repression (only in smut-susceptible plants). All changes were relative to the mock control. T – teliospore, Ap - appressorium (adapted from PETERS et al., 2017).

In brief, the oxidative burst is one of the first reactions associated with PTI and ETI in defense responses (TORRES et al., 2010; SURVILA et al., 2016). ROS in plant cells are produced by plasma membrane-localized NADPH oxidase, class III peroxidases, pathways, like photosynthesis, photorespiration and respiration (GRATÃO et al., 2005; TORRES, 2010). And the systemic propagation of ROS allows the transmission of the signals over long distances triggering specific responses adapted to the type, concentration and subcellular origin of these molecules (CHEN et al., 2015; MATTILA et al., 2015). Therefore, ROS can culminate in localized cell death (hypersensitive response – HR) in incompatible interactions, highly useful in the host defense response against biotrophic pathogens (BARNA et al., 2012).

The cell wall and the apoplastic space are active sites of ROS production. Which have a pivotal role in signaling and defense against pathogen attack, as well as it is the first barrier to penetration (DOEHLEMANN et al., 2009) and can induce, for instance, the activity of PR-proteins like the beta-1,3-glucanase (*ScGluA1 gene* - GenBank Acc No. KC848050, subfamily A), *ScGluD1* and *ScGluD2 genes* - GenBank Acc No. KC848051 and GenBank Acc No KF664181, subfamily D) in sugarcane-smut interaction (SU et al., 2013; SU et al., 2016).

Su et al. (2014), found in their studies that the catalase (*ScCAT1 gene* – GenBank Accession No. KF664183) has increased expression in the sugarcane-smut resistant variety — and is maintained at higher expression levels — as compared to susceptible variety, which suggested a positive correlation with the catalase activity for both smut resistance and abiotic stress in sugarcane. In addition, it was noted by histochemical assays that ScCAT1 acted positively in sugarcane immunity (SU et al., 2014). In other study, regarding the oxidative burst, peroxidase gene (ScSS36), poxN, was displayed upregulated in sugarcane resistant plant at 72 hours post-inoculation (hpi) whereas in susceptible plants was found as weakly induced at 24 hpi (LAO et al., 2008).

Therefore, regarding the ROS formation, multiple enzymatic reactions are responsible to produce it as a primary product or a by-product. It is remarkable that the ROS compartmentalization production and scavenging may determine their biological functions in the plant (FOYER; NOCTOR, 2016; NOCTOR; MHAMDI; FOYER, 2016). For the ROS produced during pathogen interactions, the formation can occur in different compartments in the plant cell for defense mechanism, although the primary formation after the pathogen perception occurs in the apoplasts (TORRES, 2010). Although primary targets for apoplastic ROS signals remain unclear, research progress has been made to understand the ROS signaling mediating (QI et al., 2017). Thus, the antioxidant enzymatic system composition and availability will determine the ROS longevity and concentration in the cell (MATTILA et al., 2015). Nonenzymatic system also can be used, consisting of the small soluble molecules synthesis for ROS oxidized such as glutathione and antioxidant compounds like flavonoids, carotenoids, glycosides, ergothioneine, polysaccharides, phenolic and ascorbic acid (SÁNCHES, 2017; BHUIYAN et al., 2021).

4. Dual transcriptomics in sugarcane-smut interaction: the data importance

As previously mentioned, the sugarcane varieties resistance to *S. scitamineum* can be derived from physiological and/or biochemical (internal) or morphological (external) factors and can be separated into pre-formed and post-formed. Some varieties exhibit only morphological resistance mechanisms and other varieties may exhibit mainly physiological and biochemical mechanisms, while others have both in interaction at different times (DEAN, 1982; BHUIYAN et al., 2010). The main sugarcane disease control measure currently used is the use of disease-resistant varieties. However, the genetic determinants of this resistance in breeding programs are still not entirely known, even though the importance of hereditary traits has been demonstrated (CHAO et al., 1990; MCNEIL et al., 2018).

The sugarcane-smut resistance is a quantitatively inherited trait, and it is possible to obtain both resistant and susceptible progenies by crossing two resistant varieties (CHAO et al., 1990). For resistance to be durable and effective, breeding programs use some strategies such as the use of pyramiding genes to incorporate different defense mechanisms in the host (KELLER; FEUILLET; MESSMER, 2000; MCNEIL et al., 2018). Thus, the need for genetic basis knowledge of these resistance mechanisms emerges to improve varieties and direct breeding programs (WU; HEINZE; HOGARTH, 1988; CHAO et al., 1990; MONTEIRO-VITORELLO et al., 2018).

High-throughput techniques have been used to examine the response of sugarcane - *S. scitamineum* interaction at the transcriptome level and may reveal metabolic and molecular regulatory paths involved in the pathosystem. Likewise, it is relevant to identify essential genes and define transcriptional regulation features related to sugarcane resistance to smut disease (QUE et al., 2014). Several studies have used different techniques for gene expression analyses in the interaction sugarcane-smut diseases including Subtractive Suppression Hybridisation (SSH), mRNA differential display analysis and cDNA-amplified fragment length polymorphism (HEINZE et al., 2001; THOKOANE; RUTHERFORD, 2001; BORRAS-HIDALGO et al., 2005; MCNEIL et al., 2018).

Next-generation sequencing (NGS) is a powerful technique for whole transcriptome sequencing (RNA-seq) which stands out as a fast and efficient method for studies based on gene expression data at the whole-genome level and define putative gene function (WANG; GERSTEIN; SNYDER, 2009; OZSOLAK; MILOS, 2011; SINGH; GARG; JAIN, 2013). RNAseq technique does not require extensive gene sequence knowledge for the data

investigated and provides an unbiased transcriptome view, enabling information when small gene expression changes and low-abundance transcripts are considered (`T HOEN et al., 2008). Several studies have been performed using NGS with sugarcane-smut interaction, showing a complex biological process (WU et al., 2013; QUE et al., 2014; TANIGUTI et al., 2015; SCHAKER et al., 2016; YOUXIONG et al., 2014; MCNEIL et al., 2018). Thus, the knowledge about the sugarcane resistance type, external or internal resistance mechanisms, is meaningful for breeding programs (MCNEIL et al., 2018).

Determinants set identification of host resistance and fungal pathogenicity is probably the optimal strategy to improve crop breeding programs (MONTEIRO-VITORELLO et al., 2018). RT-qPCR (Quantitative real-time PCR) technique for preselected genes can be employed to validate transcript abundance data derived from transcriptome sequencing. Differentially expressed genes (DEGs) in response to smut infection may be identified and the possible roles of these transcripts in the defense response by internal and external mechanisms in sugarcane may be elucidated (MCNEIL et al., 2018; SINGH et al., 2019; RODY et al., 2021). In the future, on an even broader stage, comparing the responses of sugarcane to all fungal phytopathogenic agents threatening the crop to find common targets to be investigated may present a major key to resistance and management of challenging fungal diseases for global sugarcane production (MONTEIRO-VITORELLO et al., 2018).

5. Objectives

This project aimed to identify ROS metabolism modulated genes in the initial *S. scitamineum*-energy cane interaction and compare the expression profiles between smut-resistant (Vx2) and -susceptible (Vx1) genotypes. Having the expression profiles of ROS-associated genes in Vx1 and Vx2, we compared the data with those previously obtained for smut-susceptible (IAC66-6) and -resistant (SP80-3280) sugarcane genotypes. We pursued the following strategies:

1. Sequence and analyze of a dual transcriptome RNAseq data from energy cane - smut interaction genotypes;
2. A comparative analysis of ROS metabolism in dual transcriptome RNAseq data from energy cane genotypes during *S. scitamineum* interaction;

3. Expression profiles evaluation of the antioxidant enzymes genes related to the oxidative burst modulation selected in a previous experiment (PETERS, 2016), using real-time qPCR;
4. Perform statistical analysis of differentially expressed genes.

Hypothesis: Energy cane modulates ROS metabolism differentially in smut-resistant- and -susceptible plants, and it is comparable to mechanisms detected in sugarcane plants.

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CHAPTER 2: COMPARATIVE ANALYSIS OF OXIDATIVE BURST MODULATION TRANSCRIPTIONAL PROFILES IN ENERGY CANE GENOTYPES DURING *S. scitamineum* INTERACTION

Abstract

Energy cane smut disease, caused by the basidiomycete fungus *Sporisorium scitamineum*, establishes a biotrophic interaction. The smut disease is one of the most important energy cane diseases and is prominent in research involving defense gene selection strategies for breeding programs. Nevertheless, knowledge about the energy cane genetic basis is still scarce compared with conventional sugarcane varieties. Thus, we propose investigate the mechanisms involved in ROS metabolism modulation in smut-susceptible (Vertix1) and -resistant (Vertix2) genotypes, by *S. scitamineum* challenging at 48 hours post-inoculation (hpi), from RNA-seq data. A total of 1,549 differentially expressed genes (DEGs) were identified, in comparison between infected and non-infected buds, 1,286 were within Vertix 1 and 263 were within Vertix 2. We observed 48 DEGs in both genotypes with contrasting expression profiles, this includes genes involved in pathogen defense, antioxidant enzymatic system and auxin response. Finally, we analyzed DEGs from defense responses specific to resistant (42 genes) and susceptible (43 genes) varieties, demonstrating responses related to smut-disease resistance for future analysis. In order to focus on ROS metabolism related DEGs, within the defense response category, we selected 20 specific genes in the resistant variety and 25 genes in the susceptible variety. We conclude that the gene expression modulation upon infection of *S. scitamineum* in energy cane genotypes used is, in general, earlier than previously observed for conventional sugarcane, with considerable differences of perception and modulation of genes related to ROS metabolism modulation.

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CHAPTER 3: GENE EXPRESSION ANALYSIS OF OXIDATIVE BURST MODULATION IN ENERGY CANE SMUT -RESISTANT AND -SUSCEPTIBLE GENOTYPES

Abstract

The smut disease, causal agent *Sporisorium scitamineum*, is an important disease in the energy cane crop. In this study, we analyzed the modulation of reactive oxygen species (ROS) metabolism through the gene expression related to the antioxidant enzyme system in response to fungal inoculation, at 48 and 72 hpi, in resistant and susceptible genotypes. We evaluate the expression gene profile of antioxidant enzymes: *superoxide dismutase* (*SOD*), *catalase 3* (*CAT3*), *catalase B* (*CATB*), *peroxidase 5-like* (*POX5*), *glutathione S-transferase t3* (*GSTt3*) and *thioredoxin h like* (*TRX*). For 48hpi time-point, the *TRX* profile appeared downregulated in susceptible genotype (Vertix1) and *SOD* was downregulated in resistant genotype (Vertix2), although the others genes profiles did not exhibited significant difference for the expression against smut inoculation in both genotypes. Conversely, for 72hpi time-point, the results did not show significant difference in profile expression of these ROS modulation genes in both genotypes against the fungal presence. In conclusion, we only observed a significant difference in expression profile of *TRX* for Vertix1 and *SOD* for Vertix2, at 48hpi, both downregulated. Regardless, we suggest that further detailed analyses for ROS modulation-related genes expression and the presence of antioxidant enzymes in infected tissues should be performed to improve the ROS metabolism understanding, in the same experimental conditions.

Keywords: Energy cane; smut disease; ROS metabolism modulation; antioxidant enzyme; gene expression.

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