University of São Paulo
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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
2022

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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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

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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, em que a atividade de SOD (superóxido dismutase) foi reprimida na variedade Vertix 2 e de TRX (Thiredoxina) reprimida na variedade Vertix1. 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 biotype, 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% (moderatelyresistant), 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).

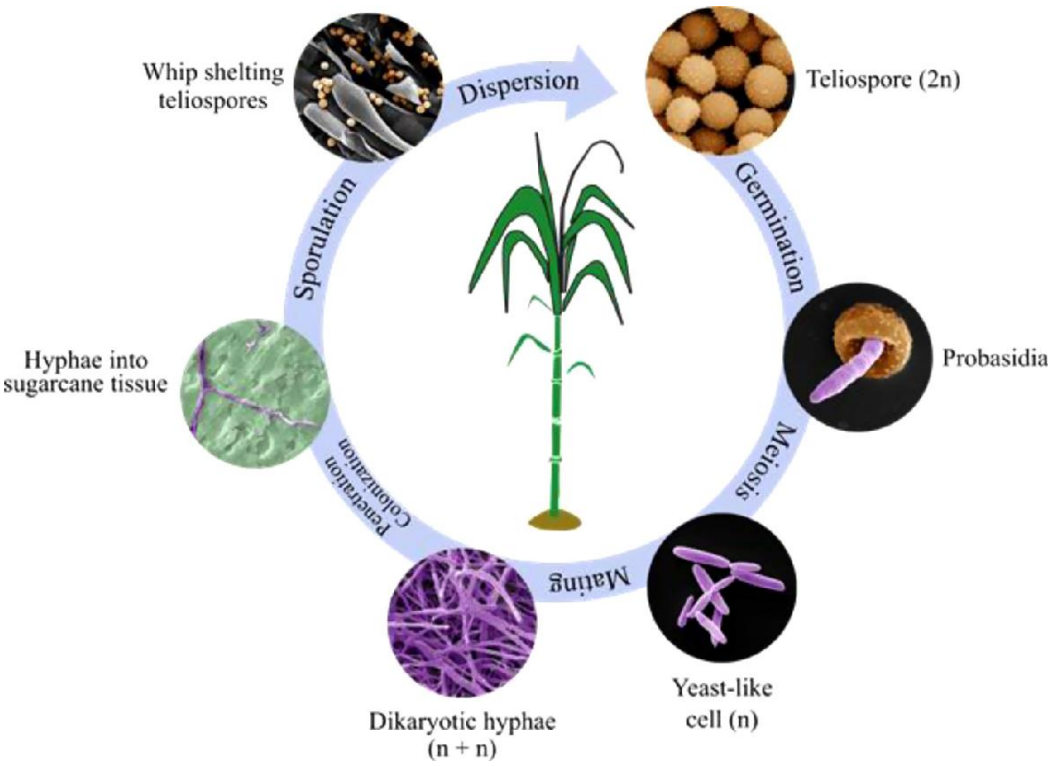

Figure 1. *Sporisorium scitamineum* life-cycle developmental structures in various stages and within host tissues (adapted from MONTEIRO-VITORELLO et al., 2018).

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).

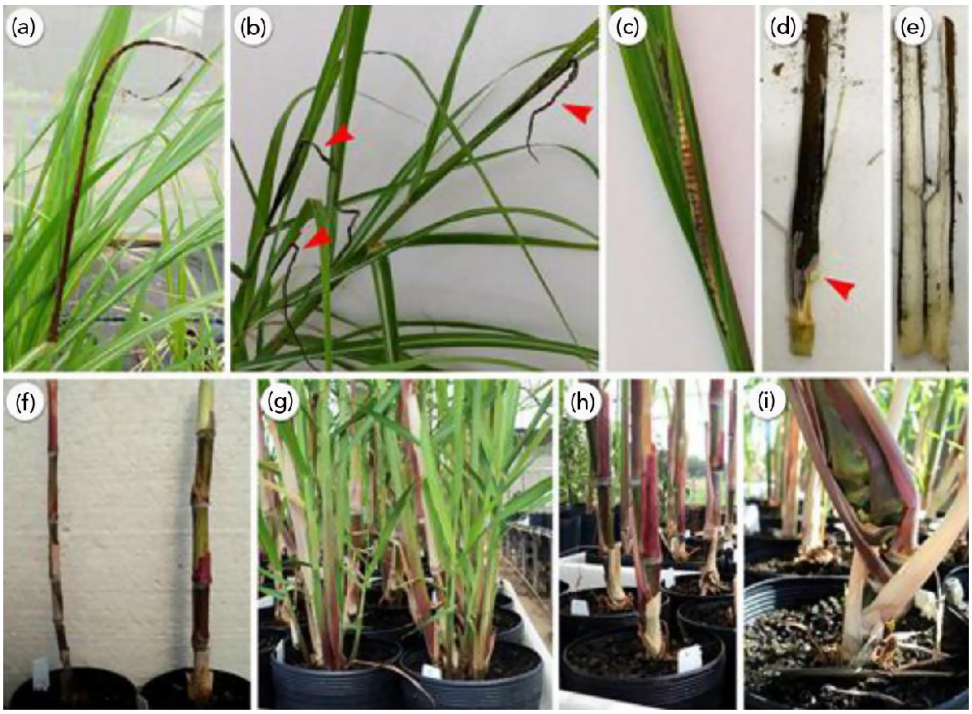

Figure 2. The sugarcane-smut signals and symptoms: (a) Single whip-like structure from sugarcane shoot apical meristem; (b) three whips emerging from sugarcane lateral tillers (red arrows); (c) tumor-like gall developed over a leaf midrib; (d) basal enlargement of a whip (red arrow); (e) longitudinal 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 culm (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).

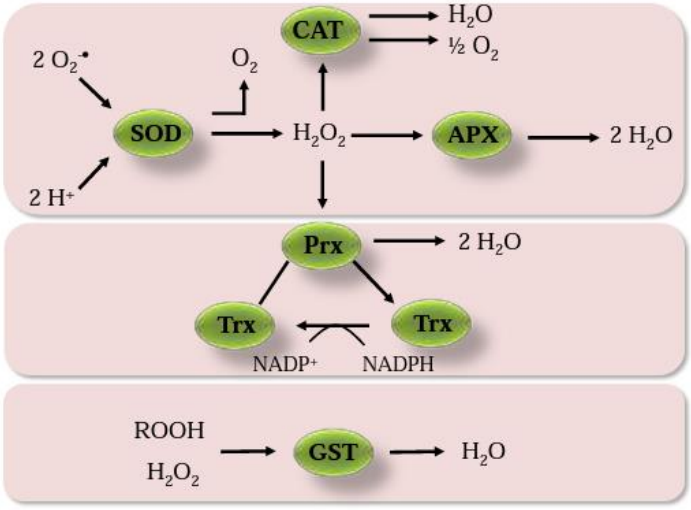

Figure 3. Mechanisms for reactive oxygen species-scavenging examples in plants. Antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (Prx), thioredoxin (Trx) and glutathione S-transferase (GST) (adapted from PETERS, 2016).

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).

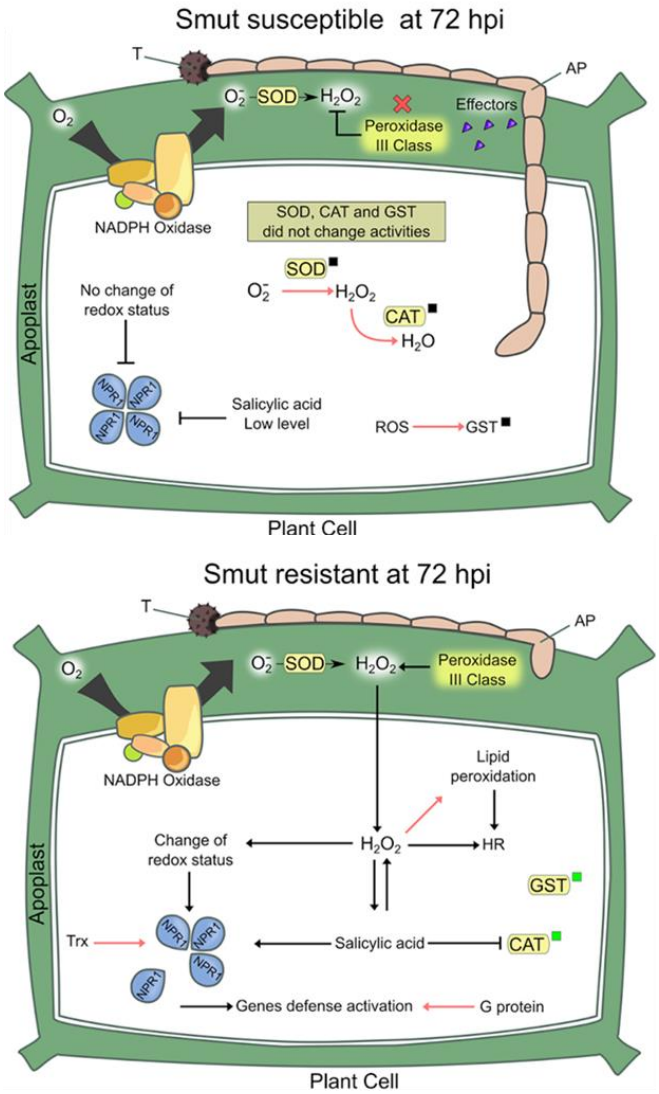

Figure 4. Overview of mechanisms associated with ROS and antioxidant enzymes in susceptible and resistant sugarcane inoculated with *S. scitaminum* at 72 hpi. *Red arrows* represent results from their study and *green squares* indicate decreases in enzymatic activity; *black squares* indicate no alterations. *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.

1. Introduction

Developing resistant genotypes is the most reliable and durable way to secure plants from pathogens. However, since pathogens usually adapt to resistance promoted by 'major resistance genes' (R genes), quantitative resistance has many advantages (PILET-NAYEL et al., 2017). The bases of diagnostic markers for quantitative resistance contemplate the search of variation in genes involved directly in pathogen recognition or related processes ('candidate gene approach') or an untargeted method such as comparing RNAseq data of resistant versus susceptible plants (MOSQUERA et al., 2016).

Over the past years, the molecular events related to sugarcane smut disease caused by *Sporisorium scitamineum*, a Basidiomycete biotrophic pathogen have been investigated (MONTEIRO-VITORELLO et al., 2018). The sugarcane smut disease can cause severe losses and for very susceptible genotypes, they can reach 100%. In addition, infected plants may experience a decrease in sucrose content (Brix) and fibers in percentage, changing dramatically the plant architecture showing thinner stems and tillering (WADA et al., 2016).

Sugarcane genetic breeding programs aim to develop varieties for sugar production and first-generation ethanol (1G) purposes, showing higher sucrose than fibers contain. However, a new cultivar biotype called energy cane, considering the high potential of sugarcane for bioenergy production, has been developed by new breeding programs (CURSI; HOFFMANN; BARBOSA, 2021). Granbio Investimentos S.A., a Brazilian company, leads one of the most important energy cane breeding programs globally with varieties called Vertix[2]. In this context, one of the three major problems in selection genotypes for energy purpose is the high incidence of smut disease (DINIZ et al., 2019).

Considering the importance of *S. spontaneum* ancestor in energy cane genotypes genetic background, the ancestor composes 50% of cane energy components, is noted that the smut-susceptibility can be transferred from this parental into the breeding development (DA SILVA, 2017; MONTEIRO-VITORELLO et al., 2018; SAKAIGAICHI et al., 2019). However, Sakaigaichi et al. (2019) described 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. Vertix 1 is a crossing of F1 resulted of a parental female RB855465 and the *S. spontaneum* Glagah, which is susceptible to smut. Whereas Vertix2, resistant to smut, is a crossing of F1 resulted of a parental cross of IAC91-1099 and *S. spontaneum* Kratatau..

Here, we used high-throughput techniques to examine the response of energy cane - *S. scitamineum* interaction at the transcriptome level for Reactive Oxygen Species (ROS) metabolism using Vertix 1 and 2 to compare to previous results obtained for sugarcane (PETERS et al., 2017). Regarding the sugarcane-*Sporisorium scitamineum* molecular interaction, it is known that the expression of genes encoding enzymes associated with oxidative burst varies in the initial moments of infection (MENOSSI et al., 2008; YOU-XIONG et al., 2011; SCHAKER et al., 2016; PETERS et al., 2016). This topic was reported by Peters et al. (2017), where early accumulation of H_2O_2, and a reduction in both the activity of antioxidant enzymes and the expression of these genes in resistant plants when compared to susceptible plants. The oxidative burst coincides with some of the fungal development stages, which are: germination, appressorium formation and fungal colonization in plant

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

tissues. This alteration in the reactive oxygen species (ROS) metabolism is delayed in susceptible plants. Potentially, this would be the first defense response of sugarcane when coming into contact with the pathogen, leading to the activation of mechanisms related to resistance.

2. Materials and methods

2.1 Plant material

Two energy cane genotypes (Vertix1 and Vertix2), susceptible and resistant (Figure 5) to smut, were used in this study. We sampled three biological replicates of single-bud sets of 10-month-old healthy plants obtained from GranBio Investimentos S.A. and inoculated using *S. scitamineum* SSC04 teliospores following as previously described by Taniguti et al. (2015). Spores viability was confirmed as >95% and used to inoculate single budded sets of each genotype (60 buds of each genotype, inoculated and mock-inoculated, in triplicates with 10 buds each). Before inoculation, plants were surface disinfected following the same process described by Taniguti et al. (2015). Puncture method was used for inoculation (10^7 teliospores. mL-1 in saline solution - NaCl 0.85M). Mock-inoculated plants were prepared with saline solution (control plants). Samplings were made from buds 48 hours post-inoculation. All samples were frozen in liquid nitrogen immediately after collection and stored at -80°C. Infected plants were compared to control samples of the same age. No special permits were necessary for teliospores and genotypes used, because this project was developed in collaboration with Granbio and IAC-Centro de Cana researchers.

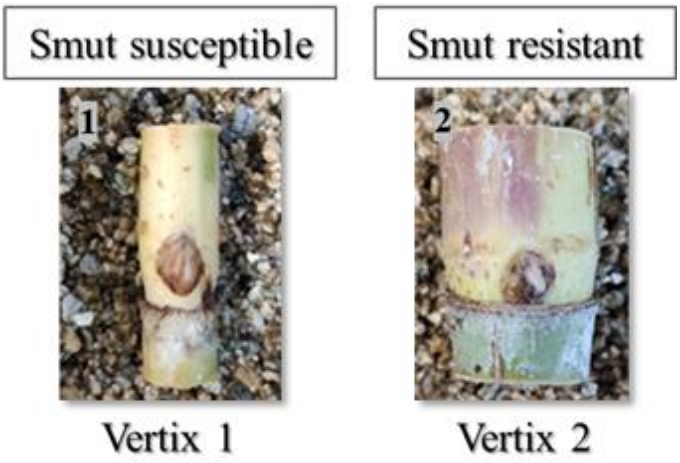

Figure 5. Energy cane collected from 10 months old plants from genotypes: 1) Vertix 1 (smut susceptible genotype) and 2) Vertix 2 (resistant genotype).

2.2 DNA extraction and quantification of *S. scitamineum* DNA

Real-time qPCR was used to confirm and quantify *S. scitamineum* infection in each biological replicate. CTAB method was used for DNA extraction (DOYLE; DOYLE, 1990). qPCRs were made using as target the ribosomal Intergenic Spacer region (IGS) from *S. scitamineum* genome (PETERS, 2016). Reactions consisted of 100 ng of total DNA, 0.2 µM of each primer, and 1× LuminoCt SYBR Green qPCR ReadyMix (Sigma-Aldrich), in a total volume of 12.5 µL. Cycling parameters were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. All reactions were run in an ABI 7500 Fast real-time PCR detection system (Applied Biosystems) in technical duplicates. Fluorescence (520 nm) was detected at the end of the elongation phase for each cycle. To evaluate amplification specificity, melt curve analysis was performed at the end of each PCR run. The quantity of *S. scitamineum* DNA in each sample was determined by absolute quantification based on a standard curve obtained using DNA extracted from mixed cultures of *S. scitamineum* SSC04A and *S. scitamineum* SSC04B isolate. Quantifications were statistically analyzed using t-test (p-value < 0.05).

2.3 RNA extraction, library preparation and sequencing

Total RNA was extracted from the samples as described by Taniguti et al. (2015). The quality of the total RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA), and the libraries were constructed using a “TruSeq Stranded mRNA”

Illumina kit as described in the manufacturer's instructions (Illumina, San Diego, CA). The libraries were paired-end sequenced using the NextSeq Illumina system.

2.4 Pre-processing, mapping the Illumina reads, and differentially expressed genes

These analyses were performed by the bioinformatics team coordinated by Dr. Hugo Rody (FAPESP 2016/17545-8) of the Genomics Group. The Illumina reads were treated as previously described (Taniguti *et al.*, 2015). The reference genome sequence used for mapping the RNAseq data was the complete set of predicted genes (83,826) of the tetraploid genome of *S. spontaneum* AP85-441 (ZHANG et al., 2018). Shortly, a total of 1,21,926,287 paired-end raw reads (an average of 10,000,000 reads per treatment per replicate) were firstly checked using the FastQC v0.11.5 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adaptors in reads were filtered out using the Cutadapt v1.18 software (MARTIN, 2011). Only reads with no N bases, length > 20 bp, and average Q > 20 were kept (99% of all reads). Reads passing our previous quality and trimming steps were mapped against the obtained reference genome of *S. spontaneum* using the HISAT2 v2.1.0 software (KIM; LANGMEAD; SALZBERG, 2015) with default parameters. Of all bases, 87,6% were aligned to the *S. spontaneum* predicted genes and the information used for further analysis. FeatureCounts software, from Subread package (LIAO; SMYTH; SHI, 2014) was used to parse the HISAT2 mapping BAM outputs and obtain the mapped reads counting tables. The EdgeR software from the Bioconductor package (ROBINSON; MCCARTHY; SMYTH, 2010) was used to identify the Differentially Expressed Genes (DEGs). Only genes having CPM (counts per millions) values greater than one in the three biological replicates were considered as expressed. DEGs were considered statistically significant if FDR (false discovery rate) < 0.05 and were represented as values of a Log2 Fold Change (inoculated/control). Because the genome reference used in our analysis was tetraploid, where one locus may express four different alleles, we from now on in the document use genes and CDSs (coding sequences) as interchangeable names to include all the alleles. A total of 41,701 CDSs expressed in the susceptible genotype corresponding to close 50% of all the CDSs belonging to the tetraploid (83,826) following criteria described above. A total of 9,960 were considered DEGs (FDR<0,05). A total of 9,960 were considered DEGs (FDR<0,05). 43,205 CDSs were expressed for the resistant genotype, and 2,219 considered

DEGs. We proceed with the analysis of ROS metabolism and related processes from these data.

2.5 *S. spontaneum* GO annotation

A database of CDSs (83,826) from tetraploid *S. spontaneum* previously annotated with GO annotated and inferred with BLASTP, where sequences passing the E-value cutoff of e-05, having a minimum of 40% of identity and 80% of query coverage were declared as orthologs (SsponGO dataset). This database was used in search of terms selected in item **2.6** and presented in Table 2 (in item **3.2** and in **Supplementary Table 1**) and DEGs of both Vertix 1 and Vertix 2 experiments in item **2.4**.

2.6 ROS related GO terms dataset

We only considered DEGs *S. spontaneum* CDSs related to ROS to downstream analysis. First, we manually curated a list of Gene Ontology (GO) terms related to ROS, having as a starting point a search with GO term of "GO:0000302 response to reactive oxygen species" on QuickGO website, which is part of EMBL-EBI Institute (https://www.ebi.ac.uk/QuickGO/). Then, we further filtered child GO terms possible for plant systems. GO is composed of three ontologies: Biological Process (BP), Molecular Functions (MF), and Cellular Components (CC). However, we used only the BP ontology (Figure 6).

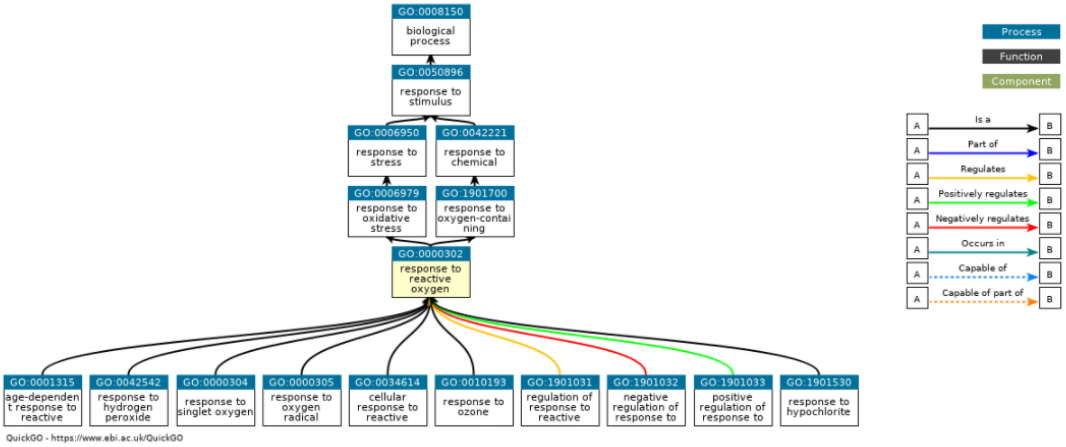

Figure 6. Biological Process Ontology for GO terms related to GO:0000302 response to reactive oxygen species (parents and childs) from QuickGO website, which is part of EMBL-EBI Institute (https://www.ebi.ac.uk/QuickGO/).

2.7 Overview: Material and Methods procedures

We present in Figure 7 the final pipeline used in this work, including the experimental design and procedures, sequencing, selection of DEGs, selection of GO terms, and final results.

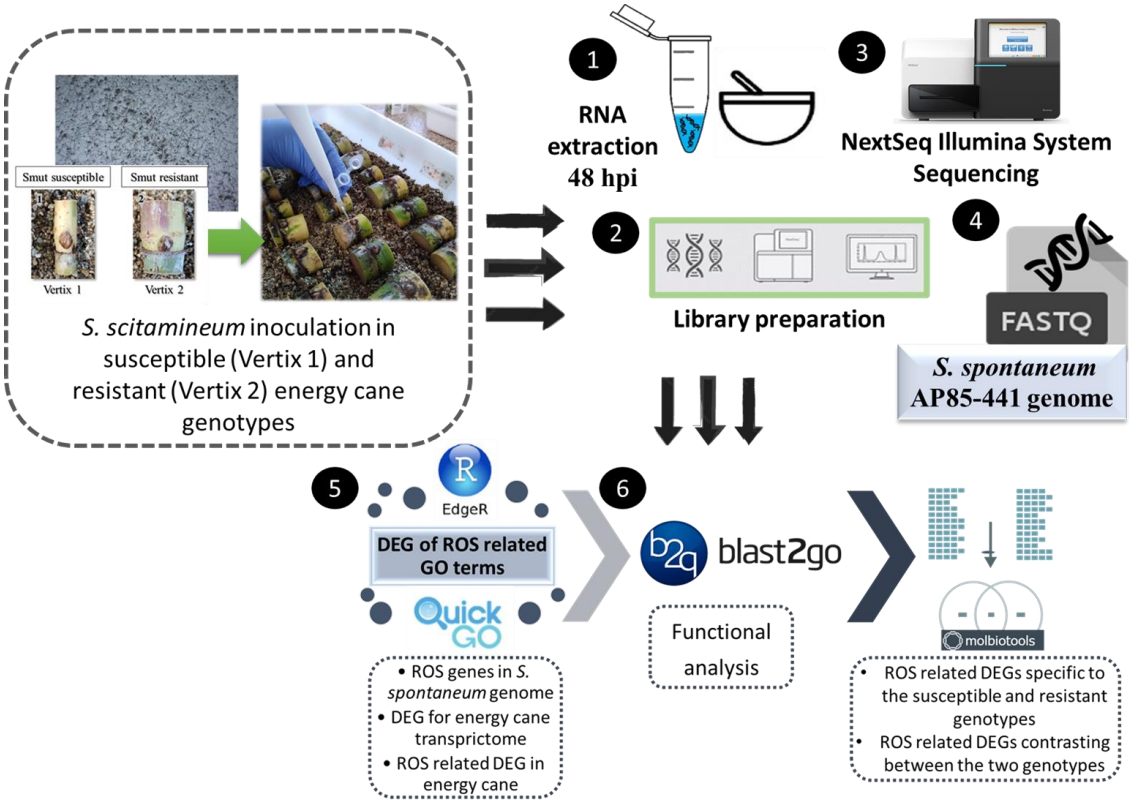

Figure 7. Workflow for biological material preparation and bioinformatics data analysis methods.

3. Results

3.1 Fungal quantification *in planta* by qPCR

To identify and quantify the presence of *S. scitamineum* in infected sugarcane, we used the qPCR protocol designed in this study for bud-infected fungus. The results showed the S. scitamineum presence in the inoculated Vertix 1 and Vertix 2 experiments, at 48hpi, as required. Furthermore, we determined that there are no significant differences between susceptible (Vertix1) and resistant (Vertix2) plants regarding fungal infection (Figure 8).

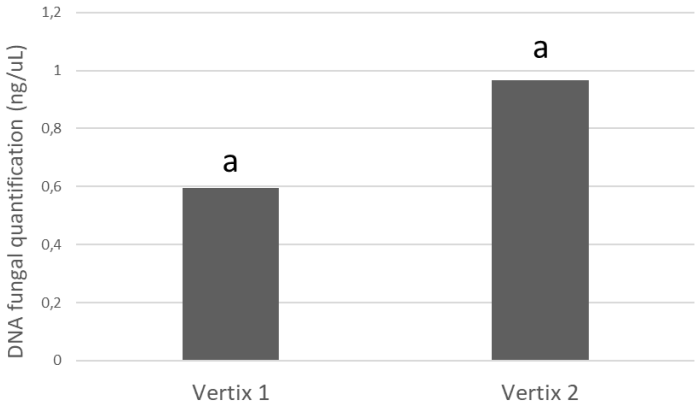

Figure 8. DNA quantify (ng) of *S. scitamineum* assessed by qPCR using IGS primer pair. A) Infected smut-susceptible (Vertix1) and -resistant (Vertix2) energy cane genotype at 48hpi (hours post-inoculation). B) Infected smut-susceptible (Vertix1) and -resistant (Vertix2) energy cane genotype at 72 hpi (hours post-inoculation). Values of DNA quantify represent the means from three biological replicates. Means with the same letters are not significantly different (P≤0.05) by Test-t Student analysis.

3.2 Overview of ROS related GO terms dataset

We identified 7,209 CDSs (coding sequence) in the SsponGO dataset of the *S. spontaneum* tetraploid genome (83,826 CDSs), where GO terms related to ROS metabolism (Table 2) were assigned. The sequence of these CDSs submitted to Blast2GO considering only functions described in monocots predicted 1,683 different functions. Within this group of CDSs, 5,130 terms assigned were within the three ontologies (Biological Processes; Molecular Functions; Cellular Components). From these, five terms were assigned to 639 genes within Molecular Functions, including 335 with the term GO:0050896 (Response to Stimulus) (Table 3). All others were metabolic processes modulated by ROS, where transcription regulation of events related to ROS included 179 transcription regulators.

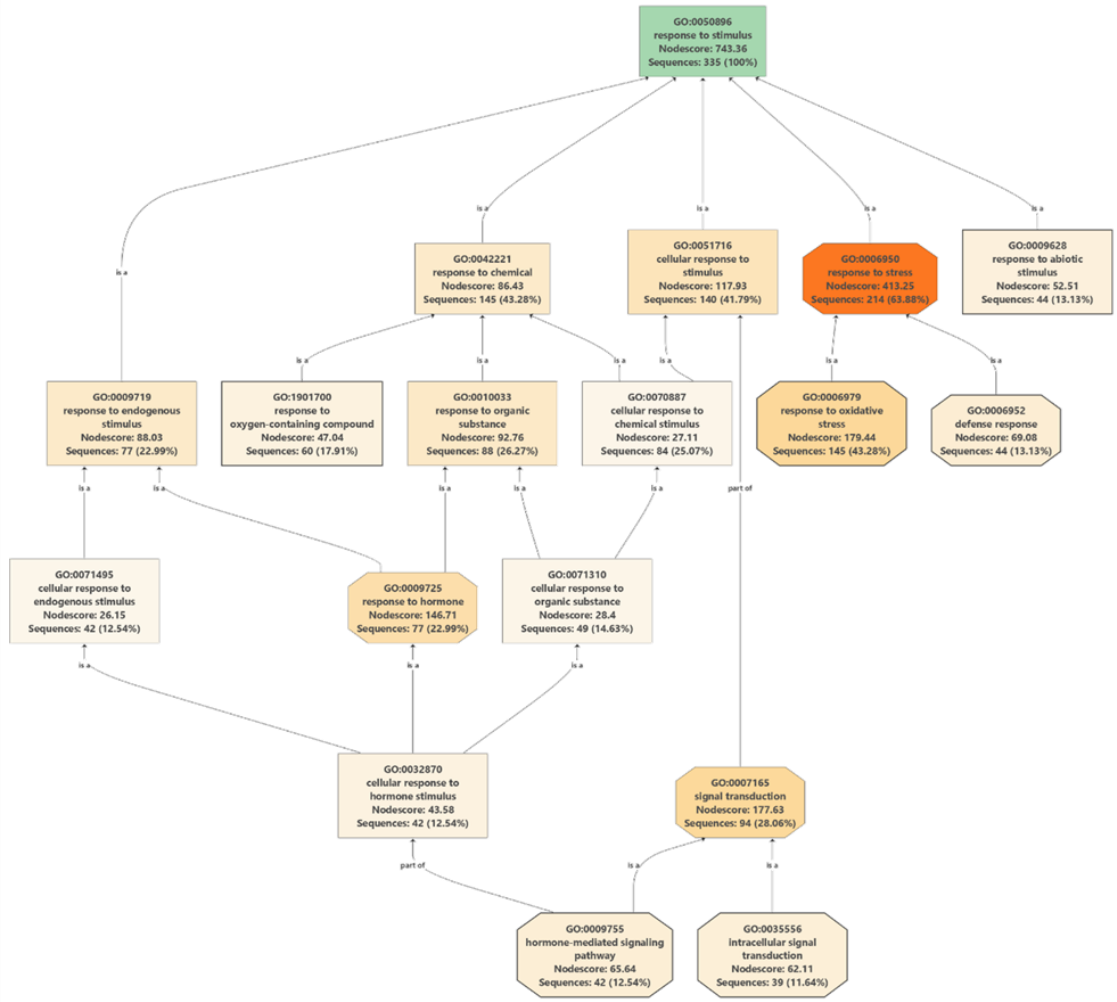

Figure 9. Gene ontology Biological Processes of all GO terms related to ROS and response to stimulus. (Blast2GO analysis of terms defined for monocots).

Table 2. List of Gene Ontology (GO) terms (Child Terms) related to ROS. Starting point a search: "GO:0000302 response to reactive oxygen species" GO term on QuickGO website, which is part of EMBL-EBI Institute (https://www.ebi.ac.uk/QuickGO/).

GO:0000302	**Response to reactive oxygen species**		**Biological Process**
Child Term			
GO Terms	**Name**	**Anotation**	**Relationship to GO:0000302**
GO:0000304	response to singlet oxygen	Biological process	is_a
GO:0042542	response to hydrogen peroxide	Biological process	is_a
GO:1901032	negative regulation of response to reactive oxygen species	Biological process	negatively_regulation
GO:1901031	regulation of response to reactive oxygen species	Biological process	Regulation
GO:1901033	positive regulation of response to reactive oxygen species	Biological process	positively_regulation
GO:0001315	age-dependent response to reactive oxygen species	Biological process	is_a
GO:0000305	response to oxygen radical	Biological process	is_a
GO:0034614	cellular response to reactive oxygen species	Biological process	is_a

Table 3. Terms (child and parents) of GO:0050896 defined for total sequences directly involved with ROS metabolism in the genome of *S. spontaneum*.

Level	GO ID	GO Name	Parents (ACC)	Parents (Name)	#Seq
1	GO:0050896	response to stimulus			335
2	GO:0042221	response to chemical	GO:0050896	response to stimulus	145
2	GO:0009628	response to abiotic stimulus	GO:0050896	response to stimulus	44
2	GO:0006950	response to stress	GO:0050896	response to stimulus	214
2	GO:0009719	response to endogenous stimulus	GO:0050896	response to stimulus	77
2	GO:0051716	cellular response to stimulus	GO:0050896	response to stimulus	140
3	GO:0007165	signal transduction	GO:0051716	cellular response to stimulus	94
3	GO:0070887	cellular response to chemical stimulus	GO:0042221, GO:0051716	response to chemical, cellular response to stimulus	84
3	GO:0010033	response to organic substance	GO:0042221	response to chemical	88
3	GO:0006952	**defense response**	GO:0006950	response to stress	44
3	GO:1901700	response to oxygen-containing compound	GO:0042221	response to chemical	60
3	GO:0009725	**response to hormone**	GO:0009719, GO:0010033	response to endogenous stimulus, response to organic substance	77
3	GO:0071495	cellular response to endogenous stimulus	GO:0009719	response to endogenous stimulus	42
3	GO:0006979	**response to oxidative stress**	GO:0006950	response to stress	145
4	GO:0035556	**intracellular signal transduction**	GO:0007165	signal transduction	39
4	GO:0032870	**cellular response to hormone stimulus**	GO:0009725, GO:0071310, GO:0071495	response to hormone, cellular response to organic substance, cellular response to endogenous stimulus	42
4	GO:0009755	**hormone-mediated signaling pathway**	GO:0007165, GO:0032870	signal transduction, cellular response to hormone stimulus	42
4	GO:0071310	cellular response to organic substance	GO:0070887, GO:0010033	cellular response to chemical stimulus, response to organic substance	49

3.3 Differential expression analysis and functional categorization

From the RNAseq data, we selected only CDSs of the experiments (Vertix 1: inoculated/control; Vertix 2: inoculated/control), where the GO terms assigned were related to ROS according to Table 2. We count all the alleles DEs of each locus with a function described for monocots. Of the Vertix 1 experiment, 1,286 DEs fitted the conditions, and for the Vertix 2, only 263. To improve our understanding of the differences detected between the two experiments, we constructed a Venn diagram (Figure 10) based on the gene and alleles names (for example, Sspon.07G0002670-2B and Sspon.07G0002670-3C are one gene and two alleles DEs).

The results showed 47 coding sequences (CDSs) shared between the two experiments. We then analyzed the expression of each CDS, building a heatmap (Figure 11). Most of the CDSs followed the same expression pattern in both experiments, which means that those genes behaved up or downregulated in both genotypes, resistant and susceptible when inoculated with smut compared to their respective controls. Only 15 CDSs had contrasting expressions, of which ten downregulated the expression and five upregulated in the resistant genotype (Table 4). Relevant to mention an ortholog of the calmodulin-binding transcription activator 3, a transcription activator biotic defense responses, upregulated, and a peroxidase 43-like downregulated in the resistant genotype.

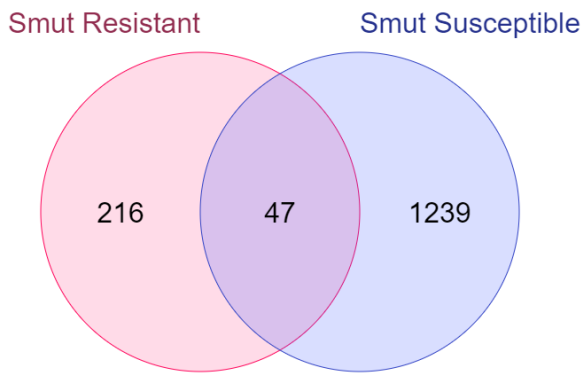

Figure 10. Venn diagram between differentially expressed genes (DEs) of Vertix 1 (smut-susceptible) and Vertix 2 (smut-resistant) RNAseq experiments with GO terms related to Reactive Oxygen Species (ROS). The digram was built with https://molbiotools.com/listcompare.php.

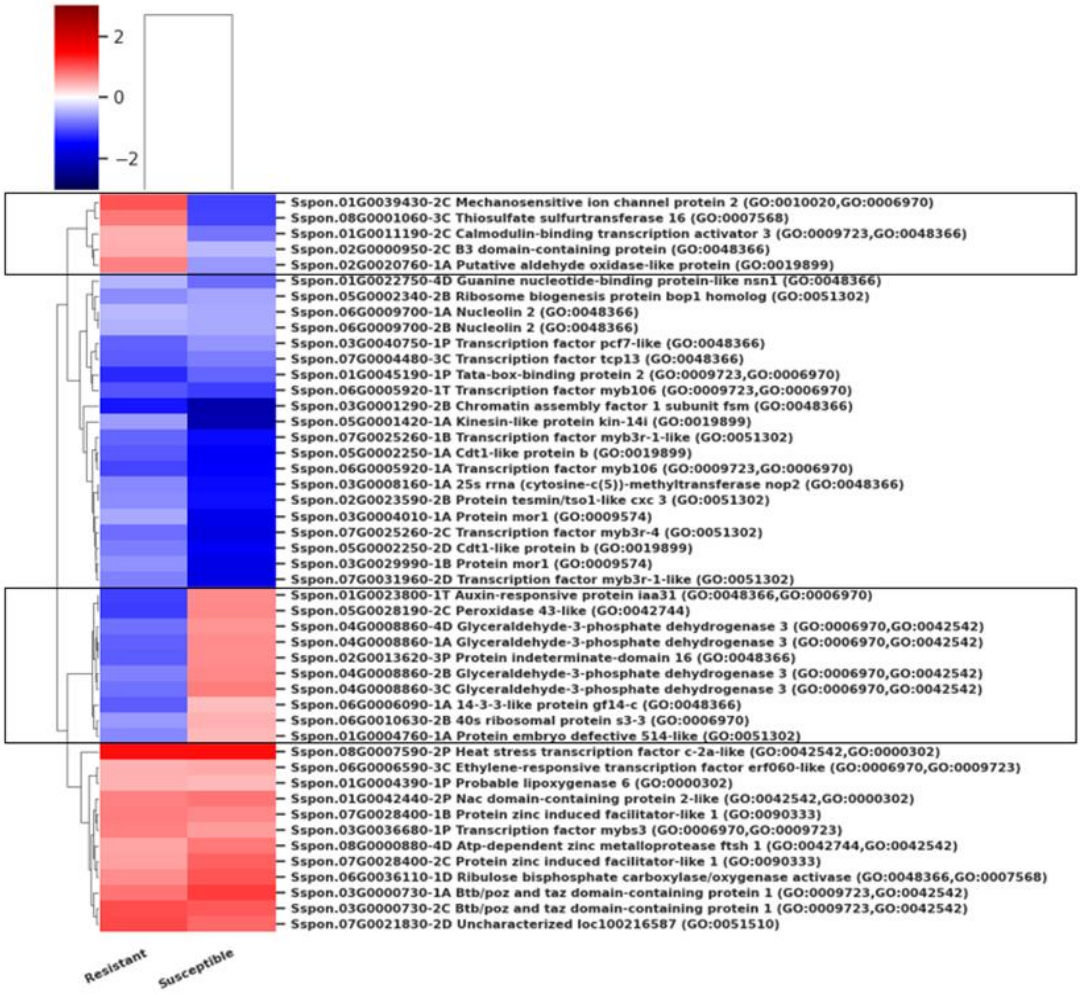

Figure 11. Clustered heatmap of differentially expressed genes (DEs) shared between the Vertix 1 and Vertix 2 RNAseq experiments with GO terms related to Reactive Oxygen Species (ROS). The black frame means the genes with contrasting expression profiles between Vertix1 and Vertix2. The clustermap was obtained with Python3 and the seaborn library (https://seaborn.pydata.org/generated/seaborn.clustermap.html).

Table 4. Genes differentially expressed (Inoculated X Control) found in both resistant and susceptible plants with contrasting expression profiles with functions related to ROS (see Figure 11).

SeqName	Description	Expression	Functional Category
Sspon.06G0006090-1A	14-3-3-like protein GF14-C	↓ resistant	Negative Regulator of flowering
Sspon.06G0010630-2B	40S ribosomal protein S3-3	↓ resistant	Translation
Sspon.01G0023800-1T	auxin-responsive protein IAA31	↓ resistant	Auxin-activated signaling pathway
Sspon.04G0008860-1A	glyceraldehyde-3-phosphate dehydrogenase 3	↓ resistant	Glucose metabolic process
Sspon.04G0008860-2B	glyceraldehyde-3-phosphate dehydrogenase 3	↓ resistant	Glucose metabolic process
Sspon.04G0008860-3C	glyceraldehyde-3-phosphate dehydrogenase 3	↓ resistant	Glucose metabolic process
Sspon.04G0008860-4D	glyceraldehyde-3-phosphate dehydrogenase 3	↓ resistant	Glucose metabolic process
Sspon.05G0028190-2C	**peroxidase 43-like**	**↓ resistant**	**Response to oxidative stress**
Sspon.01G0004760-1A	protein EMBRYO DEFECTIVE 514-like	↓ resistant	Nucleologenesis
Sspon.02G0013620-3P	protein indeterminate-domain 16	↓ resistant	Regulation of transcription (morphogenesis)
Sspon.02G0000950-2C	B3 domain-containing protein	↑ resistant	Repressor of the sugar-inducible genes
Sspon.01G0011190-2C	**calmodulin-binding transcription activator 3**	**↑ resistant**	**Transcription activator biotic defense responses**
Sspon.01G0039430-2C	mechanosensitive ion channel protein 2	↑ resistant	Cell division
Sspon.02G0020760-1A	putative aldehyde oxidase-like protein	↑ resistant	Non-specific oxidoreductase activity
Sspon.08G0001060-3C	thiosulfate sulfurtransferase 16	↑ resistant	Thiosulfate sulfurtransferase activity

Expression of specific CDS in resistant and susceptible genotypes

The resistant genotype-specific CDSs (216) were also submitted to Blast2GO, only considering defined monocot functions, resulting in 154 annotated CDSs. Within this group of genes, 537 terms assigned were within the three ontologies (Biological Processes; Molecular Functions; Cellular Components). Besides genes encoding proteins directly related to ROS metabolism, were proteins responsive to oxidative stress, such as sugar metabolism, auxin-responsive proteins, proteases, and phosphatases. For the susceptible genotype experiment, 115 CDSs were DE with log2FC greater than one fold (1,238 DE, FDR >0.05; Figure 10).

We sorted the specific DEGs to Vertix 1 and Vertix 2 experiments according to categories: Sugar metabolism; General replication, Replication, Transcription, and

Translation; Meristem functions; Hormone-related; Defense response and Cell cycle. We chose these functional categories to sort our DEGs based on previous studies of the sugarcane-smut interaction (SHACKER et al., 2016). Then, we manually curated the annotations considering these seven major function categories and the expression of the respective CDSs (Figure 12).

Within specific CDSs expressed of Vertix 1 were the plant NADPH oxidase (NOX) respiratory burst oxidase homologs (RBOHs) and various proteins related to controlling the pathogen, such as chitinases and proteases (Figure 17). On the other hand, Vertix 2, besides chitinases and proteases in a more significant proportion, induced catalases (CATs). Resistant plants also repressed superoxide dismutases (SOD) (Figure 17). One significant result is the downregulation of a calreticuli. Modulation of meristem and flowering functions were specifically active in Vertix 2. We identified 17 CDSs related mainly to control meristem identity and proliferation (Figure 12).

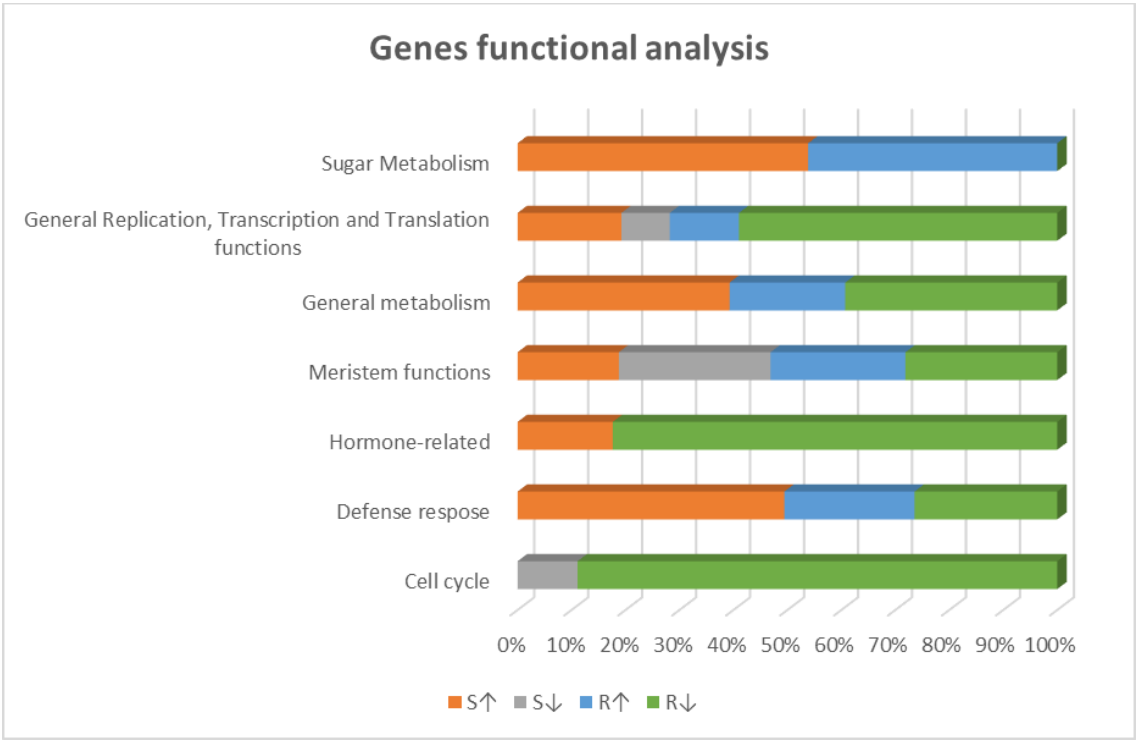

Figure 12. Major function categories grouping specific CDSs differentially expressed (FDR >0,05) of RNAseq experiments with the susceptible (S) or resistant (R) genotypes, up ↑ or ↓ down regulated.

The Vertix 1 specific defense response (43 CDSs), involving the following sub-categories: ROS metabolism modulation; Chaperones; PR-proteins; Negative regulation of programmed cell death (Figure 13), were all upregulated. Conversely, Vertix 2 showed 22 defense response DEGs downregulated and 20 upregulated, sorted in ROS metabolism modulation; Chaperones; PR-proteins; Negative regulation of programmed cell death; Signaling (Figure 14) (Supplementary Figures: 1, 2, 3).

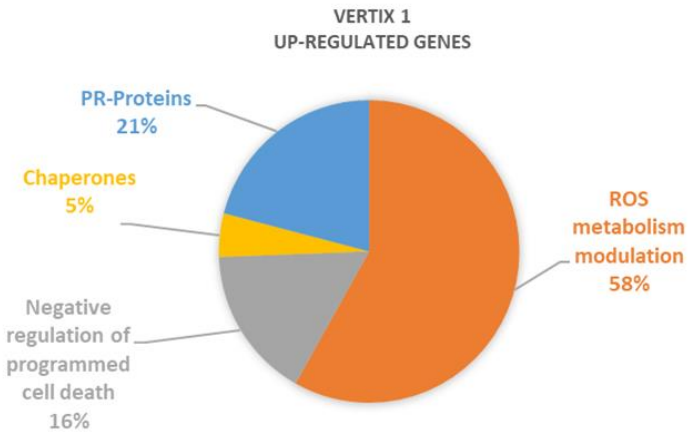

Figure 13. Percentage of genes distributed in for categories related to Response to Stimulus, in susceptible genotype (Vertix 1).

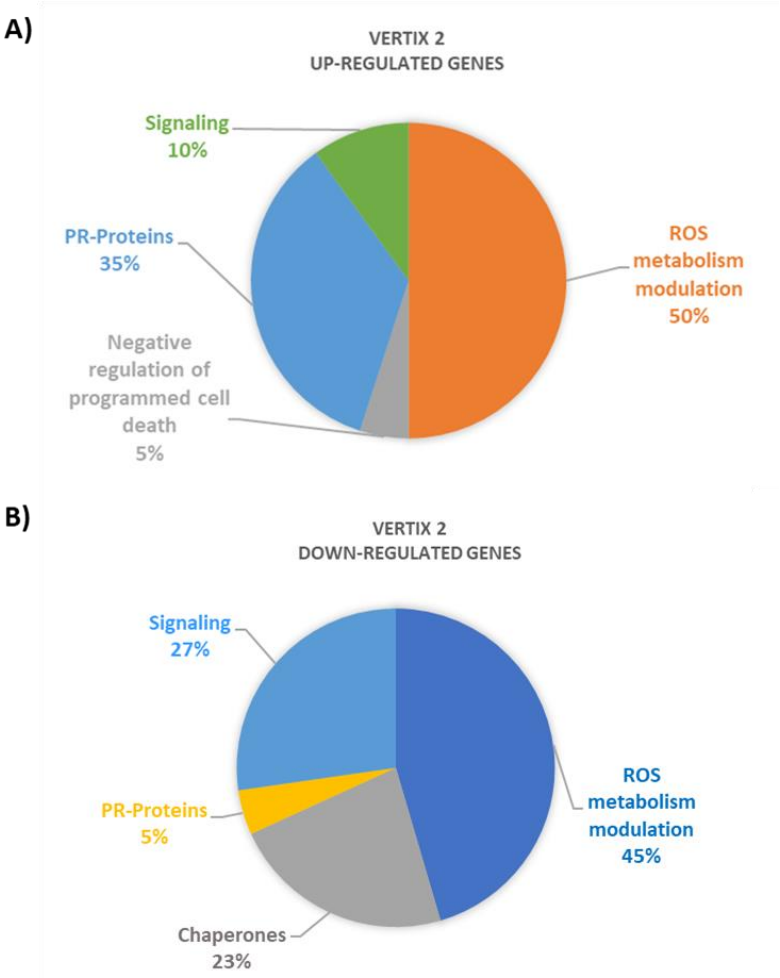

Figure 14. Percentage of genes distributed in for categories related to Response to Stimulus, in resistant genotype (Vertix 2).

Data showed that specific DEGs of the susceptible genotype have more upregulated defense genes than the resistant genotype and, in contrast to the resistant variety, none of the DEGs analyzed in the susceptible variety showed downregulation at 48hpi (Figure 15).

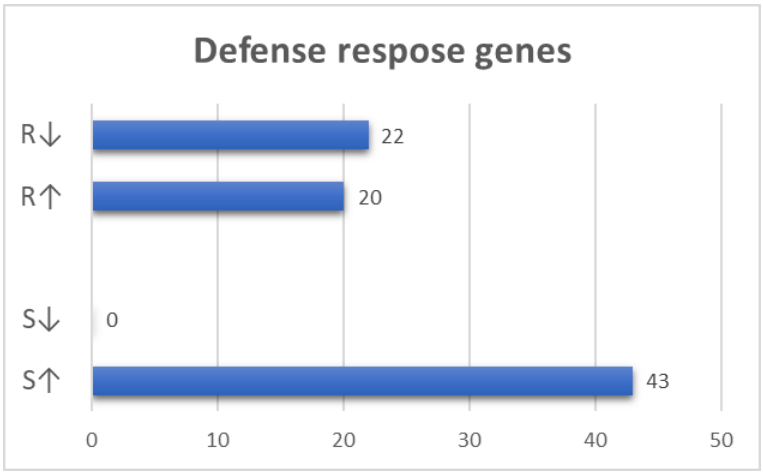

Figure 15. Percentage of genes related to defense response upregulated and downregulated, in susceptible (Vertix 1) and resistant genotype (Vertix 2).

Among the defense response DEGs we selected the ROS metabolism modulation sub-category to identify functional patterns observed in energy cane-smut interaction at 48hpi (Supplementary Figures: 5, 6). The results indicated a higher number of upregulated ROS-related functions in the susceptible genotype (25 genes) compared to the resistant genotype (10 genes), while no DEGs were observed downregulated in the susceptible, only in the resistant with 10 downregulated genes (Figure 16).

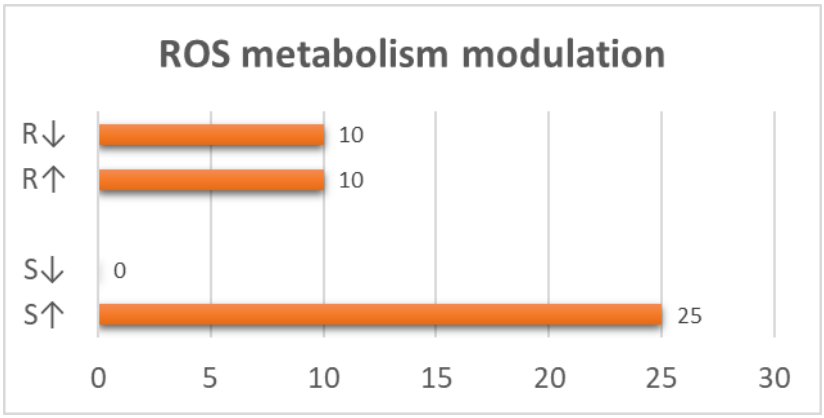

Figure 16. Percentage of genes related to ROS metabolism modulation upregulated and downregulated, in susceptible (Vertix 1) and resistant genotype (Vertix 2).

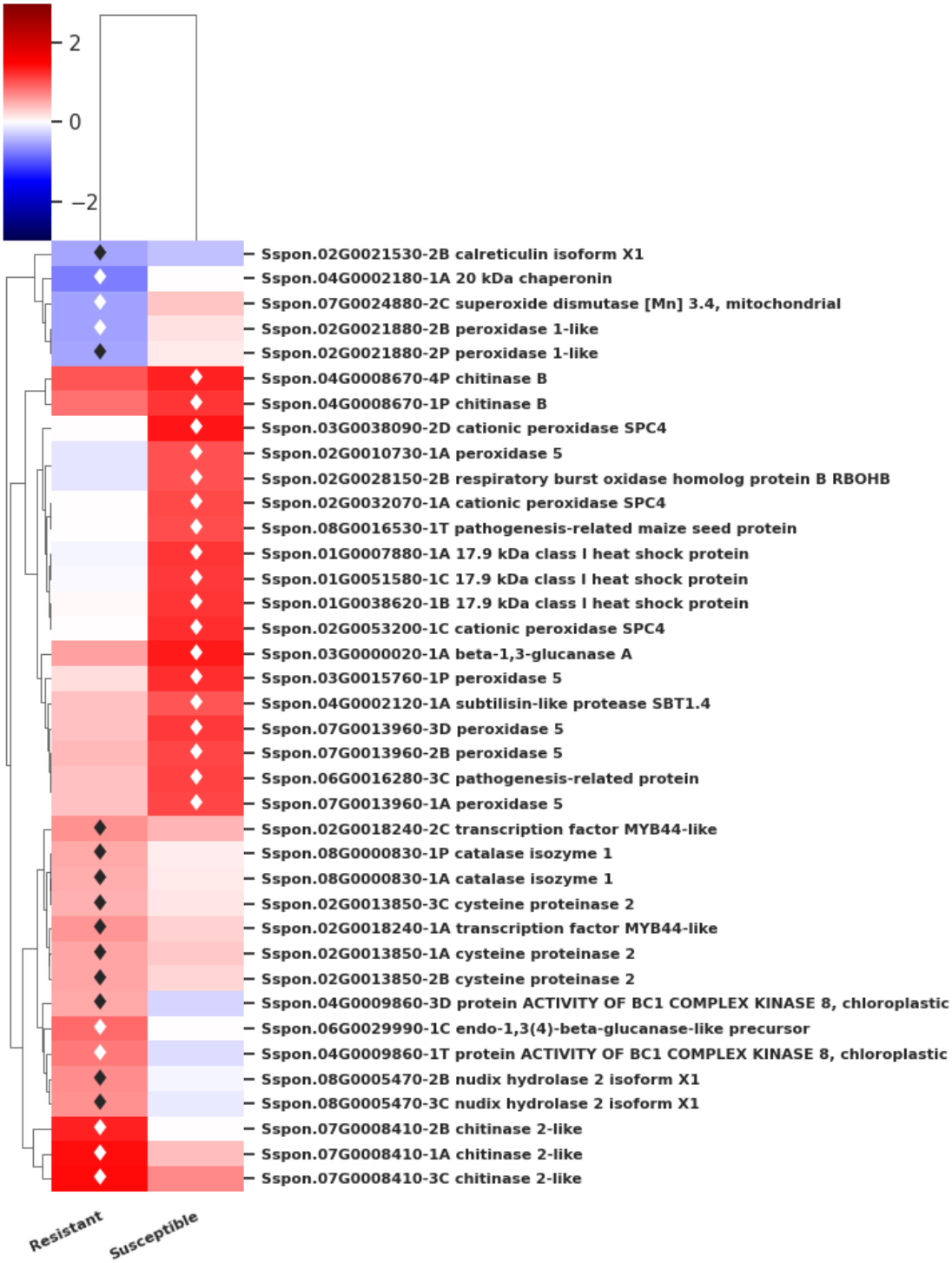

Figure 17. Clustered heatmap of differentially expressed genes (DEs) specific to Vertix 1 and Vertix 2 RNAseq experiments with GO terms related to Reactive Oxygen Species (ROS). The black and white diamonds represent the differential expression genes in Vertix1 or Vertix2 genotypes. The clustermap was obtained with Python3 and the seaborn library (https://seaborn.pydata.org/generated/seaborn.clustermap.html).

4. Discussion

We used RNAseq experiments with four treatments and three replicates, consisting of two energy cane genotypes (smut-resistant and -susceptible), inoculated and mock-inoculated to produce an expression profile of ROS metabolism-related. To investigate putative functions related to ROS we selected GO terms and searched the DEGs results. The experiments produced a higher DEGs number when susceptible plants were inoculated with *S. scitamineum.* Surprisingly, we detected the induction of the respiratory burst oxidase homolog protein B (RBOHB)- NADPH oxidase- (Sspon.02G0028150-2B), which is involved in the massive phase II oxidative burst induced by pathogen infection. Earlier work has shown that RBOH is a main component in ROS production during biotic stresses and was first studied in rice (GROOM et al., 1996). This is a plasma membrane enzyme predicted to have six transmembrane-spanning domains carrying an N-terminal extension comprising two EF-hand motifs, suggesting that Ca2+ regulates its activity (MARINO et al., 2011). We expected to see the activity of RBOH in resistant plants from 48 hours and on reaching its peak at 72 hours post-inoculation. This is the time-point determined by Peters et al. (2017) in a time-course experiment with resistant and susceptible sugarcane plants of genotypes SP80-3280 and IAC66-6, respectively. Sugarcane plants of the IAC66-6 did not induce the production of hydrogen peroxide in the experiments run by Peters et al. (2017). Differently, based on expression data, the susceptible energy cane genotype Vertix 1, seems to induce an oxidative burst and therefore have the pathogen perception. We suspect that in the resistant energy cane genotype, the burst is earlier than the described for the SP80-3280. Experiments of time-course and proving the presence of peroxide should provide a better overview of the role of RBOH as a candidate for priming the plant immune system in cane energy. To detoxify hydrogen peroxide, which has its entry facilitated by various aquaporins (also upregulated in Vertix 1) (Sspon.04G0006710-1P; Sspon.02G0019440-1A; Sspon.02G0019440-2B; Sspon.02G0019440-3C; Sspon.02G0019440-4D), plant cells produce peroxidases to cope directly with it. In addition, to cope indirectly with ROS, were identified chaperones (Sspon.04G0008980-1A), associated with protecting molecules against oxidation directed by misfolding; lipocalin (Sspon.04G0008400-3D; Sspon.04G0008400-1A; Sspon.04G0008400-2C; Sspon.06G0005730), to prevent lipid peroxidation; and negative regulation factors of programmed cell death (Sspon.06G0025280-2C - Vertix1; Sspon.04G0016560 - Vertix2) (recently review by DUMANOVIĆ et al., 2021). The production of ROS inducing an enzymatic antioxidant system to protect cell macromolecules against oxidative damage was

induced by Vertix 1 in the presence of the pathogen. The expression profile of other candidate genes goes along with this hypothesis, suggesting that the Vertix 1 indeed perceives the presence of the pathogen. Known genes encoding for proteins to contain fungal pathogens such as chitinases, $\boldsymbol{\beta}$-1,3-glucanases, proteases, and other proteins with antifungal activity (JASHNI et al., 2015) were also upregulated in the susceptible genotype (Sspon.03G0000020-1A; Sspon.04G0008670; Sspon.08G0016530-1T; Sspon.06G0016280; Sspon.04G0002120-1A).

The expression profile suggests a moment further perception of the pathogen for the resistant genotype. Besides the induction of genes encoding genes to cope with the oxidative burst (catalase, $\boldsymbol{\beta}$-1,3-glucanases, proteases, and other proteins with antifungal activity) with a different pattern (different genes and alleles), it is relevant the expression of proteins involved in signal transduction. A high-affinity Ca2+-binding protein, calreticulin, functions as a secondary messenger regulating plant defense against biotrophic pathogens (QIU et al., 2012). In Vertix 2, one allele of locus Sspon.02G0021530 with repressed expression encoded one isoform of calreticulin (calreticulin isoform X1). Studies in Arabidopsis showed that calreticulin isoforms have variant responses considering the immune system. While *atcrt1* and *atcrt2* mutant plants are more resistant to the *Pseudomonas syringe* DC3000 infection than wild-type plants, *atcrt3* mutants are more sensitive (QIU et al., 2012). More recently, Pröbsting et al. (2020) showed that the loss of a calreticulin function (*CRT1a)* resulted in activation of the ethylene signaling pathway, contributing to reduced susceptibility of *Brassica napus* towards *Verticillium longisporum* (*Vl43*).

Another remarkable CDS, upregulated in the resistant genotype, encodes a member of the MYB transcription factors family (TF), the MYB44 (Sspon.02G0018240). MYBs are TFs involved in plant development and defense responses, including cell cycle, cell morphogenesis, central circadian oscillator, and regulation of stress signaling (CAARLS et al., 2015). Along with TF families WRKY and MADS-box, MYBs activate unique cellular-level abiotic and biotic stress-responsive strategies, which play determinant roles in defense and developmental plant processes (CAARLS et al., 2015; TSUDA; SOMSSICH, 2015). More specifically, MYBs and WRKYs in Arabidopsis modulate an antagonistic interaction between salicylic acid and jasmonic acid signaling. The over-expression of *AtMYB44* down-regulated defense responses against the necrotrophic pathogen *Alternaria brassicicola*, whereas upregulated *WRKY70* and *PR* genes, leading to enhanced resistance to the biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (SHIM et al., 2013; ZOU et al., 2013).

MYB44 and WRK70 have also been implicated in the initial cross-talk communication during *Plasmopara viticola* and *Vitis vinifera* leading to grapevine resistance (GUERREIRO et al., 2016).

Smuts are biotrophic fungi that mostly use host floral structures as their reproduction site, modulating the flowering pathway positively or negatively depending on the host species (GLASSOP et al., 2014; FAN et al., 2016; SCHMITZ et al., 2018). For instance, in rice, plants infected with *Ustilaginoidea virens* downregulation of flowering-related genes, whereas in maize plants, *Ustilago maydis* induces flowering-related genes (BREFORT et al., 2009; FAN et al., 2016; SCHMITZ et al., 2018). Previously, we proposed that *S. scitamineum* modulates meristem functions upon infection (SHACKER et al., 2016), repressing vegetative to flowering transition upregulated in resistant sugarcane plants (SP80-3280) in early stages after inoculation (48 hai, ongoing experiments). In agreement, we also identified a larger number of CDSs up or downregulated in resistant energy cane related to meristem and flowering functions than in the susceptible genotypes. One of the most significant results is the transcription factors repression of the PCF (Proliferating Cell Factors) family (Sspon.01G0029720-1A; Sspon.03G0040750 (2 alleles); Sspon.07G0004480-1P; Sspon.03G0040750-2D). PCFs are proteins identified in rice, part of a family unique to plants, the TCP transcription factor family named after the teosinte branched 1 (**t**b1, *Zea mays* (Maize), **c**ycloidea (cyc) (*Antirrhinum majus*, Garden snapdragon), and **P**CF in rice (*Oryza sativa*) (Transcription factor, TCP IPR005333). These transcription factors play a significant role in plant developmental regulation (cell proliferation and differentiation, branching, leaf development, floral morphology, circadian clock regulation, seed germination, mitochondrial biogenesis, and hormone signaling). PCFs and 14-3-3 proteins are interactors of FT-like proteins (Flowering Locus T), which control the floral transition (JAEGER; WIGGE, 2007). Our data also revealed 14-3-3 CDSs (Sspon.06G0006090, three alleles) downregulated in Vertix 2 experiment. These data suggest that the control of meristematic functions may be a defense mechanism against the development symptoms of smut disease.

In summary, we suggest that the gene expression modulation upon infection of *S. scitamineum* in energy cane genotypes used is, in general, earlier than previously observed for sugarcane SP80-3280 and IAC66-6. In addition, the pathogen's perception is much more robust in Vertix1 than IAC66-6. In all cases for energy cane, we observed modulation of genes related to ROS metabolism, however, with different intensities and functions. Besides, we suspect that, as a mechanism related to the defense response in resistant genotypes, the

plants alter meristem functions, repressing phase transition. Calreticulin is an interesting candidate for involvement in plant resistance and also the transcription factor 44 of an MYB family, associated with the cross-talking of defense mechanisms mediated by the hormones salicylic acid and jasmonate.

Although, the conclusions taken within this study were based on the genes and alleles expressed using the *S. spontaneum* genome as a reference. Vertix 1 and Vertix 2 are genetic hybrids with 50% of the *S. spontaneum* genome. Nonetheless, so far, there is no better reference for analysis such as ours. We intend to further define a reference for Vertix 1 and Vertix 2 by a *de novo* assembly with all the sequence reads we generate. It is also relevant to validate the expression profiles of all the genes described here by using RT-qPCR. All of which are ongoing projects.

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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.

1. Introduction

Sugarcane is a widely spread crop in several countries, and especially in Brazil, with high relevance in the economy and agribusiness. Brazil stands out as the world's largest producer of sugarcane and has a large share in producing renewable biofuels, such as ethanol, contributing to the reduction of pollutants generated from energy production. The pursuit of more sustainable production systems for the environment highlights the vast potential of sugarcane cultivation for this purpose, uniting economic and social development (MATSUOKA et al., 2014; NEVES et al., 2011; SILVEIRA et al., 2014).

Given these concepts, a Brazilian private company, GranBio Investimentos SA, developed energy cane varieties named Vertix – Vx, with higher fiber content and lower sucrose content, characteristics regarded as ideal for energy-producing varieties. However, some diseases can impair their productivity, including the smut disease, caused by the fungus

Sporisorium scitamineum. Among abiotic stresses, drought is the most relevant to reducing sugarcane productivity. In previous studies, the company observed some susceptibility of Vertix 1 to interaction with *S. scitamineum*, as well as intolerance to drought. In the case of Vx2, resistance to smut disease, high number of rhizomes, and excellent sprouting of ratoons were noted.[3]

The drought effects on plants include reducing CO_2 assimilation, stomatal conductance, plant water potential, transpiration, leaf cell size, and growth. Also, the water deficit affects productivity influencing the development and the final height of the stalks (LAWLOR, 2013; SUGIHARTO, 2004). *Sporisorium scitamineum* is one of the most relevant fungal diseases that affect sugarcane and can reduce production by more than 60% in favorable environmental conditions (SUNDAR et al., 2012). The sugarcane smut management counts on resistance genes introgression through genetic breeding. However, even highly resistant varieties occasionally produce whips maintaining low levels of inoculum in the fields (CARVALHO et al., 2016; RAGO, 2005).

The use of biotechnology in sugarcane breeding programs to generate genetically modified plants is a viable alternative to obtain cultivars within an economically sustainable context. The Centro Avançado de Pesquisa Tecnológica do Agronegócio de Cana IAC/APTA and the Genomics ESALQ/USP research groups have been using global expression tools to prospect and characterize sugarcane genes associated with drought tolerance, smut resistance and cell wall composition, toward increasing sugarcane productivity in a sustainable context.

It is known that enzymes of oxidative stress constitute one of the first responses to biotic and abiotic stresses. Peters et al. (2017) observed an increase in hydrogen peroxide (H_2O_2) levels in a smut-resistant sugarcane genotype, which also showed a higher number of superoxide dismutase (SOD) isoforms and a significant reduction in catalase (CAT) and glutathione peroxidase (GST) activities, corroborating the increased lipid peroxidation. In this study, it was suggested that the high level of H_2O_2 is related to signaling and triggering of the plant reaction to inoculation with *S. scitamineum*, while the susceptible genotype seems unable to detect the fungal infection and trigger a similar response (PETERS et al., 2017).

[3] Information provided in the presentation: "Vertix varieties for restrictive environments" at the “Simpósio: Integração da pesquisa pública em cana-de-açúcar no Brasil”, by Bressiani J. A., on may 15th, 2018.

ROS production can regulate the expression of resistance genes and proteins associated with pathogenicity and participate in the signaling network of ethylene, jasmonic acid, and salicylic acid hormones (TORRES et al., 2006). Hormones are known to be involved in plant-pathogen resistance. In this project, we evaluated the *S. scitamineum*-energy cane interaction for the initial defense response generated shortly after *S. scitamineum* infection and compared the gene expression of antioxidant enzymes of oxidative bust in smut susceptible - Vertix 1 and resistant - Vertix 2 varieties at 48hpi and 72hpi.

2. Material and Methods

2.1 Biological material and inoculation procedure

The Vertix 1 and Vertix 2 buds were provided by Granbio S.A., by project partner enterprise. Before inoculation of 30 buds for each variety and treatment, the material was free of pathogens that may interfere with the results, for which thermal and chemical treatment were performed. The bud sets were immersed in water at 52°C for 30 minutes, followed by disinfection with sodium hypochlorite 4% for 10 minutes and washed in distilled water.

The inoculation was conducted in buds immediately after the sprouting. The bud sets were planted in vermiculite substrate, 2 cm deep, and incubated at 28°C until the time of inoculation. The suspension of teliospores was prepared at a concentration of 5×10^7 spores/mL (at NaCl 0.85 %), and the control received deionized water + NaCl 0.85 %. The inoculation were did by applying a drop with 10 µl of the spore suspension from *S. scitamineum* to the base of the bud and with the aid of a needle. *S. scitamineum* SSC04 teliospores were obtained from a diseased plant of the intermediate-resistant variety RB925345 (sprout rate >90%) and were maintained for subsequent experiments in the Genomics Laboratory (ESALQ, USP) (PETERS, 2016). After inoculation, inoculated and control treatments were kept in BOD with controlled conditions of temperature and humidity. The samples were collected from three independent biological replicates for each variety (with 10 buds each), at predefined time points of 48 hai and 72 hai, and kept at -80°C for further analysis.

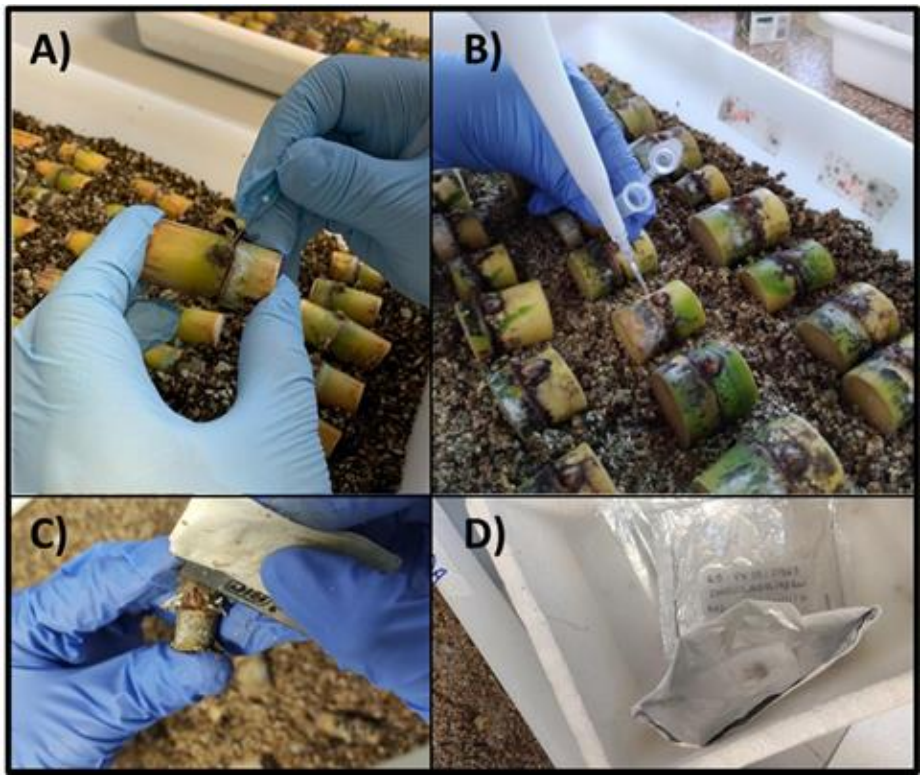

Figure 18. Workflow for biological material preparation. A) and B) Biological material fungal inoculation C) sample collection; D) biological material storage in -80ºC.

2.2 Time-points

The selected time points for the expression analysis were 48 hours post-inoculation (hpi) and 72 hpi corresponded to appressorium formation of *S. scitamineum* in sugarcane susceptible genotypes and the increase of H_2O_2 and lipid peroxidation in the resistant genotype, successively (PETERS et al., 2017). Therefore, the selection of these time points provides the possibility of comparing energy cane varieties with other sugarcane varieties already described in literature regarding ROS, in addition to the comparison between the two energy cane varieties.

2.3 RNA extraction and gene expression analysis

The RNA extraction was performed using Trizol (Sigma) and the Zymo Research Direct-zol™ RNA Miniprep Kit, following the manufacturer's instructions. After extraction, the total RNA was treated with DNAse (Sigma) and the RNA quality was verified by agarose gel. The cDNA was prepared using the GoScript Reverse Transcription System cDNA

(Promega) according to the manufacturer's recommendations, using 800ng of RNA input for the reaction. And the gene expression analysis for the different varieties were done by RT-qPCR (Reverse Transcription Quantitative-PCR). The RT-qPCR reactions were conducted in the 7300 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA) using the GoTaq® Two-Step RT-qPCR System Kit (Promega, Madison, WI).

The genes for analysis (Table 5) were selected in previous studies according to differential expression determined from RNAseq from plants infected with *S. scitamineum* (SHACKER et al., 2016) and also from previous proteomics trials in which the presence or absence of differentially abundant proteins was observed (PETERS et al., 2017). The designed primers had their specificity evaluated through the dissociation curve of each reaction. The genes used for normalization were the tubulin (TUB) and eEF1α endogenous genes.

Table 5. Primers used in RT-qPCR experiments.

Name	Gene ID/ Gene Reference	Sequence
Catalase (CAT3)	comp189288_c1_seq1	F GATCCCACCAAGTTCCGTCC R CTTCTCGATCAGGTGGTAGTCC
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 (GSTt3)	comp179663_c0_seq1	F TTCGGAACCTTCGCCTTGTC R TCAGCCAGGGGAAGCACTAC
Thioredoxin h-type (TRX)	evm.model.scga7_unitig_341686.1	F CCAAGAAGAACCCCAGCGTG R CACCCTGTCCTTCACGTCGG
Tubulin (TUB)	DA SILVA SANTOS et al., 2021.	F CTCCACATTCATCGGCAACTC R TCCTCCTCTTCTTCCTCCTCG

eEF1α	HUANG et al., 2018.	F TTTCACACTTGGAGTGAAGCAGAT R GACTTCCTTCACAATCTCATCATAA

PCR efficiency and Ct values were obtained using the LinReg PCR program (RAMAKERS et al., 2003). The relative expression change was calculated using the REST software (PFAFFL et al., 2004). Three biological replicates were evaluated, and the control (non-inoculated plants) were used for calibration. The Student test (t-test) ($p < 0.05$) were used to determine the significance of relative expression levels.

2.4 DNA extraction of *S. scitamineum* and inoculated buds

DNA extraction of *S. scitamineum* (SSC04) was conducted from 50mg of yeast cells grown to exponential phase in Yeast Medium (YM) and using QIAGEN® Genomic Tip 20G DNA, according to the manufacturer's recommendations. Before DNA extraction, the fungal cell was placed in an overnight shaker at 28º C at 150 rpm for growth and then observed under a microscope to verify that there was no contamination. The plant DNA extraction was performed using the CTAB method from 50mg plant tissues (DOYLE; DOYLE, 1987). DNA concentration and quality were verified by spectrophotometer (NanoDrop® 1000 - Thermo Scientific) and by gel electrophoresis.

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

Fungal quantification in the plant samples was performed using the qPCR molecular technique with the set of primers SSC-C target the IGS region (*Intergenic Spacer*) of *S. scitamineum* genome, the fungal sequence used in this analysis (Forward - CGGCTATTGTCGCACATCTC; Reverse - CCAAACGCAGGTCACAGTCT). The reaction was conducted with the Sigma SYBR Green PCR kit used to quantify target DNA, according to the protocol supplied by the manufacturer, with 5ul of DNA by serial dilution, for both time-points. Standard curve was generated by plotting the threshold cycles (Ct) versus the logarithmic values of known quantities of target fungal DNA. Tukey's test ($P \leq 0.05$) was used to compare the DNA quantity of *S. scitamineum* in the two genotype infected with the pathogen at 48 hpi and 72 hpi

3. Results

3.1 Gene expression analysis by RT-qPCR

ROS-related marker genes were obtained by previous studies from RNAseq data (SCHAKER et al., 2016) that were used by Peter et al. (2017) in analyses for identification of protein sequences induced and repressed by the fungus. A total of six antioxidant enzyme genes were analyzed at two time points: 48hpi (Figure 19) and 72hpi (Figure 20). These time points were selection according with appressorium formation, 24hpi in sugarcane smut-susceptible genotype, and coincided with the increase in H_2O_2 and lipid peroxidation hates in sugarcane smut-resistant genotypes (PETERS et al., 2017). At 48hpi, for Vertix1 (smut susceptible genotype) only the gene encoding TRX showed a decrease in expression, in which was down-regulated triggered by *S. scitamineum* (Figure 19a). And, at the same time-point, for Vertix2 (smut-resistant genotype) just the SOD gene encoding was significantly down-regulated in the smut interaction (Figure 19b). The other genes analyzed *POX5, CAT3, CATB, GST3* did not demonstrate differences against smut inoculation. At 72hpi, both energy cane genotypes did not display any significant differences for any of the ROS-scavenging related genes selected for this study in response to *S. scitamineum* inoculation (Figure 20).

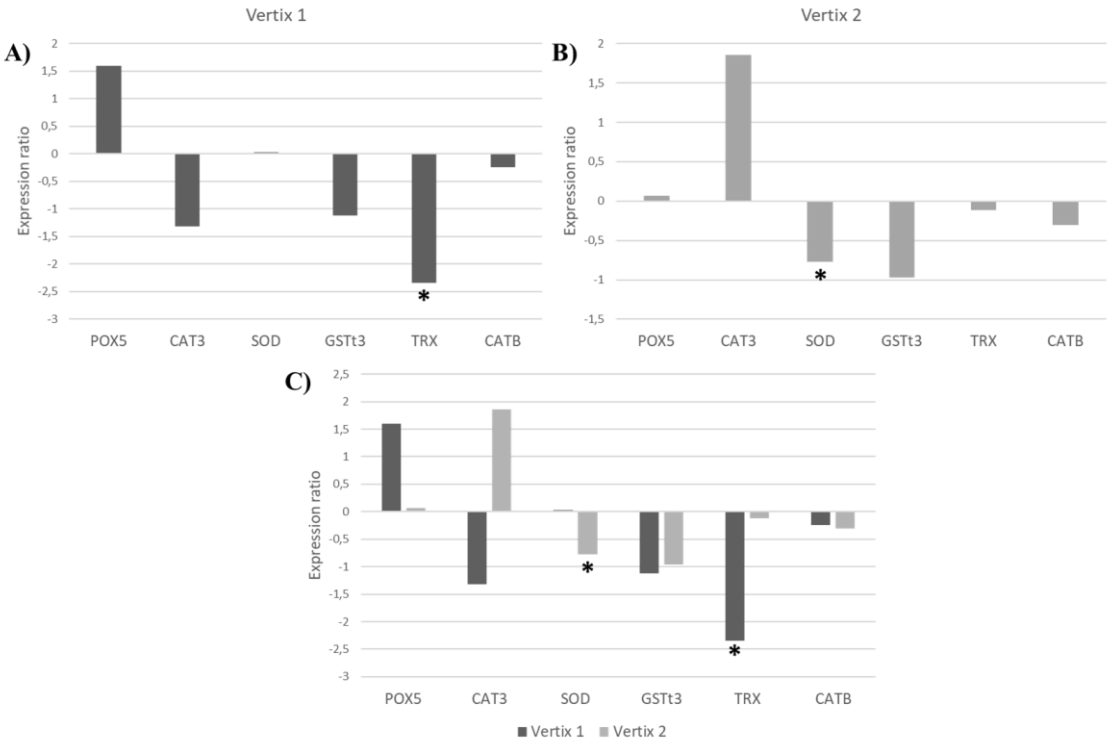

Figure 19. Expression profiles of superoxide dismutase (SOD-comp186491_c0_seq 1), catalase 3 (CAT3-comp189288_c1_seq 1), catalase B (CATBcomp191235_c0_seq 1), peroxidase 5-like (POX5-comp127311_c0_seq 1), glutathione S-transferase t3 (GST t3- comp198747_c0_seq 1) and thioredoxin h like (TRX h-evm.model.scga7_unitig_341686.1) genes associated with the antioxidant system in (a) smut-susceptible (Vertix1) and (b) -resistant (Vertix2) genotypes by RT-qPCR analysis and (c) the two genotypes. Gene expression at 48 hpi (hours post-inoculation). Statistical analysis was performed using the REST_ software. Asterisk represents genes differentially expressed by RT-qPCR ($P < 0.05$).

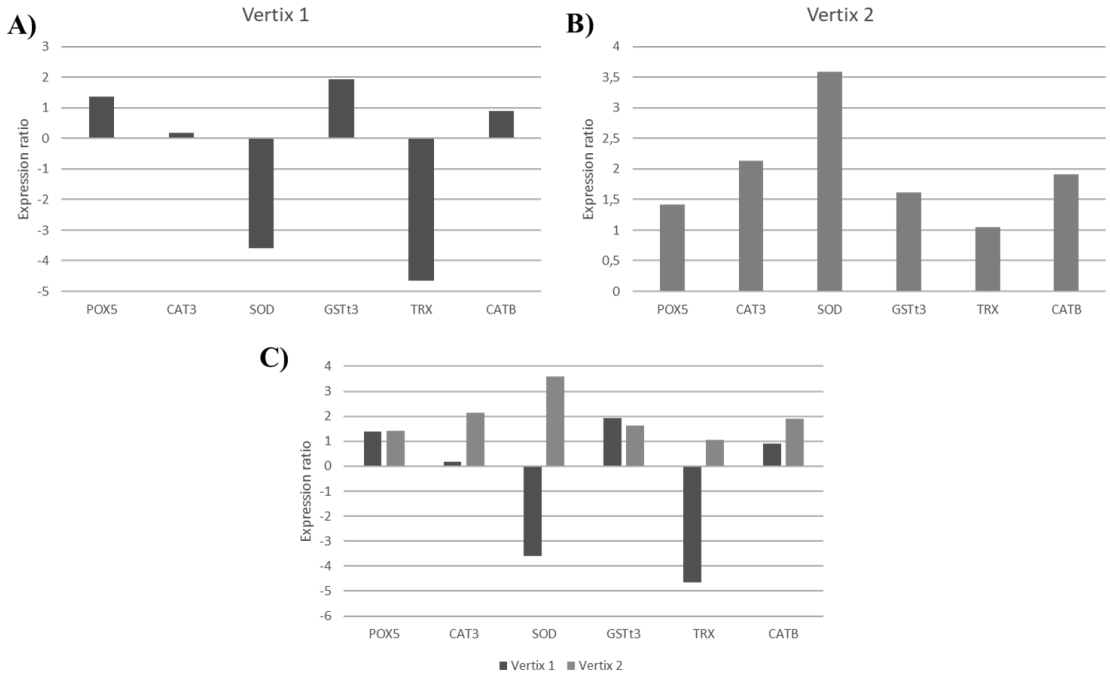

Figure 20. Expression profiles of superoxide dismutase (SOD-comp186491_c0_seq 1), catalase 3 (CAT3-comp189288_c1_seq 1), catalase B (CATB-comp191235_c0_seq 1), peroxidase 5-like (POX5-comp127311_c0_seq 1), glutathione S-transferase t3 (GST t3- comp198747_c0_seq 1) and thioredoxin h like (TRX h-evm.model.scga7_unitig_341686.1) genes associated with the antioxidant system in (a) smut-susceptible (Vertix1) and (b) -resistant (Vertix2) genotypes by RT-qPCR analysis and (c) the two genotypes. Gene expression at 72 hpi (hours post-inoculation). Statistical analysis was performed using the REST_ software. No asterisk represents no genes differentially expressed by RT-qPCR ($P < 0.05$).

3.2 Fungal quantification *in planta* by qPCR

In order to identify and quantify the presence of *S. scitamineum* in infected sugarcane, we used the qPCR protocol developed in this study for bud infected fungus. The results showed the *S. scitamineum* presence in the inoculated samples (at 48hpi and 72 hpi), as required. In addition, we observed a higher amount of fungus on the resistant varieties compared to the susceptible varieties for both time-points measured (Figure 21). However, no significant differences were observed for the fungus quantification in susceptible (Vertix1) and resistant (Vertix2) varieties, in both time-points.

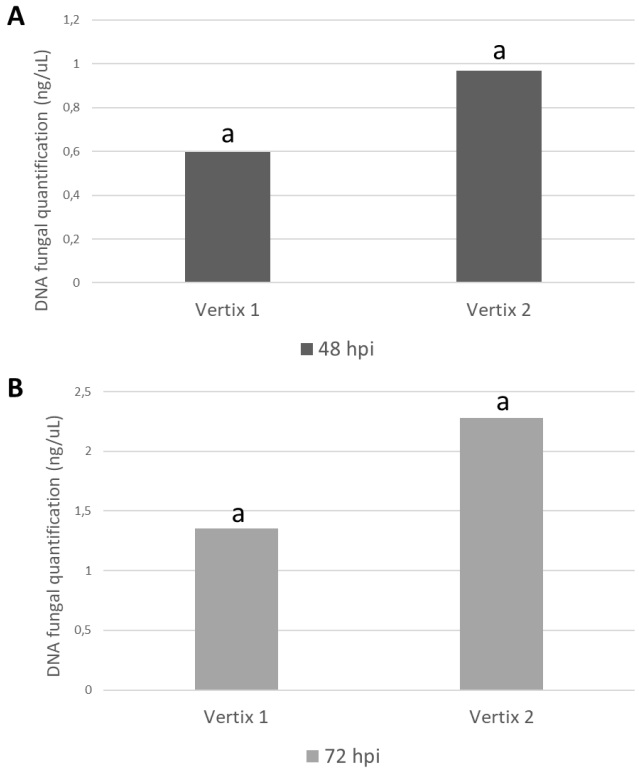

Figure 21. DNA quantify (ng) of *S. scitamineum* assessed by using IGS primer pair. A) Infected smut-susceptible (Vertix1) and -resistant (Vertix2) energy cane genotype at 48hpi (hours post-inoculation). B) Infected smut-susceptible (Vertix1) and -resistant (Vertix2) energy cane genotype at 72 hpi (hours post-inoculation). Values of DNA quantify represent the means from three biological replicates. Means with the same letters are not significantly different (P≤0.05) by Test-t Student analysis.

4. Discussion

In an attempt to improve knowledge of the host defense mechanisms present in energy cane against the *S. scitamineum*, causing smut disease, the expression of ROS-related genes in smut-resistant (Vertix2) and smut-susceptible (Vertix1) varieties at the interaction early stages was evaluated. As reported by Peters et al., (2017), ROS compound (mainly H_2O_2) produced as an interaction response starts earlier at 6hpi (hours post-inoculation) in resistant plants, along with teliospore germination, and increases until 72 hpi, the high levels were at 48 hpi and 72 hpi. In sugarcane resistant genotypes the appressorium formation rate is lower and occurs later than in susceptible genotypes, and, at 72 hpi, the formation of an extensive network of filaments in both genotypes was observed (PETERS et al., 2017). According to Peters et al. (2017), 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 - however, for the resistant varieties, H_2O_2 accumulation was initiated earlier at 6 hpi along with teliospore germination. According to this finding, 48 hpi

and 72 hpi were selected as time points for energy cane analysis, to reach the main defense times in both genotypes (Vertix1 and Vertix2), regarding ROS.

This study is the first one involving the defense response expression genes, such as ROS-related genes, in energy cane varieties. Energy cane varieties have considerable differences when compared to conventional sugarcane in several features, especially given the genetic background from the crossing to obtain genotypes with higher fiber contents and lower sucrose contents (DINIZ et al., 2019). In addition to these commercially significant differences may also be considered differences regarding defense responses derived from *S. scitamineum* infection. *S. scitamineum*, likewise to other smut species, is a biotrophic fungus that during the early stage of infection penetrates plant tissues colonizing the primary meristem (SUNDAR et al. 2012).

As a baseline for this study, the differences observed between the two smut-resistant (Vertix2) and smut-susceptible (Vertix1) energy cane varieties regarding the gene expression for antioxidant enzymes in ROS modulation should be discussed. Also, the potential differences between the contrasting energy cane varieties and conventional sugarcane varieties, previously studied in terms of ROS modulation (PETERS et al., 2017), will be explored. The conventional sugarcane used for Peters et al., (2017) was a resistant genotype, SP80-3280, 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).

Systems for ROS-scavenging play a significant role in ROS managing in the plant-pathogen interaction complex (TORRES, 2010) and have also been reported in higher activity in water deficit conditions (JAIN et al., 2015). SOD enzyme catalyses the dismutation of superoxide anion to H_2O_2 and O_2 and performs the first defense line against ROS (GRATÃO et al., 2005). The gene expression results by RT-qPCR in energy-cane - smut interaction, at 48hpi, revealed no significant differences in SOD gene expression in susceptible genotypes inoculated when compared to control treatment. The opposite was found in resistant genotypes, at the same time-point, in which SOD gene activity was down-regulated with significant difference, showing a similar result to that observed in the sugarcane varieties (resistant and susceptible) by Peters et al. (2017). Other study on different sugarcane varieties have shown a similar increase response of SOD in resistant (F134) and susceptible (NCo310) sugarcane genotypes, in later infection times (30 to 180 days after inoculation), by reporting a

positive response against smut diseases (SINGH et al., 2019). Therefore, the two energy cane genotypes exhibited a different role for SOD expression, in which susceptible genotypes showed an increase in SOD expression, without differences concerning smut inoculation, while the resistant genotypes exhibited a decrease, resulting from interaction (downregulated). This energy cane scenario is distinct to sugarcane one (SP80-3280 and IAC66-6 varieties), wherein the SOD gene expression was down-regulated and did not display any major alterations in the contrasting genotypes due the *S. scitamineum.*

Thioredoxin (TRX) is a critical antioxidant enzyme involved in many plants process as in the H_2O_2 eliminate, connecting with Trx-dependent peroxidases (PETERS et al., 2016), in the control of protein S-nitrosation in plant root development, photosynthetic light harvesting proteins translation and immune responses (JEDELSKÁ; LUHOVÁ; PETˇRIVALSKÝ, 2020). TRX are required in plant-pathogen interaction to catalyse the conversion of the SA-induced nonexpressor of pathogenesis-related (PR) genes 1 (NPR1) into a monomer and turn on the plant defense responses (TADA et al., 2008), and sugarcane in interaction with *S. scitamineum* shows NPR1 up-regulated (CHEN et al. 2012). Therefore, G proteins are related to plants defense signaling (LIU et al. 2013) and these proteins activation occurs by pathogen elicitors responses, resulting in the increase of ROS (TORRES et al., 2013) and PR-proteins synthesis (BEFFA et al., 1995). In our analysis, only the susceptible genotype (VERTIX 1), at 48hpi, shows difference for TRX gene expression by smut inoculation, which was downregulated. These results suggest a contrasting pattern with those observed in conventional sugarcane for TRX gene expression, in which the SP80-3280 (smut-resistant) was up-regulation and the IAC66-6 (smut-susceptible) did not show difference by smut inoculation at 48hpi - but was up-regulation at 72hpi (PETERS et al., 2017). These findings suggested that energy cane may respond differently in smut interaction than previously observed by conventional sugarcane smut-resistant and -susceptible.

We conclude, under these experimental conditions, significant differences were observed in the selected genes expression at the 48hpi, TRX for Vertix1 and SOD for Vertix2, both downregulated, while for 72hpi none of the genes exhibited a significant difference triggered by *S. scitamineum* challenging. Regarding SOD, the result validates the profile earlier shown in the RNA-seq data for the Vertix2 variety. For the susceptible genotype, Vertix1, the TRX downregulated shows an important evidence to be investigated is the role of NRP1 monomers internalized in the nucleus, responsible for the defense signaling via salicylic acid inactivation. This is an effect that needs to be explored further and the different

enzymes analyzed may respond distinctly depending on the inoculation, period tested, and genotype selected. Regardless, we suggest that further detailed analyses for ROs modulation-related genes expression and for the presence of antioxidant enzymes in infected tissues should be performed to improve the understanding of defense responses around ROS production and the enzymatic antioxidant system, in the two contrasting energy cane genotypes by smut interaction. In addition, we intend to select endogenous genes from the transcriptome obtained for these energy cane varieties for use in further analysis, with the aim of improving the results obtained through relative gene expression experiments by RT-qPCR in this context.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Terms (co-occurring Terms) of GO:0050896 defined for total sequences directly involved with ROS metabolism in the genome of *S. spontaneum*.

Co-occurring Terms (The top 100 of 1,047 co-occurring terms)		
GO Terms	**Name**	**Anotation**
GO:0000302	response to reactive oxygen species	Biological process
GO:0000303	response to superoxide	Biological process
GO:0034599	cellular response to oxidative stress	Biological process
GO:0042542	response to hydrogen peroxide	Biological process
GO:0030308	negative regulation of cell growth	Biological process
GO:0051216	cartilage development	Biological process
GO:0006970	response to osmotic stress	Biological process
GO:0060588	negative regulation of lipoprotein lipid oxidation	Biological process
GO:0071638	negative regulation of monocyte chemotactic protein-1 production	Biological process
GO:0033906	hyaluronoglucuronidase activity	Molecular function
GO:0001666	response to hypoxia	Biological process
GO:0030294	receptor signaling protein tyrosine kinase inhibitor activity	Molecular function
GO:2000405	negative regulation of T cell migration	Biological process
GO:0010764	negative regulation of fibroblast migration	Biological process
GO:0010642	negative regulation of platelet-derived growth factor receptor signaling pathway	Biological process
GO:0019899	enzyme binding	Molecular function
GO:0042308	negative regulation of protein import into nucleus	Biological process
GO:0007568	aging	Biological process
GO:0000425	pexophagy	Biological process
GO:0061099	negative regulation of protein tyrosine kinase activity	Biological process
GO:0010020	chloroplast fission	Biological process
GO:0043407	negative regulation of MAP kinase activity	Biological process
GO:0006027	glycosaminoglycan catabolic process	Biological

		process
GO:0009723	response to ethylene	Biological process
GO:0048366	leaf development	Biological process
GO:0051898	negative regulation of protein kinase B signaling	Biological process
GO:0032364	oxygen homeostasis	Biological process
GO:0038060	nitric oxide-cGMP-mediated signaling pathway	Biological process
GO:0008074	guanylate cyclase complex, soluble	Cellular component
GO:0042744	hydrogen peroxide catabolic process	Biological process
GO:0030139	endocytic vesicle	Cellular component
GO:0004415	hyaluronoglucosaminidase activity	Molecular function
GO:0001315	age-dependent response to reactive oxygen species	Biological process
GO:0006110	regulation of glycolytic process	Biological process
GO:0010575	positive regulation of vascular endothelial growth factor production	Biological process
GO:0010120	camalexin biosynthetic process	Biological process
GO:0061419	positive regulation of transcription from RNA polymerase II promoter in response to hypoxia	Biological process
GO:0032909	regulation of transforming growth factor beta2 production	Biological process
GO:0051302	regulation of cell division	Biological process
GO:2000434	regulation of protein neddylation	Biological process
GO:0009864	induced systemic resistance, jasmonic acid mediated signaling pathway	Biological process
GO:0090333	regulation of stomatal closure	Biological process
GO:0097468	programmed cell death in response to reactive oxygen species	Biological process
GO:0009707	chloroplast outer membrane	Cellular component
GO:0010082	regulation of root meristem growth	Biological process
GO:0000268	peroxisome targeting sequence binding	Molecular function
GO:0071467	cellular response to pH	Biological process
GO:1900063	regulation of peroxisome organization	Biological process
GO:0045785	positive regulation of cell adhesion	Biological process

GO:0009574	preprophase band	Cellular component
GO:0010229	inflorescence development	Biological process
GO:0009410	response to xenobiotic stimulus	Biological process
GO:0050679	positive regulation of epithelial cell proliferation	Biological process
GO:0062151	catalase complex	Cellular component
GO:0080136	priming of cellular response to stress	Biological process
GO:0051510	regulation of unidimensional cell growth	Biological process
GO:0061692	cellular detoxification of hydrogen peroxide	Biological process
GO:0090575	RNA polymerase II transcription regulator complex	Cellular component
GO:0030307	positive regulation of cell growth	Biological process
GO:0010183	pollen tube guidance	Biological process

Supplementary Table 2. Terms (child and parents) of GO:0050896 (response to stimulus) defined for total sequences directly involved with ROS metabolism DE exclusive for each inoculated X control experiment of the susceptible (Vertix 1) and resistant (Vertix 2).

				# Sequences		
Level	GO ID	GO Name	Parents (GO ID)	*S. spontaneum*	Vertix 1	Vertix 2
1	**GO:0050896**	**response to stimulus**		**335**	16	-
2	GO:0042221	response to chemical	GO:0050896	145	2	-
2	GO:0009628	response to abiotic stimulus	GO:0050896	44	1	-
2	GO:0006950	response to stress	GO:0050896	214	12	-
2	GO:0009719	response to endogenous stimulus	GO:0050896	77	1	-
2	GO:0051716	cellular response to stimulus	GO:0050896	140	4	-
3	GO:0007165	signal transduction	GO:0051716	94	4	-
3	GO:0070887	cellular response to chemical stimulus	GO:0042221 GO:0051716	84	1	-
3	GO:0010033	response to organic substance	GO:0042221	88	1	-
3	GO:0006952	defense response	GO:0006950	44	2	-
3	GO:1901700	response to oxygen-containing compound	GO:0042221	60	-	-
3	GO:0009725	response to hormone	GO:0009719, GO:0010033	77	1	-
3	GO:0071495	cellular response to endogenous stimulus	GO:0009719	42	1	-
3	GO:0006979	response to oxidative stress	GO:0006950	145	9	-
4	GO:0000302	response to reactive oxygen species	GO:0006979	31	-	-
5	GO:0042542	response to hydrogen peroxide	GO:0000302	20	-	-
4	GO:0035556	intracellular signal transduction	GO:0007165	39	4	-
4	GO:0032870	cellular response to hormone stimulus	GO:0009725, GO:0071310, GO:0071495	42	1	-
4	GO:0009755	hormone-mediated signaling pathway	GO:0007165, GO:0032870	42	1	-
3	**GO:0072593**	**reactive oxygen species metabolic process**	**GO:0044237**	**80**	**9**	**2**
4	GO:0042743	hydrogen peroxide metabolic process	GO:0072593	76	9	0
5	GO:0042744	hydrogen peroxide catabolic process	GO:0042743	76	9	2

Supplementary Figure 1. Upregulated genes related to defense response in Vertix 1 genotype. Genes manually selected and separated into the subcategories: ROS metabolism modulation; Negative regulation of programmed cell death; Chaperones; PR-proteins.

Vertix 1 - UP-REGULATED DEFENSE GENES

Gene	Protein name	# Seq	References	Function (by similarity)	GO
ROS metabolism modulation					
Sspon.01G0007880-1A; Sspon.01G0038620-1B; Sspon.01G0051580-1C	17.9 kDa class I heat shock protein	3	UniProt KB; Kang et al., 2021 (DOI:10.3390/horticulturae7090312)	Under heat stress, HSPs, as molecular chaperones, bind to heatdenatured proteins and mediate refolding, assembly for repairing denatured proteins or degradation of misfolded proteins to maintain homeostasis of proteins	response to hydrogen peroxide, GO:0042542
Sspon.01G0030330-3P	AAA-ATPase At3g28580	1	Baruah et al., 2012 (DOI:10.1007/s11103-009-9491-0)	singlet O2-responsive AAA-ATPase gene (At3g28580) but not by superoxide or hydrogen peroxide.	response to singlet oxygen, GO:0000304
Sspon.04G0006710-1P; Sspon.02G0019440-1A; Sspon.02G0019440-2B; Sspon.02G0019440-3C; Sspon.02G0019440-4D	aquaporin PIP1-1	5	Li et al., 2020 (DOI:10.3390/plants9091134	Water channel required to facilitate the transport of water across cell membrane. In plants, AQPs can mediate H2O2 transport across plasma membranes (PMs) and contribute to the activation of plant defenses by inducing pathogen-associated molecular pattern (PAMP)-triggered immunity and systemic acquired resistance (SAR), followed by downstream defense reactions.	water channel activity; GO:0015250
Sspon.02G0032070-1A; Sspon.02G0053200-1C; Sspon.03G0038090-2D	Cationic peroxidase SPC4	3	UniProt KB	Removal of H2O2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress. These functions might be dependent on each isozyme/isoform in each plant tissue.	response to oxidative stress, GO:0006979
Sspon.07G0013960-1A; Sspon.07G0013960-2B; Sspon.03G0015760-1P; Sspon.07G0013960-3D; Sspon.02G0010730-1A	peroxidase 5	5	Uniprot KB	Removal of H2O2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress.	response to oxidative stress, GO:0006979
Sspon.02G0028150-2B	Respiratory burst oxidase homolog protein B RBOHB	1	Uniprot KB	Calcium-dependent NADPH oxidase that generates superoxide. Involved in the massive phase II oxidative burst induced by pathogen infection.	NAD(P)H oxidase H2O2-forming activity, GO:0016174; peroxidase activity; GO:0004601
Sspon.04G0008400-3D; Sspon.04G0008400-1A; Sspon.04G0008400-2C; Sspon.06G0005730	temperature-induced lipocalin-1	7	Uniprot KB	Lipocalin that confers protection against oxidative stress caused by heat, freezing, paraquat and light	positive regulation of response to oxidative stress, GO:1902884
Negative regulation of programmed cell death					
Sspon.06G0025280-2C	E3 ubiquitin-protein ligase BOI	1	UniProt KB	E3 ubiquitin-protein ligase involved in the regulation of pathogen and abiotic stress responses by facilitating degradation of MYB108/BOI. Attenuates cell death by preventing caspase activation. Has no effect on the stability of the DELLA proteins.	negative regulation of programmed cell death, GO:0043069
Chaperones					
Sspon.04G0008980-1A	BAG family molecular chaperone regulator 1	2	Doukhanina et al., 2006 (DOI:10.1074/jbc.M511794200)	Co-chaperone that regulates diverse cellular pathways, such as programmed cell death and stress responses. Plant BAG family members are also multifunctional and remarkably similar to their animal counterparts, as they regulate apoptosis-like processes ranging from pathogen attack to abiotic stress and development.	protein binding, GO:0005515
PR- proteins					
Sspon.03G0000020-1A	beta-1,3-glucanase A	2	UniProt KB	Implicated in the defense of plants against pathogens.	defense response; GO:0006952
Sspon.04G0008670	chitinase B	2	UniProt KB	Random endo-hydrolysis of N-acetyl-beta-D-glucosaminide (1->4)-beta-linkages in chitin and chitodextrins	carbohydrate metabolic process, GO:0005975; chitin catabolic process, GO:0006032
Sspon.08G0016530-1T	pathogenesis-related maize seed protein	1	UniProt KB; Majumdar, et al.,, 2017 (DOI: 0.3389/fpls.2017.01758)	Probably involved in the defense reaction of plants against pathogens. The Pathogenesis-Related Maize Seed (PRms) Gene Plays a Role in Resistance to Aspergillus flavus Infection and Aflatoxin Contamination	defense response, GO:0006952 / beta-glucanase activity, GO:0052736
Sspon.06G0016280	pathogenesis-related protein	3	Uniprot KB	Shows antifungal activity towards B.cinerea and towards the wheat-specific pathogenic fungi F.culmorum and F.graminearum (groups 1 and 2).	defense response, GO:0006952
Sspon.04G0002120-1A	subtilisin-like protease SBT1.4	1	Figueiredo et al., 2014 (DOI: 10.3389/fpls.2014.00739)	Subtilisin-like proteases in plant–pathogen recognition and immune priming: a perspective.	serine-type endopeptidase activity, GO:0004252

Supplementary Figure 2. Upregulated genes related to defense response in Vertix 2 genotype. Genes manually selected and separated into the subcategories: ROS metabolism modulation; Negative regulation of programmed cell death; PR-proteins; Signaling.

Vertix 2 - UP-REGULATED DEFENSE GENES

Gene	Protein name	# Seq	Reference	Function (by similarity)	GO
ROS metabolism modulation					
Sspon.07G0019470	aldehyde dehydrogenase family 3 member H1-like	1	Stiti et al., 2011 (DOI: 10.1042/BJ20101337)	Involved in oxidative stress tolerance by detoxifying reactive aldehydes derived from lipid peroxidation. Medium- to long-chain saturated aldehydes are preferred substrates, while the short-chain aldehyde propanal is a weak substrate.	defense response; GO:0006952
Sspon.08G0000830	catalase isozyme 1	2	Du et al, 2008 (10.1111/j.1744-7909.2008.00741.x)	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide.	response to oxidative stress; GO:0006979
Sspon.08G0005470	nudix hydrolase 2 isoform X1	2	Ogawa et al., 2009 (DOI: 10.1111/j.1365-313X.2008.03686.x)	Overexpression of NUTD2 confers enhanced tolerance to oxidative stress.	Overexpression of NUTD2 confers enhanced tolerance to oxidative stress.
Sspon.01G0024190	ornithine aminotransferase, mitochondrial	2	Senthil-Kumar & Mysore, 2012 (DOI:10.1111/j.1365-3040.2012.02492.x	Plays a role in non-host disease resistance by regulating pyrroline-5-carboxylate metabolism-induced hypersensitive response.	arginine catabolic process to glutamate; GO:0019544
Sspon.04G0009860	Protein ACTIVITY OF BC1 COMPLEX KINASE 8, chloroplastic	2	UniProt, Manara et al., 2014 (DOI: 10.1111/nph.12533)	Involved in resistance to oxidative stress (e.g. hydrogen peroxide H2O2), high light and heavy metals (e.g. cadmium ions Cd2+)	cellular response to oxidative stress, GO:0034599
Sspon.01G0036870-1T	purple acid phosphatase 17	1	UniProt KB	Metallo-phosphoesterase involved in phosphate metabolism. Has a peroxidase activity.	response to hydrogen peroxide, GO:0042542
Negative regulation of programmed cell death					
Sspon.04G0016560	cyclase-like protein 1	1	UniProt KB; Qin et al., 2015 (DOI:10.1016/j.jplph.2015.03.018)	Acts as a negative regulator of fumonisin B1- and pathogen-induced programmed cell death (PCD), and regulates pathogen-induced symptom development. May function redundantly with CYCLASE2 for normal plant growth, development and viability (Probable). The overexpression of one stress-responsive gene OsCYL4a in rice resulted in decreased tolerance to salt, drought, cold, and oxidative stress.	defense response; GO:0006952
PR-protein					
Sspon.07G0008410	chitinase 2-like	3	UniProt KB	Hydrolyzes chitin and plays a role in defense against fungal pathogens containing chitin. Its overexpression confers enhanced resistance to sheath blight pathogen (R.solani).	carbohydrate metabolic process; GO:0005975
Sspon.02G0013850	cysteine proteinase 2	3	UniProt KB	Specific inhibitor of cysteine proteinases. Probably involved in the regulation of endogenous processes and in defense against pests and pathogens (By similarity).	defense response; GO:0006952; cystein-type peptidase activity GO:0006508
Sspon.06G0029990	endo-1,3(4)-beta-glucanase-like precursor	1	UniProt KB	Hydrolysis of (1->3)-beta-D-glucosidic linkages in (1->3)-beta-D-glucans	defense response; GO:0006952
Signaling					
Sspon.02G0018240	transcription factor MYB44-like	2	UniProt KB	Activates salicylic acid (SA)- mediated defenses and subsequent resistance to biotrophic pathogen P.syringae pv. tomato DC3000, but represses jasmonic acid (JA)-mediated defenses responses against the necrotrophic pathogen A.brassicicola	regulation of transcription, GO:0006357; defense response to fungus, GO:0050832

Supplementary Figure 3. Downregulated genes related to defense response in Vertix 2 genotype. Genes manually selected and separated into the subcategories: ROS metabolism modulation; Chaperones; PR-proteins; Signaling.

Vertix 2 - DOWN-REGULATED DEFENSE GENES					
Gene	**Protein name**	**# Seq**	**Reference**	**Function (by similarity)**	**GO**
ROS metabolism modulation					
Sspon.04G0002180	20 kDa chaperonin	2	Kuo et al., 2013 (DOI: 10.1111/j.1469-8137.2012.04369.x)	Required to activate the iron superoxide dismutases (FeSOD)	Positive regulation of superoxide dismutase activity; GO:1901671
Sspon.06G0013710	deoxyhypusine synthase	1	UniProt KB	Catalyzes the NAD-dependent oxidative cleavage of spermidine and the subsequent transfer of the butylamine moiety of spermidine to the epsilon-amino group of a specific lysine residue of the eIF-5A precursor protein to form the intermediate deoxyhypusine residue. Also able to produce homospermidine from putrescine (By similarity).	protein maturation; GO:0051604
Sspon.01G0014210	nucleoside diphosphate kinase 1	4	UniProt KB; Fukamatsu et al., 2013 (DOI: 10.1093/pcp/pcg140)	Plays a role in response to reactive oxygen species (ROS) stress. Plants over-expressing NDK1 are more tolerant to paraquat and have increased ability to eliminate exogenous H2O2	cellular response to hydrogen peroxide; GO:0070301
Sspon.02G0021880	peroxidase 1-like	2	UniProt KB	Removal of H2O2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress. These functions might be dependent on each isozyme/isoform in each plant tissue. There are 73 peroxidase genes in A.thaliana.	response to oxidative stress, GO:0006979
Sspon.07G0024880-2C	superoxide dismutase [Mn] 3.4, mitochondrial	1	UniProt KB	Destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems.	superoxide metabolism process; GO:0006801
Chaperones					
Sspon.02G0021530	calreticulin isoform X1	1	Qiu et al., 2012 (DOI:10.4161/psb.20721)	Recent studies suggest that both isoforms of plant CRTs (AtCRT1/2 and AtCRT3) are involved in regulating plant defense against biotrophic pathogens.	protein folding; GO:0006457
Sspon.08G0018860	patellin-4	3	UniProt KB; Zhou et al., 2019 (DOI: 10.1016/j.jplph.2019.01.012)	Carrier protein that may be involved in membrane-trafficking events associated with cell plate formation during cytokinesis. Binds to some hydrophobic molecules such as phosphoinositides and promotes their transfer between the different cellular sites. The elucidation of PATLs' biological function in plants will provide new insights on plant membrane trafficking and its regulatory roles in either plant growth or environmental stress response signaling networks.	lipid metabolism; GO:0008289; cellular response to auxin stimulus, GO:0071365
Sspon.03G0006840-2B	profilin-A	1	UniProt KB; Sun et al., 2018 (DOI: 10.1016/j.cub.2018.04.045)	Binds to actin monomers and regulates the organization of the actin cytoskeleton. Inhibits cell growth of various pathogenic fungal strains. May play a role as antifungal proteins in the defense system against fungal pathogen attacks	actin polymerization or depolymerization; GO:0008154
PR-protein					
Sspon.03G0003670	non-specific lipid-transfer protein 1-like	1	UniProt KB; Liu et al., 2015 (DOI:10.1093/jxb/erv313)	Plant non-specific lipid-transfer proteins transfer phospholipids as well as galactolipids across membranes. Binds cis-unsaturated fatty acids and jasmonic acid with a higher affinity than linear chain fatty acids. Formation of the complex with jasmonic acid results in a conformational change facilitating the LPT1 binding on the elicitin plasma membrane receptor that is known to be involved in plant defense induction. May also play a role in wax or cutin deposition in the cell walls of expanding epidermal cells and certain secretory tissues.	lipid metabolic process; GO:0006629
Signaling					
Sspon.07G0002670	guanine nucleotide-binding protein subunit beta-like protein A	3	UniProt KB	The heterotrimeric G-protein controls defense responses to necrotrophic and vascular fungi probably by modulating cell wall-related genes expression (e.g. lower xylose content in cell walls); involved in resistance to fungal pathogens such as Alternaria brassicicola and Fusarium oxysporum. Modulates root architecture (e.g. lateral root formation). Acts with XGL3 in the positive regulation of root waving and root skewing. Involved in the asymmetric division of zygote and specification of apical and basal cell lineages	defense response; GO:0006952
Sspon.02G0044620	myb-related protein MYBAS2-like isoform X2	2	UniProt KB; Katiyar et al., 2012 (10.1186/1471-2164-13-544)	Acts redundantly with MYR1 as a repressor of flowering and organ elongation under decreased light intensity. MYB transcription factors are involved in plant development, secondary metabolism, hormone signal transduction, disease resistance and abiotic stress tolerance.	regulation of transcription; GO:0006355
Sspon.01G0022950	peptidyl-prolyl cis-trans isomerase E	1	UniProt KB	PPIases regulate the molecular interaction and enzymatic reaction, and could act as the molecular timer in various physiological and pathological processes	regulation of transcription; GO:0006355

Supplementary Figure 4. Upregulated genes related to defense response in Vertix 1 genotype. Genes manually selected and separated into the subcategory: ROS metabolism modulation.

VERTIX 1 - UP-REGULATED ROS METABOLISM MODULATION GENES					
Gene	Protein name	# Seq	Reference	Function (by similarity)	GO
Sspon.01G0030330-3P	AAA-ATPase At3g28580	1	Baruah et al., 2012 (DOI:10.1007/s11103-009-9491-0)	singlet O2-responsive AAA-ATPase gene (At3g28580) but not by superoxide or hydrogen peroxide.	response to singlet oxygen, GO:0000304
Sspon.04G0006710-1P; Sspon.02G0019440-1A; Sspon.02G0019440-2B; Sspon.02G0019440-3C; Sspon.02G0019440-4D	aquaporin PIP1-1	5	Li et al., 2020 (DOI:10.3390/plants9091134	Water channel required to facilitate the transport of water across cell membrane. In plants, AQPs can mediate H2O2 transport across plasma membranes (PMs) and contribute to the activation of plant defenses by inducing pathogen-associated molecular pattern (PAMP)-triggered immunity and systemic acquired resistance (SAR), followed by downstream defense reactions.	water channel activity; GO:0015250
Sspon.02G0032070-1A; Sspon.02G0053200-1C; Sspon.03G0038090-2D	Cationic peroxidase SPC4	3	UniProt KB	Removal of H2O2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress. These functions might be dependent on each isozyme/isoform in each plant tissue.	response to oxidative stress, GO:0006979
Sspon.07G0013960-1A; Sspon.07G0013960-2B; Sspon.03G0015760-1P; Sspon.07G0013960-3D; Sspon.02G0010730-1A	peroxidase 5	5	Uniprot KB	Removal of H2O2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress.	response to oxidative stress, GO:0006979
Sspon.02G0028150-2B	Respiratory burst oxidase homolog protein B RBOHB	1	Uniprot KB	Calcium-dependent NADPH oxidase that generates superoxide. Involved in the massive phase II oxidative burst induced by pathogen infection.	NAD(P)H oxidase H2O2-forming activity, GO:0016174; peroxidase activity; GO:0004601
Sspon.04G0008400-3D; Sspon.04G0008400-1A; Sspon.04G0008400-2C; Sspon.06G0005730	temperature-induced lipocalin-1	7	Uniprot KB	Lipocalin that confers protection against oxidative stress caused by heat, freezing, paraquat and light	positive regulation of response to oxidative stress, GO:1902884
Sspon.01G0007880-1A; Sspon.01G0038620-1B; Sspon.01G0051580-1C	17.9 kDa class I heat shock protein	3	UniProt KB; Kang et al., 2021 (DOI:10.3390/horticulturae7090312)	Under heat stress, HSPs, as molecular chaperones, bind to heatdenatured proteins and mediate refolding, assembly for repairing denatured proteins or degradation of misfolded proteins to maintain homeostasis of proteins	response to hydrogen peroxide, GO:0042542

Supplementary Figure 5. Upregulated genes related to defense response in Vertix 2 genotype. Genes manually selected and separated into the subcategory: ROS metabolism modulation.

VERTIX 2 - UP-REGULATED ROS METABOLISM MODULATION GENES					
Gene	Protein name	# Seq	Reference	Function (by similarity)	GO
Sspon.07G0019470	aldehyde dehydrogenase family 3 member H1-like	1	Stiti et al., 2011 (DOI: 10.1042/BJ20101337)	Involved in oxidative stress tolerance by detoxifying reactive aldehydes derived from lipid peroxidation. Medium- to long-chain saturated aldehydes are preferred substrates, while the short-chain aldehyde propanal is a weak substrate.	defense response; GO:0006952
Sspon.08G0000830	catalase isozyme 1	2	Du et al, 2008 (10.1111/j.1744-7909.2008.00741.x)	Occurs in almost all aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide.	response to oxidative stress; GO:0006979
Sspon.08G0005470	nudix hydrolase 2 isoform X1	2	Ogawa et al., 2009 (DOI: 10.1111/j.1365-313X.2008.03686.x)	Overexpression of NUTD2 confers enhanced tolerance to oxidative stress.	Overexpression of NUTD2 confers enhanced tolerance to oxidative stress.
Sspon.01G0024190	ornithine aminotransferase, mitochondrial	2	Senthil-Kumar & Mysore, 2012 (DOI:10.1111/j.1365-3040.2012.02492.x	Plays a role in non-host disease resistance by regulating pyrroline-5-carboxylate metabolism-induced hypersensitive response.	arginine catabolic process to glutamate; GO:0019544
Sspon.04G0009860	Protein ACTIVITY OF BC1 COMPLEX KINASE 8, chloroplastic	2	UniProt, Manara et al., 2014 (DOI: 10.1111/nph.12533)	Involved in resistance to oxidative stress (e.g. hydrogen peroxide H2O2), high light and heavy metals (e.g. cadmium ions Cd2+)	cellular response to oxidative stress, GO:0034599
Sspon.01G0036870-1T	purple acid phosphatase 17	1	UniProt KB	Metallo-phosphoesterase involved in phosphate metabolism. Has a peroxidase activity.	response to hydrogen peroxide, GO:0042542

Supplementary Figure 6. Downregulated genes related to defense response in Vertix 2 genotype. Genes manually selected and separated into the subcategory: ROS metabolism modulation.

DOWN-REGULATED					
Gene	Protein name	# Seq	Reference	Function (by similarity)	GO
Sspon.04G0002180	20 kDa chaperonin	2	Kuo et al., 2013 (DOI: 10.1111/j.1469-8137.2012.04369.x)	Required to activate the iron superoxide dismutases (FeSOD)	Positive regulation of superoxide dismutase activity; GO:1901671
Sspon.06G0013710	deoxyhypusine synthase	1	UniProt KB	Catalyzes the NAD-dependent oxidative cleavage of spermidine and the subsequent transfer of the butylamine moiety of spermidine to the epsilon-amino group of a specific lysine residue of the eIF-5A precursor protein to form the intermediate deoxyhypusine residue. Also able to produce homospermidine from putrescine (By similarity).	protein maturation; GO:0051604
Sspon.01G0014210	nucleoside diphosphate kinase 1	4	UniProt KB; Fukamatsu et al., 2013 (DOI: 10.1093/pcp/pcg140)	Plays a role in response to reactive oxygen species (ROS) stress. Plants over-expressing NDK1 are more tolerant to paraquat and have increased ability to eliminate exogenous H2O2	cellular response to hydrogen peroxide; GO:0070301
Sspon.02G0021880	peroxidase 1-like	2	UniProt KB	Removal of H2O2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress. These functions might be dependent on each isozyme/isoform in each plant tissue. There are 73 peroxidase genes in A.thaliana.	response to oxidative stress, GO:0006979
Sspon.07G0024880-2C	superoxide dismutase [Mn] 3.4, mitochondrial	1	UniProt KB	Destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems.	superoxide metabolism process; GO:0006801