

University of São Paulo  
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Unraveling sugarcane-*Diatraea saccharalis*-opportunistic fungi interaction in  
sugarcane

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

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## RESUMO

**Desvendando a interação cana-de-açúcar-*Diatraea saccharalis*-fungos oportunistas em cana-de-açúcar**

As plantas respondem ao ataque de insetos e patógenos induzindo e acumulando um grande conjunto de proteínas de defesa. A colonização do caule de cana por fungos oportunistas, como *Fusarium verticillioides* e *Colletotrichum falcatum*, geralmente ocorre após o ataque de lagartas de *Diatraea saccharalis* (Lepidoptera: Crambidae), resultando no aumento do dano causado pelo inseto. Dois homólogos da proteína BARWIN foram identificados em cana-de-açúcar, SUGARWIN1 e SUGARWIN2. A expressão desses genes é induzida em resposta ao ferimento mecânico e ao ataque de *Diatraea saccharalis*, entretanto, a proteína não afeta o desenvolvimento do inseto, mas promove alterações morfológicas e fisiológicas significativas em *Fusarium verticillioides* e *Colletotrichum falcatum*, causando a morte destes fungos por apoptose. Esses dados indicam que as SUGARWINs podem funcionar como uma defesa inicial contra a infecção fúngica. Neste estudo, aprofundamos nosso entendimento do papel das SUGARWINs na defesa de plantas e os mecanismos moleculares pelos quais essas proteínas afetam os fungos, elucidando seus alvos moleculares. Nossos resultados mostraram que as SUGARWINs desempenham um papel importante na defesa da planta contra patógenos oportunistas. Foi demonstrado que essas proteínas também são induzidas por *C. falcatum* em cana-de-açúcar, e sua indução pode variar entre as variedades de cana-de-açúcar. A variedade de cana-de-açúcar que apresentou o maior nível de indução de SUGARWINs apresentou uma redução considerável na infecção por *C. falcatum*. Além disso, SUGARWIN1 exibiu atividade de ribonuclease e quitinase, enquanto que SUGARWIN2 exibiu apenas atividade de quitinase. Esta especificidade enzimática parece ser o resultado da composição divergente de aminoácidos no sítio de ligação do substrato. Além disso, as plantas atacadas por insetos e patógenos exibem profundas alterações fisiológicas, morfológicas e químicas ou adaptações, que podem resultar em atração ou repelência do organismo, dessa forma, estudamos também a associação inseto-fungos na cana-de-açúcar, e o papel dos compostos voláteis fúngicos nessa associação. Nossos resultados mostraram que *D. saccharalis* influencia positivamente a infecção por *C. falcatum* em cana-de-açúcar, induzindo crescimento rápido do fungo quando comparado ao tratamento com *C. falcatum* sem ataque de *D. saccharalis*. Além disso, ambos os fungos, *C. falcatum* e *F. verticillioides*, mostraram um efeito duplo sobre lagartas de *D. saccharalis*, promovendo uma forte atração desses insetos devido à emissão de compostos orgânicos voláteis e influenciando positivamente a alimentação de *D. saccharalis* e ganho de peso em dietas suplementadas com fungos. Os compostos orgânicos voláteis fúngicos de *C. falcatum* e *F. verticillioides* foram identificados e quantificados; acoradieno e acorenol foram especificamente induzidos pelos fungos. Estes dados sugerem uma interação sinérgica, mediada por compostos orgânicos voláteis, entre *D. saccharalis* e os fungos *C. falcatum* e *F. verticillioides* em cana-de-açúcar.

Palavras-chave: Cana-de-açúcar; SUGARWIN; BARWIN; *Colletotrichum falcatum*; *Fusarium verticillioides*; Quitinase; RNase; Broca da cana; Interação planta-inseto-fungo

## ABSTRACT

**Unraveling sugarcane-*Diatraea saccharalis*-opportunistic fungi interaction in sugarcane**

Plants respond to insect and pathogen attack by inducing and accumulating a large set of defense proteins. Colonization of sugarcane stalk by opportunistic fungi, such as *Fusarium verticillioides* and *Colletotrichum falcatum*, usually occurs after *Diatraea saccharalis* (Lepidoptera: Crambidae) caterpillars attack increasing the damage caused by the borer. Two homologous of BARWIN protein were identified in sugarcane, SUGARWIN1 and SUGARWIN2. Their gene expression is induced in response to wound and *Diatraea saccharalis* damage. However, the recombinant SUGARWIN protein does not affect insect development; but promotes significant morphological and physiological changes in *Fusarium verticillioides* and *Colletotrichum falcatum*, which lead to fungal cell death via apoptosis, indicating that SUGARWINs may work as a first layer of defense against the fungi infection. In this study, we deepen our understanding of the role of SUGARWINs in plant defense and the molecular mechanisms by which these proteins affect fungi by elucidating their molecular targets. Our results show that SUGARWINs play an important role in plant defense against opportunistic pathogens. We demonstrated that *SUGARWINs* are induced by *C. falcatum*, and the induction of *SUGARWINs* can vary among sugarcane varieties. The sugarcane variety exhibiting the highest level of *SUGARWIN* induction exhibited a considerable reduction in *C. falcatum* infection. Furthermore, SUGARWIN1 exhibited ribonuclease and chitinase activity, whereas SUGARWIN2 exhibited only chitinase activity. This variable enzymatic specificity seems to be the result of divergent amino acid composition within the substrate-binding site. Additionally, plants attacked by insects and pathogens display profound physiological, morphological and chemical changes or adaptations, which may result in organism attraction or avoidance. In this study, we also aimed to understand the insect-fungi association in sugarcane and the role of fungal volatile compounds in this association. Our results have shown that *D. saccharalis* positively influences *C. falcatum* infection on sugarcane, inducing a fast growing when compared to *C. falcatum* treatment without *D. saccharalis* attack. In addition, both fungi, *C. falcatum* and *F. verticillioides*, have been shown a double effect on *D. saccharalis* caterpillar, they promoted a strong attraction for insects due volatile organic compound emission and positively influenced *D. saccharalis* feeding and weight gain in diets supplemented with fungi. Fungal volatile organic compounds from *C. falcatum* and *F. verticillioides* were identified and quantified; acoradiene and acorenol were specifically induced by the fungi. These data suggest a synergistic interaction, mediated by organic volatile compounds, between *D. saccharalis* and the fungi *C. falcatum* and *F. verticillioides* in sugarcane.

Keywords: Sugarcane; SUGARWIN; BARWIN; *Colletotrichum falcatum*; *Fusarium verticillioides*; Chitinase; RNase; Sugarcane borer; Plant-insect-fungus interaction

## 1. INTRODUCTION

Plants are constantly submitted to a wide range of biotic stresses simultaneously and developed strategies to recognize insects attack and pathogen infections in order to reduce its damages [1-7]. Damage on plant by herbivore or pathogens can cause variation in plant chemical profile and metabolites, affecting the plant organic volatile and non-volatile compounds production and the nutrients profile from plants in addition to visual cues [8-10]. These changes can influence the insect behavior and pathogen infections, affecting the plant fitness [9-13].

Insects have been evolving sophisticated sensorial systems, which allow them to find sexual partners, food sources, habitat, oviposition sites and to escape from predators [14]. The olfactory system permits the insect to detect, identify and develop a behavior depending on a mixture of volatile compounds [15-17]. On the other hand, microorganisms such as fungi can directly produce volatile organic compounds (VOCs) or can indirectly induce plants to produce VOCs, which can affect insect attraction or repellence [18-20].

In addition to olfactory cues, visual cues are also important for insects to assist in the identification of food sources and oviposition places [21-24]. The infection by pathogens can interfere on plant color and morphology, which in turn affect insect choice [21, 25]. Some pathogens have also the ability to mimic parts of the plant to attract insects that will disseminate them [26, 27]. In other situations, plants can mimic fungus-infected foliage to attract pollinators, as such in the orchid *Cypripedium fargesii*, that shows blackish hairy spots on the upper surface of foliage to imitate black mold spots, using it as short-term visual cues [28].

Plant pathogens can affect herbivorous insects directly when they feed on mycelia or spores, absorbing their toxins, or indirectly due to changes on plant nutritional quality, being able to modify calcium, phosphorous, nitrogen levels, and amino acid composition [10, 29-31]. Furthermore, fungal enzymes, including those involved in the external digestion of plant polymers, have a role in plant-fungi-insect interaction, facilitating insect digestibility [32, 33].

Changes in plant metabolism can be favorable to insects [34]. For example, in peanut plants, the beet armyworm *Spodoptera exigua* prefer leaf tissue infected with white mold (*Sclerotium rolfsii*) due an increase in soluble sugars when compared to control plants [35]. The plant infection by pathogens can also negatively affect the associated insect resulting in avoidance of oviposition, decrease of larval development and increase of larval and pupal mortality [36]. The avoidance of insects to plant infected by pathogens can be result of plant metabolic changes, or due toxins produced by fungi, such as destruxins produced by *Alternaria brassicae* [37], enniatins produced by *Fusarium avenaceum*, and deoxynivalenol and zearalenone produced by *F. culmorum* [18]. Furthermore, the pathogen infection can result in plant nutritional deficiency, resulting in slower insect

development, when compared to healthy plants [38]. Another important nutritional compound for insects are the sterols. They are important to insects lipid biostructures as precursors to steroid hormones and as regulators of developmental processes [39]. However, insects are not able to produce sterols, therefore, they have to obtain it from their diets [39] or from fungi [40-42].

Plants response to pathogens and herbivore insects depends on the timescale, order of attack and plant genotype [43-45]. Rice plants infected by *Xanthomonas oryzae* pv. *oryzae* spend three days to show increased resistance to the insect *Nilaparvata lugens*, [21]. Furthermore, in herbivore-susceptible genotypes of cottonwood trees, the fungi *Drepanopeziza populi* infection reduced the herbivory more than in herbivore-resistant genotypes assuming that differences in resistance to herbivory was eliminated due the pathogen infection [45]. The combined impact on plant performance by herbivores and phytopathogens is usually additive [46]. In this way, plants can activate the defense against phytopathogens even when was first attacked by herbivorous insect [47].

In sugarcane, the attack of sugarcane borer, *Diatraea saccharalis*, precedes the infection by two opportunistic fungi *Fusarium verticillioides* and *Colletotrichum falcatum*. Sugarcane-attacked plants induce a large set of defense proteins, including proteins called SUGARWINs. SUGARWIN1 and SUGARWIN2 are defense proteins from sugarcane that show a signal peptide and a BARWIN-like domain [47]. BARWIN-like proteins have been described in a several plant species [47-59], and some of them have shown antifungal activity [52, 53, 55, 57, 58, 60, 61]. SUGARWIN1 and 2 are secreted proteins and are induced in response to *D. saccharalis*, methyl jasmonate and wound [47]. SUGARWIN2 does not affect insect development, however it promotes significant morphological and physiological changes in *Fusarium verticillioides* and *Colletotrichum falcatum*, which lead to fungal cell death by apoptosis, and may work as a first layer of defense against the fungi infection [47, 62].

The understanding of microorganism-insect-plant interactions is crucial, not only to assist the development of new genetic varieties but also to integrated pest management programs. In the first chapter of this study, we deepen our understanding of the role of SUGARWINs in plant defense and the molecular mechanisms by which these proteins affect fungi by elucidating their molecular targets. We also aimed to identify differences in *SUGARWIN* induction using different sugarcane varieties, and evaluated the gene induction in response to *C. falcatum* infection. We investigated if sugarcane varieties with a higher induction of *SUGARWIN*s are less susceptible to infection by *C. falcatum*. In the second chapter of this study, we investigated the close association between the opportunistic fungi and the sugarcane borer. We investigated whether the fungi *F. verticillioides* and *C. falcatum* establish mutualistic interaction with the sugarcane borer by testing

whether *D. saccharalis* herbivory influences fungal colonization in sugarcane; and whether fungal colonization affects *D. saccharalis* performance, feeding and olfactory behavior.

## 2. CONCLUSIONS

In the first chapter of this work, we identified that *SUGARWIN* genes are induced at different levels by *D. saccharalis* depending on the sugarcane variety. Nevertheless, *SUGARWIN* genes are also induced by *C. falcatum* infection in sugarcane. The sugarcane variety that exhibited high levels of *SUGARWIN* induction, was less susceptible to infection by *C. falcatum*, indicating that *SUGARWINs* could be linked to plant defense. Our enzymatic assays revealed that *SUGARWIN1* exhibits RNase and chitinase activity, whereas *SUGARWIN2* showed only chitinase activity. The distinct substrate specificity observed for *SUGARWIN1* and *SUGARWIN2* can be related to their divergent amino acid compositions. Taking all together, these results suggest a role of *SUGARWINs* in plant defense, by enzymatic activity.

In the second chapter of this work, we identified that *D. saccharalis* positively influences *C. falcatum* infection on sugarcane. The previously insect attack contributed to the fungus infection, causing a faster fungus growing when compared to infection in the absence of the caterpillar. Similarly, in artificial diets, the fungi *C. falcatum* and *F. verticillioides* seem to play a role on *D. saccharalis* attractiveness and feeding, the caterpillar performance was better in fungus-colonized diet relative to the control, nevertheless the *A. nidulans*-colonized diet showed a negative influence in *D. saccharalis* attraction and performance, indicating a specificity in insect-fungi interaction. *D. saccharalis* caterpillars are highly attracted to volatile compounds emitted by *F. verticillioides* and *C. falcatum*, such as acoradiene and acorenol. Our results showed a strong synergistic interaction between *F. verticillioides* and *C. falcatum* with *D. saccharalis* in sugarcane colonization, including a role of fungal VOCs in this association. These knowledge enhance our understanding of plant-insect-fungal interaction and may influence the use of integrated pest and disease management.

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