University of São Paulo
"Luiz de Queiroz" College of Agriculture

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

Flávia Pereira Franco

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

Piracicaba
2017

Flávia Pereira Franco
Agronomist

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Advisor:
Prof. Dr. **MARCIO DE CASTRO SILVA FILHO**

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Franco, Flávia Pereira

Unraveling sugarcane-*Diatraea saccharalis*-opportunistic fungi interaction in sugarcane / Flávia Pereira Franco. - - Piracicaba, 2017.

64 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Cana-de-acúcar 2. SUGARWIN 3. BARWIN 4. *Colletotrichum falcatum* 5. *Fusarium verticillioides* 6. Quitinase 7. RNase 8. Broca da cana 9. Interação planta-inseto-fungo I. Título

I dedicate:

To my parents Irairdes and Guido,

For the example of life, constant support

And unconditional love.

ACKNOWLEDGMENT

I thank my supervisor Prof. Dr. Marcio de Castro Silva Filho, for the reception, support, teachings, for sharing his enthusiasm and vision and, mainly, for the confidence deposited in me.

To Prof. Dr. José Mauricio S. Bento, Prof. Dr. Walter S. Leal and Prof. Dr. Daniel S. Moura for the enormous participation in this work, and for the great discussions during this time.

I thank all the others contributors envolved in these studies: Renata O. Dias, Adelita C. Santiago, Flávio Henrique-Silva, Maria Fernanda G.V. Peñaflor, Felipe G. Gonçalves. Without them I couldn´t have completed this work.

I also thank Rafael Colombi for technical assistance with sugarcane plantlets. Neide G. Zério and José R. P. Parra from the University of São Paulo for providing *D. saccharalis* caterpillars and S. M. Chabregas and C. Gonçalves of the Centro de Tecnologia Canavieira for providing sugarcane plantlets. Professor Gustavo H. Goldman from University of São Paulo and Dr. Paul Daly for providing the ball-milled sugarcane bagasse used in the diets and the technician Arodi Prado Favaris, and the trainee Emile Goutorbe for collaboration in bioassays involving volatile organic compounds.

I thank all the friends from the laboratory, that passed or that are still there, whom made the work environment a pleasant and joyful place, sharing protocols, experiences, knowledge and discussions as well as laughter and companionship. In special Thais P. Souza, Karina L. Lopes and Augusto Lima Diniz, that became special friends.

I thank the financial support, by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant 2014/50275-9 and 2013/12577-0, and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grant 482737/2012-3. In addition, the National Institute of Science and Technology (INCT) Semiochemicals in Agriculture (FAPESP 2008/57701-2 and CNPq Proc. 502 573761/2008-6).

Finally, I thank my family and my friends, for the support, love and fellowship in every moment of my life.

EPÍGRAFE

“Research is to see what everybody else has seen, and think what nobody has thought.

Genius is seeing what everyone else sees and thinking what no one else has thought.

Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

The task is, not so much to see what no one has seen yet; but to think what nobody has thought yet, about what everybody sees”.

Arthur Schopenhauer

SUMÁRIO

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: Cambridae), 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 *SUGARWIN*s 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 sinergistica, 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: Cambridae) 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 *SUGARWIN*s 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].

Furtermore, 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 phythopathogens even when was first attacked by herbivorous insect [47].

In sugarcane, the attack of sugarcane borer, *Diatraea sachharalis,* preceeds 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.

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2. STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF SUGARWINS AND THEIR ROLE IN PLANT DEFENSE

ABSTRACT

SUGARWIN1 and 2 are defense proteins from sugarcane. Their gene expression is known to be induced in response to wound and *Diatraea saccharalis* damage. Although the recombinant SUGARWIN protein does not affect insect development, it promotes significant morphological and physiological changes in *Fusarium verticillioides* and *Colletotrichum falcatum*, which lead to fungal cell death via apoptosis. 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 *SUGARWIN*s 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.

Keywords: Sugarcane; BARWIN; *C. falcatum*; Chitinase; RNase

2.1. INTRODUCTION

The plant defense system is under constant selective pressure to improve its response to pathogens and insect damage [1]. Pathogen recognition by plants activates the host defense response, resulting in cell wall fortification via callose and lignin synthesis, production of secondary metabolites such as phytoalexins that exhibit an antimicrobial effect, and accumulation of pathogenesis-related proteins (PR proteins) [2].

The pathogenesis-related protein-4 (PR-4) family is a group of proteins equipped with a BARWIN-like domain. This domain can be associated with a chitin-binding domain, also well known as the hevein-like domain. This association separates the family into PR4 classes I (with the hevein-like domain) and II (without the hevein-like domain) [3, 4].

BARWIN is a protein induced in barley by wounding or pathogens [5, 6]. Homologs of BARWIN have been identified in several plants, including tobacco [7], tomato [8], *Arabidopsis* [9], wheat [10], *Wasabia japonica* [11], maize [12], rice [13, 14], *Lycoris radiata* [15], apple [16], cacao [17], and pepper [18]. Our previous studies have identified two homologs of BARWIN in sugarcane: SUGARWIN1 and SUGARWIN2 [19].

In many plant species, homologs of the BARWIN protein are associated with the plant response to fungal infection and mechanical wounding [5, 8, 10, 12, 13, 15]. In addition to SUGARWIN, antifungal activity has been described to BARWIN-like proteins found in barley [5], *Wasabia japonica* [11], wheat [20], maize [12], rice [14], apple [16] and cacao [17].

PR-4 proteins are classified as chitinases [4, 21]; however, several studies have also reported RNase activity for BARWIN-like proteins [16-18, 22-25]. RNA-binding site have been described for WHEATWIN1 [22] and CARWIN [24]. Antifungal DNase activity was also observed, together with RNase activity, for the *Capsicum chinense* PR-4 protein [23] and the *Theobroma cacao* TcPR-4b protein [17].

The SUGARWIN1 and SUGARWIN2 proteins (*sugar*cane *w*ound-*in*ducible proteins) are believed to be part of a defense mechanism against pathogenic fungi in sugarcane plants [19, 26]. They are secreted proteins, induced in response to mechanical wounding, methyl jasmonate treatment, and *Diatraea saccharalis* attack [19]. However, recombinant SUGARWIN2 [19] has no effect on insect development but triggers changes in the hyphal morphology of *Fusarium verticillioides* and *Colletotrichum falcatum*, including increased vacuolization, multiple points of fracture, and extensive leaking of intracellular material, leading to cell death [19, 26]. The effect of SUGARWIN2 is specific to sugarcane pathogenic fungi and is not observed in fungi such as *Saccharomyces cerevisiae* and *Aspergillus nidulans,* which are unrelated to sugarcane diseases [26].

The borer *Diatraea saccharalis* (F.) (Lepidoptera: Pyralidae) is a major problem in sugarcane fields in Brazil, resulting in direct and indirect damage. *Colletotrichum falcatum* (Went) and *Fusarium verticillioides* (Sacc.) Nirenberg are responsible for indirect damages in sugarcane. These fungi take advantage of the openings produced by *D. saccharalis* to infect the plant [27]. In Brazil, usually, the presence of these insects and fungi are correlated; however, *C. falcatum* infestation in the absence of *D. saccharalis* has been reported in other countries, including India, Australia, Thailand, Fiji, and the US [28].

The goal of this study was to investigate the roles of SUGARWIN1 and 2 in sugarcane plant defense by identifying their enzymatic activity and molecular targets. We characterized both SUGARWIN1 and 2 as DNase, RNase or chitinase. Furthermore, molecular modeling allowed the identification of relevant binding-site positions via protein three-dimensional structure prediction. We have also demonstrated that sugarcane varieties with a higher induction of *SUGARWIN*s are less susceptible to infection by *C. falcatum*.

2.2. MATERIALS AND METHODS

2.2.1. Sugarcane variety, fungus and insects

Several genotypes of sugarcane (*Saccharum officinarum* x *Saccharum spontaneum*, cv. SP80-3280, SP80-1842, SP89-1115 e SP81-3250) were obtained from the Centro de Tecnologia Canavieira, Piracicaba, SP, Brazil. One-eyed sugarcane seed sets were disinfected with 0.01% chlorine, planted using a commercial planting mix (Plantmax, Eucatex, São Paulo, Brazil), and cultivated in a greenhouse under natural conditions.

Diatraea saccharalis caterpillars were reared on an artificial diet [29] and were maintained at 25 ± 4°C and 60% ±10% relative humidity with a light phase of 14 h. *Colletotrichum falcatum* isolates were cultivated in potato dextrose (PD) (Difco™, Sparks, USA) medium and maintained at 25°C.

2.2.2. SUGARWIN gene induction in different varieties of sugarcane

Forty-day-old plants from the sugarcane genotypes SP80-3280, SP80-1842, SP89-1115 and SP81-3250 were used to identify differences in *SUGARWIN* expression. The assay was performed according to a previously published protocol [19]. Third instar *D. saccharalis* caterpillars were starved for 12 h and individually placed on sugarcane seedling stalks. Plant material was collected 48 h after larval entry into the stalk region from approximately two centimeters around the point of inoculation and was frozen immediately. The control plants were left undisturbed, and was collected at 0 h and at 48 h. A pool of six plants per treatment was used, and the experiment was repeated twice. Analysis of *SUGARWIN1* and *2* gene expression was performed as described in the next section, and the varieties exhibiting the greatest contrast in *SUGARWIN1* and *2* expression levels were selected for use in the *C. falcatum* quantification assay.

2.2.3. RNA isolation, cDNA synthesis and qRT-PCR

Total RNA from the sugarcane tissue was isolated with TRIZOL reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions, followed by DNA removal by treatment with 2 units of RNase-free DNase I (Fermentas, Vilnius, Lithuania) for 20 min at 37°C. Then, the RNA was re-extracted with TRIZOL reagent to remove any trace of DNA or

DNase. Total RNA samples were quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, USA), and their quality was assessed by agarose gel electrophoresis. First-strand synthesis was performed using ImProm-II Reverse Transcriptase (Promega Corp., Madison, USA) according to the manufacturer's instructions.

Quantitative real-time PCR was performed using a StepOne™ Real-Time PCR system (Applied Biosystems, Waltham, USA) and Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, Vilnius, Lithuania). Gene-specific primers for *SUGARWIN1*, *SUGARWIN2* and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as described in [19], the primers for rRNA 25S was used as described in [30]. GAPDH and rRNA 25S were used as endogenous controls and exhibited the same pattern of regulation; therefore, GAPDH was chosen as the reference gene. The reference genes used in this work were re-validated under experimental conditions. The amplification efficiencies and relative expression levels were calculated as described in [31] using REST 2008 software, showing primers efficiencies ranging from 90% to 99%.

2.2.4. *Colletotrichum falcatum* inoculation in different sugarcane varieties

Sixty-day-old sugarcane varieties were inoculated with $1x10^3$ *C. falcatum* conidia. The seedlings remained in a moist chamber in a greenhouse for 12 h and then were maintained for 10 days under natural conditions without supplemental artificial light. The control plants were left undisturbed, and were collected at 0 h, in the beginning of the experiment, and after 10 d of experiment. Samples were collected from the stalk region. A pool of six plants per treatment was used, and the experiment was repeated twice. The plant material collected was immediately frozen in liquid nitrogen for later transport to the laboratory and processing or storage at -80°C.

2.2.5. Plasmid standard curve for *Colletotrichum falcatum* quantification in sugarcane plants

A plasmid standard curve was used to quantify *C. falcatum* contamination in sugarcane. This methodology uses a plasmid standard curve to quantify the number of target gene copies per PCR reaction. The number of plasmid molecules was determined using the following equation: $\text{Copies}/\mu\text{L} = \text{L} \cdot \left(\frac{C}{m \cdot N}\right)$, where L represents Avogadro's constant (6.022×10^{23}

molecules/mol), C is the concentration of DNA in g/μL, m is the molecular weight of one bp of DNA (660 g/mol), and N is the size of the plasmid in base pairs.

First, *C. falcatum* conidia were grown in liquid PD medium for 48 h at 25°C and 250 rpm. Mycelia were collected by vacuum filtration, and after appropriate drying, they were frozen in liquid nitrogen and then macerated. DNA extraction was performed according to the method described in [32]. The plasmid standard curve was constructed by ligating an ITS (rDNA internal transcribed spacer) gene fragment from *C. falcatum* into the pCR2.1 commercial plasmid vector from the TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The ITS sequence was obtained from NCBI (gene bank accession number EU554112.1), and specific primers were designed using the program *OligoPerfect™ Designer* (Forward 5' - GATGAAGAACGCAGCGAAAT – 3' and Reverse 5' - AACGGATCTCTTGGTTCTGG – 3'). Plasmid DNA extraction was performed using a Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions and was quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, USA). The plasmid was sequenced to confirm its transformation and fragment orientation. Reference plasmid DNA was diluted (1:5; 1:25; 1:125; 1:625; 1:3125) to obtain plasmid genome equivalents for standard curve analysis. Quantification of *C. falcatum* in sugarcane plants was performed using specific ITS primers and the Standard Curve method using a PCR StepOne™ Real-Time PCR system (Applied Biosystems, Waltham, USA) and Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas). The standard curves consistently demonstrated correlation coefficients (R^2) of 0.99 and PCR efficiencies ranging from 90% to 100% when analyzed using StepOne™ Software, version 2.0 (Applied Biosystems, Waltham, USA).

2.2.6. Sugarcane genomic DNA extraction

Sugarcane DNA extraction was performed according to the methods described in [33] and was followed by treatment with RNase for 1 h at 37°C. DNA extraction was performed a second time using only chloroform:isoamyl alcohol (24:1) to remove the residual phenol from the samples. Total DNA was quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, USA), and the quality was assessed with agarose gel electrophoresis.

2.2.7. Ribonuclease activity assay

Ribonuclease activity was assessed as described in [34] with few modifications. Total RNA was isolated from *C. falcatum* with TRIZOL® Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The detection of RNase activity of the recombinant SUGARWIN1 and SUGARWIN2 [19] was performed at room temperature using 12 μg RNA and purified protein in amounts varying from 2 to 16 μg in 10 mM Tris–HCl, pH 7.5, with 10 mM imidazole and 5 mM NaCl. After 1 h of incubation, the results were observed on a 1% agarose gel. Heat-inactivated SUGARWIN1 and 2 were used as controls.

2.2.8. Deoxyribonuclease activity

The deoxyribonuclease activity assay was performed as described in [23] with few modifications. To determine the DNase activity of recombinant SUGARWIN1 and SUGARWIN2 [19], 12 μg of *C. falcatum* DNA was incubated with the purified protein (2 to 16 μg) in a total volume of 25 μl in the presence of 10 mM Tris-HCl, pH 7.5, with 10 mM imidazole and 5 mM NaCl, in either the presence or absence of 2.5 mM $MgCl_2$, for 1.5 h at room temperature. The results were observed on a 1% agarose gel.

2.2.9. Chitinase activity

Recombinant SUGARWIN1 and SUGARWIN2 [19] samples were added to SDS–PAGE sample buffer and heated at 100°C for 10 min. The proteins were separated on a 10% polyacrylamide gel containing 0.01% glycol chitosan (Sigma-Aldrich, St. Louis, USA). The gel was cut in two parts; the negative control was not immersed in a refolding buffer, and the other part of the gel was immersed in a refolding buffer (50 mM Tris–HCl pH 7.5, 1% Triton X-100) at 37°C overnight. The gel was washed with distilled water and then stained with 0.01% (w/v) calcofluor white M2R in 10 mM Tris–HCl (pH 7.5). After 5 min, the brightener solution was removed, and the gel was washed with distilled water. Protein activity was visualized by placing the gels on a UV transilluminator [35]. The two parts of the gel were photographed together.

2.2.10. SUGARWIN structure prediction and protein-ligand docking analysis

Three-dimensional structure prediction of both SUGARWIN proteins was performed using MODELLER software v9.14 [36]. For this analysis, the structure of a homologous *Carica papaya* BARWIN-like protein (CARWIN - PDB: 4JP7, 1.05 Å) [24] exhibiting 76 and 71% identity to SUGARWIN1 and SUGARWIN2, respectively, was used as a template. Quality assessment of the predicted structures was performed using PROCHECK software [37], and the resulting Ramachandran plots are presented in Supplementary Material 1 and Supplementary Material 2. The structures were prepared for docking analysis using UCSF Chimera software v 1.10.2 [38], in which 1000 steepest descent steps for energy minimization were conducted to remove unfavorable hydrogen contacts.

The putative mode of interaction between SUGARWINs and chitosan was predicted using AutoDock Vina v 1.1.2 software. The chitosan oligosaccharide structure was obtained from the PubChem database [39] (CID: 3086191). The protein and ligand structures were prepared for docking using AutoDockTools v1.5.6 [40]. Docking analyses were performed in two steps. First, "blind docking" was conducted, in which a large grid box (80 x 80 x 80 grid points, with a grid spacing of 1 Å) was established around the protein, allowing an unbiased search of the entire protein surface for the putative binding site. Second, "local docking" was conducted to more finely search the binding site selected in the first step. Local docking was conducted using a smaller grid box (20 x 20 x 20 grid points with 1 Å of spacing) centered on the predicted ligand-binding site. For each docking analysis, a total of 50 independent runs were performed, generating 500 putative ligand positions. The ligand position presenting the greatest predicted affinity to the target protein was selected for subsequent analysis. All structures were visualized using PyMOL software [41].

2.3. RESULTS

2.3.1. The *SUGARWIN*1 and 2 genes are differentially induced in sugarcane varieties challenged by *Diatraea saccharalis* caterpillar

Our previous work showed that *SUGARWIN*s are induced by *Diatraea saccharalis*, methyl jasmonate and wounds (Medeiros et al., 2012). In this work, we aimed to identify the influence of *SUGARWIN*s in *Colletotrichum falcatum* contamination in sugarcane; therefore, we selected sugarcane varieties with different patterns of *SUGARWIN* gene expression. We used

our previous knowledge about *SUGARWIN* gene expression in plants under *D. saccharalis* attack to select the sugarcane varieties.

To evaluate *SUGARWIN1* and *SUGARWIN2* gene induction in different sugarcane varieties, we exposed SP80-3280, SP80-1842, SP89-1115 and SP81-3250 plants to the caterpillar of *Diatraea saccharalis*. We observed differing levels of *SUGARWIN1* up-regulation in all varieties tested (Fig. 1A). After 48 h of caterpillar attack, SP80-1842, SP89-1115 and SP81-3250 plants increased their *SUGARWIN1* mRNA levels in leaf stalks by up to 523, 315 and 518 times the levels found in the leaf stalks of 0 h control plants. The variety exhibiting the smallest mRNA induction level when attacked by *D. saccharalis* was SP80-3280, which increased its *SUGARWIN1* mRNA level approximately 66 times compared with 0 h control plants. The 48 h control plants showed no significant difference when compared to the 0 h control.

The *SUGARWIN2* gene was also up-regulated following *D. saccharalis* attack in all sugarcane varieties evaluated (Fig. 1B). The increase in mRNA levels observed for the *SUGARWIN2* gene in SP80-1842 were the highest at approximately $2.5x10^3$ times the levels found in the leaf stalks of 0 h control plants. The SP89-1115, SP81-3250 and SP80-3280 varieties exhibited lower levels of *SUGARWIN2* mRNA induction following *D. saccharalis* attack, with 457, 218 and 340 times the 0 h control levels, respectively, the 48 h control plants showed no significant difference when compared to the 0 h control (Fig. 1B). Based on the up-regulation of both *SUGARWIN* genes upon caterpillar attack, we selected the sugarcane varieties SP80-3280 and SP80-1842 as the low-*SUGARWIN* and high-*SUGARWIN* varieties, respectively.

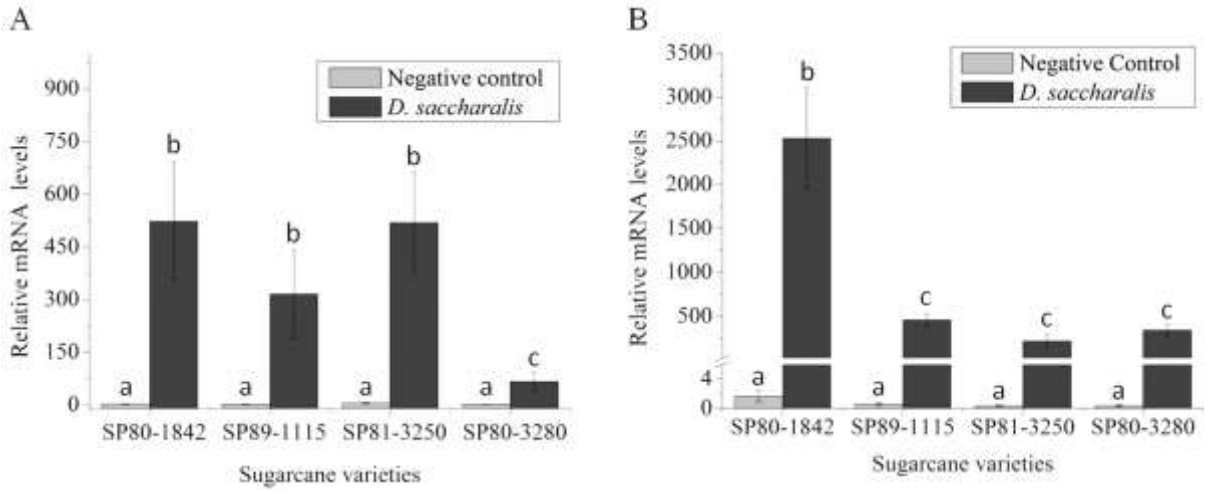

Figure 1. *SUGARWIN* gene expression in different sugarcane varieties. (A) *SUGARWIN1* and (B) *SUGARWIN2* gene expression after 48 h of *Diatraea saccharalis* infestation. Expression was quantified by qRT-PCR, and the values are presented as the mean (± standard error) transcript levels of three technical replicates normalized to the abundance of GAPDH. Gene expression was calculated using REST 2008 software [31]. Different letters indicate significant differences between treatments ($P \leq 0.05$).

2.3.2. High- and low-SUGARWIN varieties exhibit differential effects on *Colletotrichum falcatum* infection

Both the high-*SUGARWIN* (SP80-1842) and low-*SUGARWIN* (SP80-3280) varieties were infected with *C. falcatum* fungus, and the levels of *SUGARWIN1* and *2* mRNA were evaluated. The sugarcane variety SP80-1842 exhibited 2- and 100-fold inductions in *SUGARWIN1* and *SUGARWIN2* mRNA levels, respectively, after 10 d of *C. falcatum* treatment when compared with the 0 h control (Fig. 2A and B). The low-*SUGARWIN* variety exhibited 0.6- and 8-fold inductions in *SUGARWIN1* and *SUGARWIN2* mRNA, respectively, after 10 d of *C. falcatum* treatment, when compared with the 0 h control plants (Fig. 2A and 2B); however, the induction of *SUGARWIN1* in this sugarcane variety was not significantly different from the control (Fig. 2A). The 10 d control plants showed no significant difference when compared to the 0 h control. To monitor the growth of the fungus, we also performed a *C. falcatum* quantification in infected plants and control plants. In the high-*SUGARWIN*, *C. falcatum* growth was approximately half that observed in the low-*SUGARWIN* variety (Fig. 2C), showing an inverse correlation between *SUGARWIN* induction and *C. falcatum* infection.

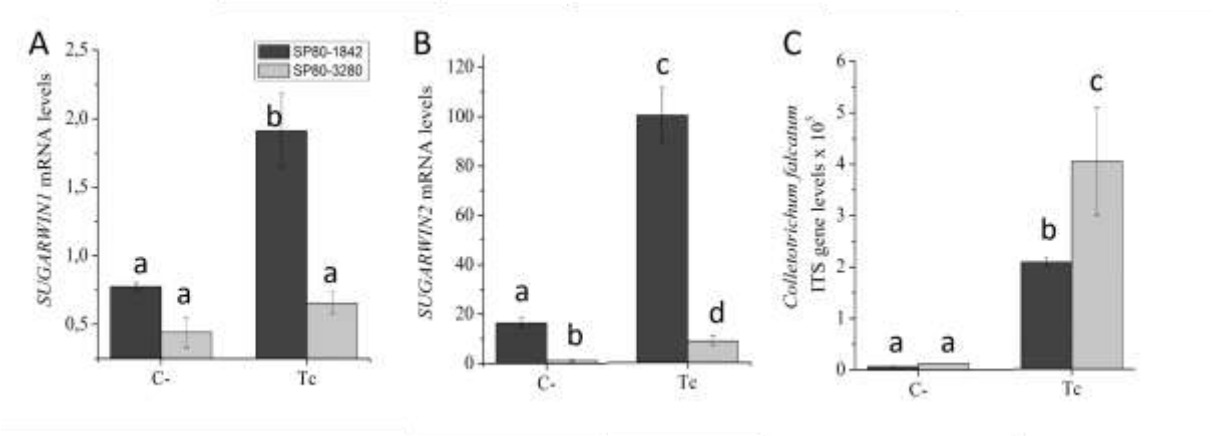

Figure 2. *SUGARWIN* gene expression and *Colletotrichum falcatum* quantification in sugarcane. (A) *SUGARWIN1* and (B) *SUGARWIN2* gene expression after 10 d of treatment with *C. falcatum* (Tc) or without any treatment (C-). Gene expression was quantified by qRT-PCR, and the values are presented as the mean (± standard error) transcript levels of three technical replicates normalized to the abundance of GAPDH. Regulation of expression was calculated using REST 2008 software [31]. Different letters indicate significant differences between treatments ($P \leq 0.05$). (C) Quantification of *C. falcatum* was performed by qRT-PCR using the standard curve method and the ITS gene. The values are the mean (± standard error) of three technical replicates. Different letters indicate significant differences between treatments, as determined using a *t*-test ($P \leq 0.05$).

2.3.3. SUGARWIN1 and SUGARWIN2 enzymatic activity

To understand the SUGARWIN mechanism of action, recombinant SUGARWIN1 and SUGARWIN2 [19] proteins were used to perform enzymatic assays for three common BARWIN molecular targets: RNA, DNA, and chitin. To perform DNAse and RNase assays, the proteins were tested in different concentrations. Only SUGARWIN1 was able to degrade RNA, with increased activity at higher protein concentrations (Fig. 3A and Supplementary Material 3). The control, using PBS or heat-inactivated protein, did not show any change in RNA integrity. Neither SUGARWIN1 nor SUGARWIN2 was able to degrade the DNA in any tested concentration (Fig. 3B).

To test the chitinase activity of the SUGARWINs, we used a glycol-chitosan substrate in an SDS-PAGE gel. (Fig. 3C - left panel) shows the SDS-PAGE stained with Coomassie blue after staining with calcofluor to detect chitinase activity (Fig. 3C - right panel). Our results showed that both SUGARWIN1 and SUGARWIN2 produced a dark band when stained with calcofluor, indicating substrate degradation and confirming chitinase activity for both proteins. It was possible to identify this activity after gel immersion in a refolding buffer. The control, heat inactivated protein that was not immersed in the refolding buffer, did not show any activity (Fig. 3C - right panel).

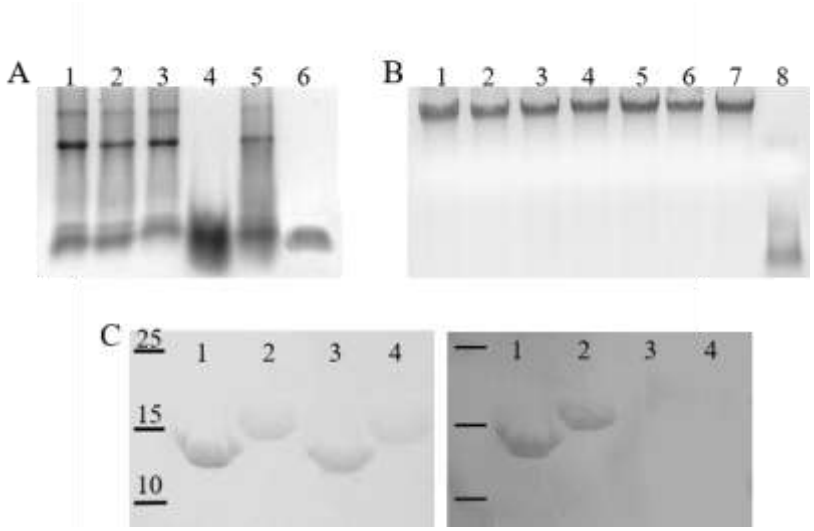

Figure 3. SUGARWIN1 and SUGARWIN2 enzymatic assay. (A) RNase assay and (B) DNase assay. For (A): (1) Negative control (PBS), (2) heat-inactivated SUGARWIN1, (3) heat-inactivated SUGARWIN2, (4) active SUGARWIN1, (5) active SUGARWIN2, and (6) positive control with RNase. For (B): (1) Negative control (PBS), (2) heat-inactivated SUGARWIN1, (3) heat-inactivated SUGARWIN2, (4) active SUGARWIN1, (5) active SUGARWIN2, (6) active SUGARWIN1 + $MgCl_2$, (7) active SUGARWIN2 + $MgCl_2$ (8) positive control with DNase. The RNase assay was performed for 1 h and the DNase assay for 1.5 h. (C) Chitinase assay. For both panels: (1) active SUGARWIN1 (14.2 kDa), (2) active SUGARWIN2 (16.4 kDa), (3) SUGARWIN1 without passing through the refolding buffer (negative control), and (4) SUGARWIN2 negative control. The left panel shows an SDS-PAGE gel for SUGARWIN1 and SUGARWIN2 stained with coomassie blue after the enzymatic assay, and the right panel shows a chitinase assay performed after SDS-PAGE containing 0.01% (w/v) glycol-chitosan substrate.

2.3.4. SUGARWIN structural characterization

To understand the different substrate specificities observed during SUGARWIN analysis, the three-dimensional structure of both proteins was predicted by homology modeling. Several divergent amino acid positions were observed between SUGARWIN1 and SUGARWIN2, some of which were located on the surface of the predicted structures (Fig. 4). The involvement of these surface positions as putative ligand-binding sites was further analyzed using docking strategies.

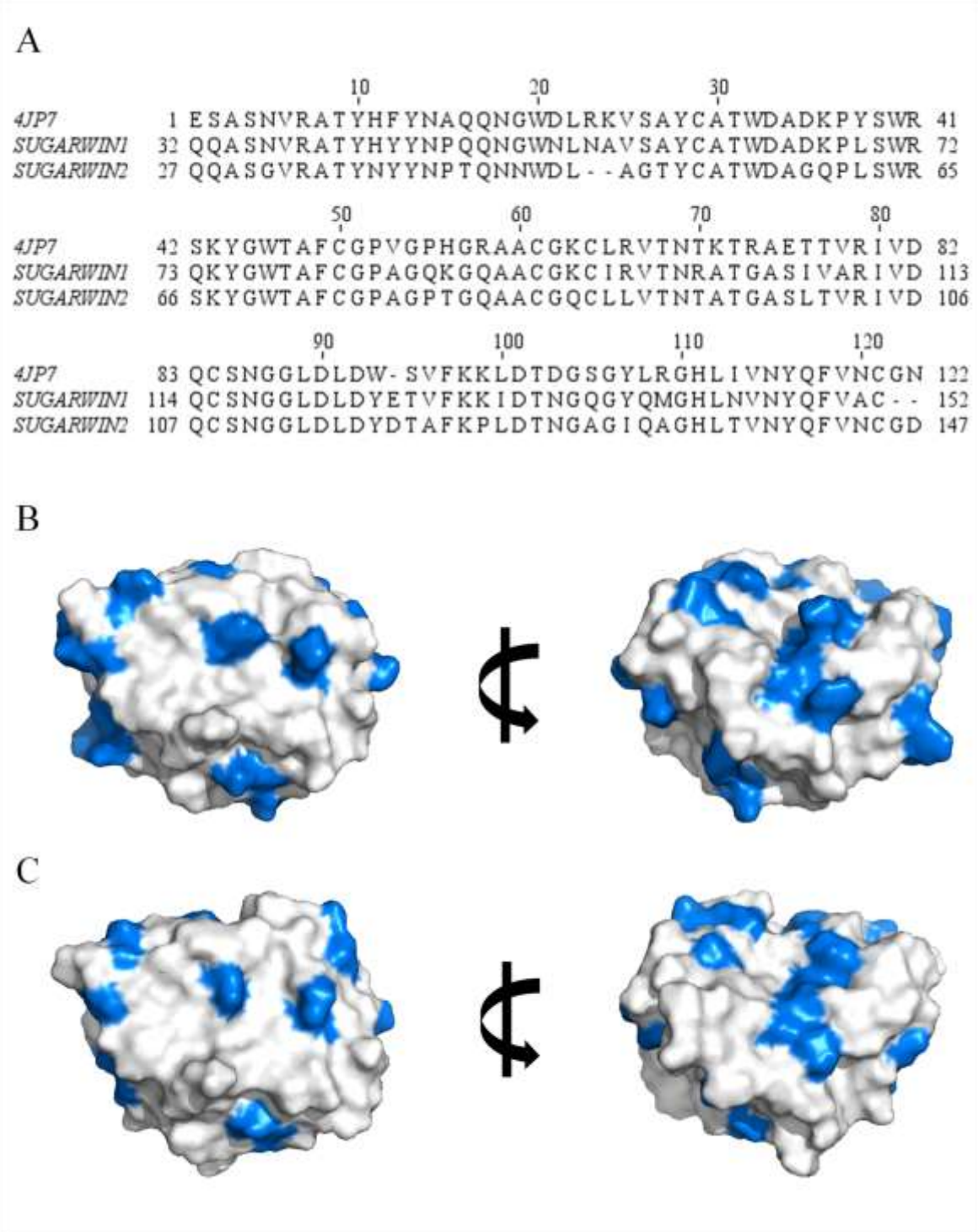

Figure 4. Comparison of the primary and tertiary protein structures of SUGARWIN1 and SUGARWIN2. (A) Amino acid sequence alignment between SUGARWINs and CARWIN (PDB: 4PJ7). The predicted tridimensional structures of (B) SUGARWIN1 and (C) SUGARWIN2. Divergent amino acid positions are highlighted in blue.

Unbiased searches for a chitosan-binding site in the predicted models produced similar results for both SUGARWINs. Eleven common amino acid positions (numbered according to the CARWIN template) – 9 (Thr), 11 (His/Asn), 13 (Tyr), 26 (Ser/Gly), 27 (Ala/Thr), 28 (Tyr),

82 (Asp), 83 (Gln), 84 (Cys), 85 (Ser) and 90 (Asp) – were contacted by chitosan in both proteins by bonded and non-bonded contacts (Fig. 5). These results highlighted the presence of a highly similar putative chitosan-binding motif in both SUGARWINs. However, some differences were observed in the docking results, with two amino acid positions exclusively contacted by chitosan in SUGARWIN1 (Arg7 and Tyr10), and two others in SUGARWIN2 (Asn86 and Leu88). The chitosan putative mode of interaction was also variable in terms of contact types between proteins and ligand. Hydrogen bonds were predicted between chitosan and the amino acid positions Arg7, Asp82, Gln83 and Ser85 of SUGARWIN1 and the positions Asn11, Tyr13, Tyr28, Asp82, Ser85 and Asn86 of SUGARWIN2 (Fig. 5).

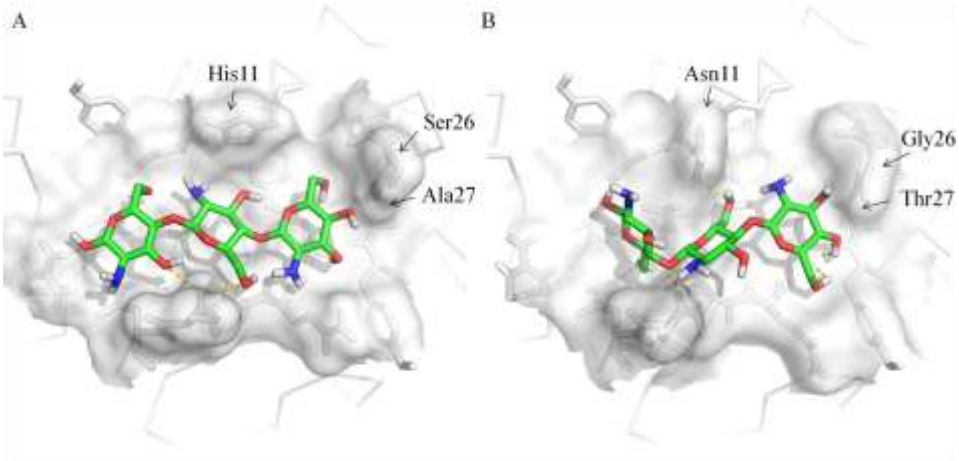

Figure 5. Putative modes of interaction of (A) SUGARWIN1 and (B) SUGARWIN2 with chitosan.

2.4. DISCUSSION

Plants are constantly being attacked by insects and pathogens and have developed sophisticated strategies to protect themselves. In a previous study, we identified two insect-induced genes homologous to *BARWIN* in sugarcane, called *SUGARWIN1* and *SUGARWIN2*. *SUGARWIN* genes are induced in sugarcane (SP80-3280) in response to insects, wounding and methyl jasmonate [19]. However, the protein causes morphological and physiological changes in *C. falcatum* and *F. verticillioides* fungi [19, 26]. In this work, we found that *SUGARWIN* genes are induced at different levels by *D. saccharalis* depending on the sugarcane variety (Fig. 1). Nevertheless, *SUGARWIN* genes are also induced by *C. falcatum* infection in the sugarcane varieties SP80-1842 and SP80-3280 (Fig 2A and 2B). Interestingly, the sugarcane variety SP80-1842, which exhibited high levels of *SUGARWIN* induction, was less susceptible to infection by *C. falcatum*, indicating that *SUGARWINs* could be linked to plant defense (Fig. 2A). This pattern

of plant response has been observed in other plants, such as *Pseudotsuga menziesii* [42], rice [43] and lentil [44]. The varieties that show a pattern of high PR4 gene induction after pathogen infection show higher tolerance to the pathogen than varieties with low gene induction [42-44]. CaPR4c, a BARWIN-like protein from pepper, was overexpressed in transgenic *Arabidopsis* plants and conferred greater resistance against pathogen infection [18]. *SUGARWIN* gene induction was lower in sugarcane plants when infected with *C. falcatum* than when attacked by *D. saccharalis*. This can be result of a higher damage caused by the caterpillar, considering that *SUGARWIN* genes are also induced by mechanical wounding [19], and the mechanical damage caused by *C. falcatum* is lower when compared to *D. saccharalis* mechanical damage.

The PR4 proteins are grouped into class I and class II based on the presence or absence of a chitin-biding domain [4]. These proteins are classified as chitinases due to the chitinase activity shown by a tobacco protein from the class I group [45]. Others works revealed that PR4 proteins from class II, which show only a BARWIN domain, exhibit RNase activity [16, 18, 22]. In some cases, the RNase and DNase activities occur in parallel [17, 23]. Cysteine proteinase inhibitor activity was also identified along with RNase activity in the pepper CaPR4c protein [18]. SUGARWINs are homologs of PR4 class II [19]; however, our enzymatic assays revealed that SUGARWIN1 exhibits RNase and chitinase activity (Fig. 3A and 3C), whereas SUGARWIN2 showed only chitinase activity (Fig. 3C). Both the RNase and chitinase assays showed that protein folding is important for enzymatic activity, since denaturation by heating caused lose of activity (Fig. 3A and 3C). DNase activity was not observed for either SUGARWIN1 or SUGARWIN2 (Fig. 3B). Divalent metal cations are usually required by plant DNases [46], however, even when in the presence of $MgCl_2$ SUGARWINs showed do not affect DNA integrity. The chitinase activity of SUGARWIN2 described in this work corroborates our previous work, in which dramatic changes, including cell wall rupture, were identified in fungi after SUGARWIN2 treatment [19]. These results differ from the results for other BARWIN-like proteins, WHEATWIN1, for example, which did not affect the fungal cell wall and exhibits only RNase activity [22]. These differences in enzymatic activity can be the result of variations in amino acid residues.

The distinct substrate specificity observed for SUGARWIN1 and SUGARWIN2 can be related to their divergent amino acid compositions. The identified chitosan binding site (Fig. 5) has already been implicated in the RNase activity of other BARWINs [22, 24]. Three amino acid positions contacted by chitosan in the docking analysis differ between SUGARWIN1 and SUGARWIN2: positions 11, 26, and 27 are composed of His, Ser and Ala in SUGARWIN1 and

Asn, Gly and Thr in SUGARWIN2, respectively (Fig. 5). These changes are capable of altering the binding site charge and shape and can be involved in SUGARWIN1 RNase activity.

BARWIN RNase activity has been correlated with the presence of two histidine residues: one at position 11 and another at position 111 (relative to the CARWIN structure) [22, 24, 34]. Two different mutations in His11 (H11G and H11L) have been shown to partially inhibit RNase activity in WHEATWIN [22], revealing the importance of this amino acid for RNase activity. Both SUGARWINs have the His111 residue; however, only SUGARWIN1 exhibits the His11 residue (Fig. 4). These observations raised the hypothesis that Asn11 is responsible for the absence of RNase activity in SUGARWIN2. Our results suggest that the ribonuclease activity of SUGARWIN1 occurs according to the classical acid-base mechanism that involves two His residues, similar to RNase A, T1, and wheatwins [34].

Taking all togheter, these results suggest a role of SUGARWINs in plant defense, by enzymatic activity. More studies involving high-SUGARWIN varieties in plant defense should be performed to consider the relevance of these proteins in controlling red rot disease caused by *C. falcatum*.

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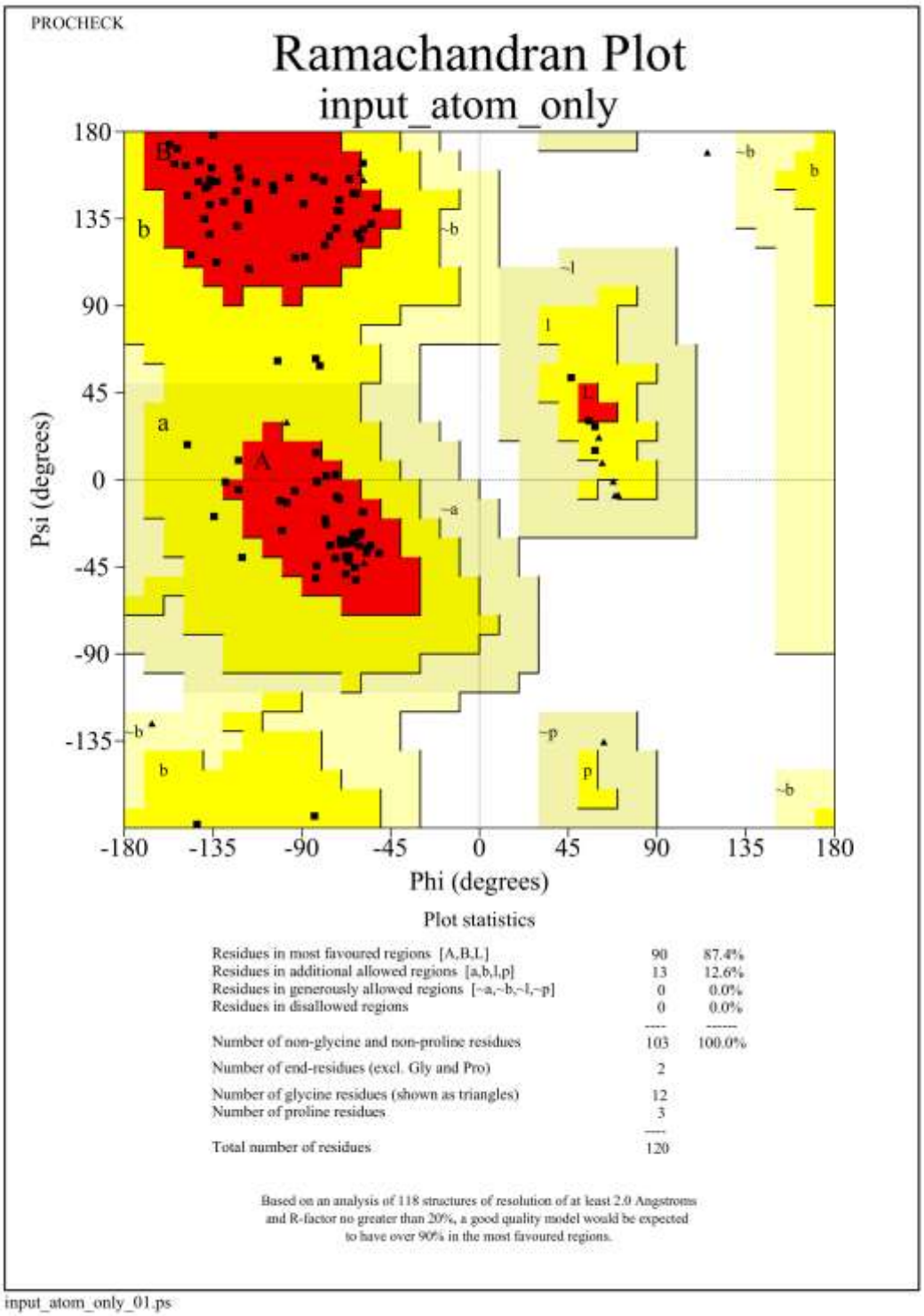
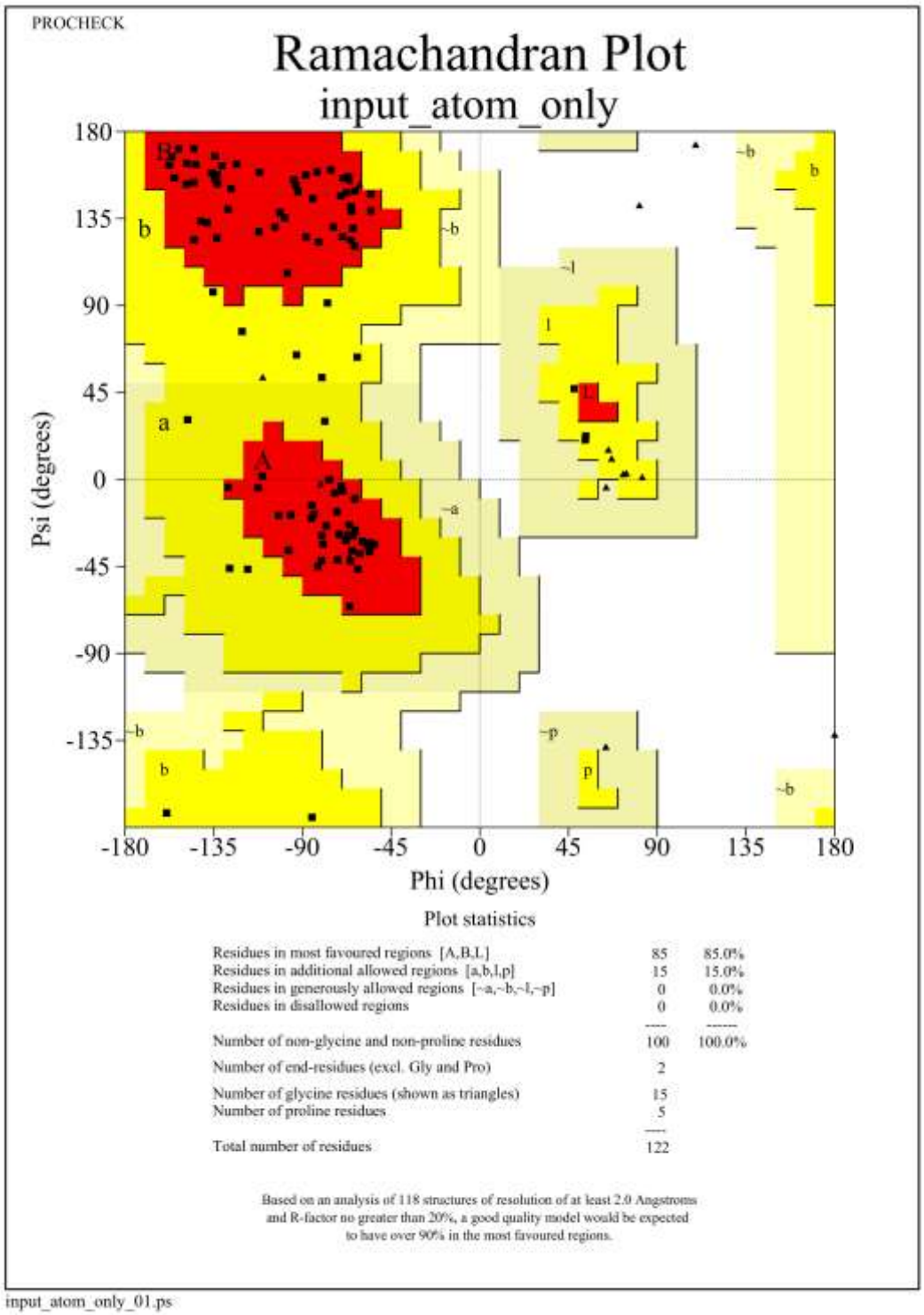
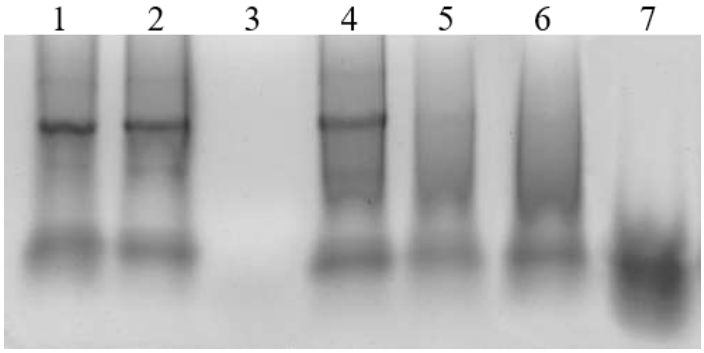
Supplementary Material 1. Ramachandran plot of the predicted SUGARWIN1 tridimensional structure.



Supplementary Material 2. Ramachandran plot of the predicted SUGARWIN2 tridimensional structure.



Supplementary Material 3. The RNase activity of SUGARWIN1. (1) Negative PBS control, (2) SUGARWIN1 (heat-inactivated), (3) Positive RNase control, (4-7) Active SUGARWIN1, (4) 2 μg (5) 4 μg, (6) 8 μg, and (7) 16 μg.



3. FUNGAL VOLATILE ORGANIC COMPOUNDS ATTRACTIVE TO *Diatraea saccharalis* (F.) (LEPIDOPTERA: PYRALIDAE) ARE PRESENT IN A SYNERGISTIC INTERACTION OF OPPORTUNISTIC FUNGI AND INSECT

ABSTRACT

Colonization of sugarcane stalk by opportunistic fungi, such as *Fusarium verticillioides* and *Colletotrichum falcatum*, usually occurs after *Diatraea saccharalis* (Lepidoptera: Cambridae) caterpillars attack. Insects and fungi interactions have been studied in different plants and some fungal volatile compounds have shown to influence insect behavior. In this study, we aimed to understand the insect-fungi association in sugarcane and the role of fungal volatile compounds in this association, investigating whether *(i) D. saccharalis* herbivory influences fungal infection in sugarcane; and *(ii)* fungal colonization affects *D. saccharalis* performance and feeding. 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: *Colletotrichum falcatum*; *Fusarium verticillioides*; Sugarcane borer; Plant-insect-fungus interaction

3.1. INTRODUCTION

Plants are constantly exposed to pathogens and insects attack, keeping the plant defense system constantly under surveillance to protect itself against aggressors. The plant-microorganism and plant-insect interaction can influence the plant defense response and, consequently, influence insect behavior or pathogen infection [1, 2].

Plants colonization by fungi can establish detrimental or beneficial associations with insect herbivores by changing their behavior and biology [3-10]. Several environmental cues affect insect behavior and these cues can be altered by plant-pathogens interaction, due to changes in plant nutritional profile, organic volatile compound emission or plant phenotype, resulting in insect attraction or repellence [7, 9-17]. Moreover, insects are not able to produce sterols, and can use fungi mycelia and spores as sterol source [18-20]. On the other hand, insect

can also have a role in fungi infection, for example, necrotrophic fungi can grow faster in tissues previously attacked by insects due to cell rupture [15]. Furthermore, insects can carry fungi spores to long distances [20-23] or can facilitate microorganism entrance, due to wound made by the insect [15, 24].

In the same plant-insect system, several species of microorganisms can differently affect insect behavior. For instance, on wheat kernels species of *Fusarium,* such as *F. proliferatum, F. poae* and *F. culmorum* are attractive to *Tenebrio molitor* larvae [9]. In contrast, *F. avenaceum* was shown to repel the same insect [9]. Repellent volatiles may represent survival threat signals for the larvae resulting in their avoidance [9]. In addition, insect attraction by fungal volatiles may represent a beneficial interaction for both organisms, in which insects find a food source and the fungi a way to disseminate itself [20].

The sugarcane infection by *Fusarium verticillioides* and *Colletotrichum falcatum* usually occurs in association with *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae). The fungi take advantage of the openings made by the borer to penetrate the sugarcane stalk and infect the plant, resulting in addition damage to the culture [25, 26]. Previously work have shown that when attacked by *D. saccharalis*, sugarcane can induce defense proteins with antifungal activity [24, 27]. Interestingly, these proteins (SUGARWIN1 and SUGARWIN2), showed to affect *F. verticillioides* and *C. falcatum* morphology causing fungi death, however, they were not able to affect *Aspergilus nidulans*, a non-sugarcane pathogenic fungi, indicating a close and specific interaction between *D. saccharalis* with *C. falcatum* and *F. verticillioides* in sugarcane [24, 27].

Because of the close association between the opportunistic fungi and the sugarcane borer, in this work we investigated whether the fungi *F. verticillioides* and *C. falcatum* establish mutualistic interaction with the sugarcane borer by testing the following hypotheses: *(i) D. saccharalis* herbivory influences positively fungal colonization; and *(ii)* fungal colonization affects *D. saccharalis* performance, feeding and olfactory behavior. We analyzed how fungi and *D. saccharalis* interact by measuring the development of *C. falcatum* in sugarcane previously attacked by *D. saccharalis* caterpillars, and the other way around, i.e. parameters of *D. saccharalis* feeding and orientation behavior to fungus-colonized diet. We tested if caterpillar orientation was mediated by fungal volatiles and characterized the volatile profile of fungus-colonized diet. Our results showed a strong influence of *D. saccharalis* in sugarcane contamination by *C. falcatum* as well as an influence of the fungi *C. falcatum* and *F. verticillioides* in *D. saccharalis* development and attractiveness by fungi volatile organic compounds affecting insect behavior. These data suggest a mutualistic interaction between these organisms.

3.2. MATERIALS AND METHODS

3.2.1. Sugarcane cultivation, fungus culture and insect rearing

Genotype of sugarcane (*Saccharum officinarum* x *Saccharum spontaneum*, cv. SP80-3280) was obtained from the 'Centro de Tecnologia Canavieira' (Piracicaba, SP, Brazil). The sugarcane one-eyed seed sets were disinfected with 0.01% chlorine, cultivated in commercial planting mix (Plantmax, Eucatex, São Paulo, Brazil) and maintained in a greenhouse for 60 days under natural oscillations of light, temperature and air humidity.

Isolates of *F. verticillioides* and *C. falcatum* were cultivated in potato dextrose (PD) medium (Difco™, Sparks, USA) and maintained at 25°C with 12h photoperiod in climatic chambers. The fungus not involved in the red rot disease, *Aspergillus nidulans*, used as a control in the assays, was cultivated in yeast glucose (YG) medium [0.5% yeast extract, 2% glucose, and 0.1% trace elements (75 mM $ZnSO_4 \cdot 7H_2O$, 180 mM H_3BO_3, 25 mM $MnCl_2 \cdot 4H_2O$, 18 mM $FeSO_4 \cdot 7H_2O$, 6 mM $CoCl_2 \cdot 5H_2O$, 6 mM $CuSO_4 \cdot 5H_2O$, 1 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 140 mM EDTA) at pH 6.7] and maintained in climatic chambers at 37°C in the dark.

The sugarcane borer caterpillars were fed on an artificial diet [28] and maintained in rooms under controlled conditions (temperature 25±4°C, relative humidity 60±10% and 14 h of light). Adults were maintained in cages covered with white paper sheets, where eggs were deposited and collected daily. Newly-hatching caterpillars were transferred to sterile artificial diet.

3.2.2. Influence of *D. saccharalis* in sugarcane infection by *C. falcatum*

We tested *C. falcatum* infection in sugarcane seedlings under two treatments: Tc: undamaged sugarcane; T(D+C): *D. saccharalis* damaged sugarcane. In Tc, 60-day-old sugarcane seedlings were inoculated with a suspension containing a total of $1x10^3$ *C. falcatum* conidia in a hole manually opened in basis of the stalk. In T(D+C), one individual of 3th instar *D. saccharalis* was placed near the stalk as previously described [24] and *C. falcatum* was inoculated in the plants 24h after *D. saccharalis* entrance in the same way described for Tc, but the hole was opened by the borer. A negative control was used without any fungal contamination (named C). Seedlings remained in a moist chamber in the greenhouse for 12 h and then for additional 10 d under natural conditions and without supplementary artificial light. Samples were collected in the stalk region, about two centimeters around the inoculation. A pool of five plants per treatment was used, and the experiment was repeated twice. The plant material was collected, flash-frozen in

liquid nitrogen (N_2) and storage at -80°C until processing samples. The analysis of *C. falcatum* infection was performed by quantitative PCR using the absolute quantification of internal transcribed sequence region of *C. falcatum* (as previously described in chapter 1 – item 2.2.5), based in a plasmid standard curve. The plasmid standard curve, quantitative PCR and sugarcane genomic DNA extraction were performed according to previously described in chapter 1 (item 2.2.5 and 2.2.6).

3.2.3. Influence of fungi on *D. saccharalis* performance

To determine the influence of *F. verticillioides* and *C. falcatum* on *D. saccharalis* feeding, 5th instar caterpillars were removed from the rearing diet and inoculated in glass tubes (8.5 cm long and 1.5 cm diameter) containing a new diet. The new diets were a minimal medium (MM) varying only the carbon source: 5% salt solution 20x [12% (p/v) $NaNO_3$; 1% (p/v) KCl; 3% (p/v) KH_2PO_4; 1% (p/v) $MgSO_4 \cdot 7H_2O$]; 0,1% traces elements 5x [75 mM $ZnSO_4 \cdot 7H_2O$, 180 mM H_3BO_3, 25 mM $MnCl_2 \cdot 4H_2O$, 18 mM $FeSO_4 \cdot 7H_2O$, 6 mM $CoCl_2 \cdot 5H_2O$, 6 mM $CuSO_4.5H_2O$, 1 mM $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, e 140 mM EDTA - pH 6.7]; 2% agar and 1% fructose (F) or ball-milled sugarcane bagasse (SCB) or sucrose (S). A total of 10^5 fungal conidia of *F. verticillioides* (F.v.), *C. falcatum* (C.f.), or *A. nidulans* (A.n.) were added to the diet five days before the assay, so the medium was already colonized with the corresponding fungus when the caterpillar was inoculated. In the negative control (C), no fungus was added. Performance of the sugarcane borer was estimated based on the weight assessed every two days for 10 days. Three replicates for each diet were used. In each replicate was add 14 caterpillars. The assay was repeated twice.

3.2.4. Olfactory preference assay

Initially, a total of 10^5 fungal conidia of *F. verticillioides*, *C. falcatum*, or *A. nidulans* were inoculated in a Falcon tube (15 mL) containing 5 mL of fructose diet. The negative control was not inoculated with any fungus. After 5 days, with the diet already completely colonized by the respective fungus, this tube was inserted to the bottom part of a Petri dish (15 cm of diameter), which was lined with moistened filter paper. Tubes containing fungus-colonized and control diet were at the opposite ends of the Petri dish bottom (Fig. 1). A group of 20 third-instar *D. saccharalis* caterpillars was released in the central region of the arena. Caterpillar choice was considered when they entered in the diet tube. Dishes were closed, sealed and kept in a dark room for 5 h at 25°C; the number of caterpillars inside the tubes was than recorded. The assay

was repeated ten times. The assay was also performed using third-instar *Spodoptera frugiperda,* to detect a possible specific attractiveness, and with 5th instar *D. saccharalis*, to find changes in insect behavior during immature stage. The assay using 5th instar *D. saccharalis* was performed with 10 caterpillar in each arena, and the assay was repeated 20 times.

To confirm the insect attraction to the fungi volatiles organic compounds (VOCs), the collected VOCs extract from *F. verticillioides* were used to attract *D. saccharalis*. This assay was performed using Petri dishes adapted with tubes containing an extract of VOCs emitted by *F. verticillioides* in fructose diet in one side, and an extract of VOCs emitted by the fructose diet (negative control) in the opposite side. 30µl of the crude extract was placed on a piece of cotton and added to the tube. The dishes were sealed and placed in the dark for 48h at 25°C. Ten 5th instar *D. saccharalis* caterpillars were released in the central region of the arena, and the assay was repeated 20 times.

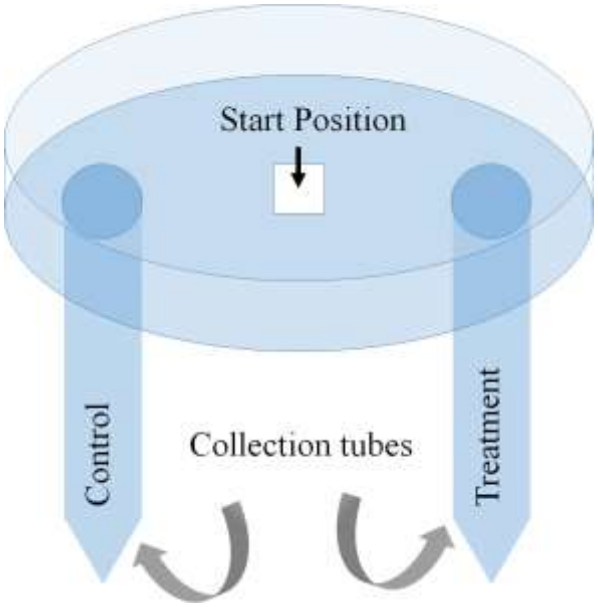


Figure 1. Design of olfactory preference assay used to caterpillars of *Diatraea saccharalis* and *Spodoptera frugiperda* in response to volatiles emitted by *Fusarium verticillioides, Colletotrichum falcatum* and *Aspergillus nidulans*. The caterpillar was placed in the start position. After 5 or 48 hours, the caterpillars inside to the collection tubes were counted and submitted to statistical analysis.

3.2.5. Volatile Collection and identification

Volatiles emitted by *F. verticillioides, C. falcatum* and *A. nidulans* in the fructose diet were collected using ARS Volatile Collection System (ARS, Gainesville, FLA, USA). Control and fungus-colonized diet were placed in fully enclosed glass chambers connected to the ARS by

Teflon® hoses. Clean and humidified air was injected into the chambers at 0.8 L/min. An adsorbent polymer column (Hayesep-Q®, 30 mg, Alltech Associates, Deerfield, IL, USA) was coupled to the other outlet of the glass chamber. After 8h of volatile collection, the polymer columns were eluted with 150 μL of hexano solvent (Sigma Aldrich, St. Louis, MO, USA) and samples stored in vials were kept in a freezer at -30°C until analysis. An aliquot of 2 μL of each sample was injected in splitless mode into a HP-5 capillary column (15 m of length 0.25 mm in diameter and 0.1 μm of thickness) in a GC-MS (Varian gas chromatograph model 3800 coupled to Varian mass spectrometer model 4000) with helium as carrier gas (24 cm/sec). The oven was held at 40°C for 5 min, raised to 150°C at 5°C/min, maintained for 1 min, then increased to 250°C at 20 °C/min. The detector signal was processed with the Workstation software version 6.9. Compounds were identified based on the mass spectra, which were compared with NIST08 library, Wiley6 library [29], Adams (2012) [30] and the pherobase [31] and confirmed by Kovats retention index. The volatile compounds quantification was performed using the GC-Fid Shimadzu, 2010 Plus equipment, based on the peak area relative to the internal standard (Nonyl acetate, Sigma Aldrich, St. Louis, MO, USA). Six repetition of each treatment were performed.

3.2.6. Statistical analysis

Statistical analysis for *C. falcatum* contamination in sugarcane assay and insect feeding on different diets were performed using *t*-test ($P \leq 0.05$) with R statistical software [32]. The olfactory preference assays were analyzed using paired *t*-test ($P \leq 0.05$) with R statistical software [32].

3.3. RESULTS

3.3.1. *D. saccharalis* positively influences the sugarcane infection by *C. falcatum*

The sugarcane infection by *C. falcatum* had an increase when in association to *D. saccharalis*. We used the fungus ITS region, a fungi barcode [33] to quantify the *C. falcatum* contamination in sugarcane. The quantification of ITS region in *D. saccharalis* plus *C. falcatum* treatment was around five times higher when compared to the *C. falcatum* treatment (Fig.2).

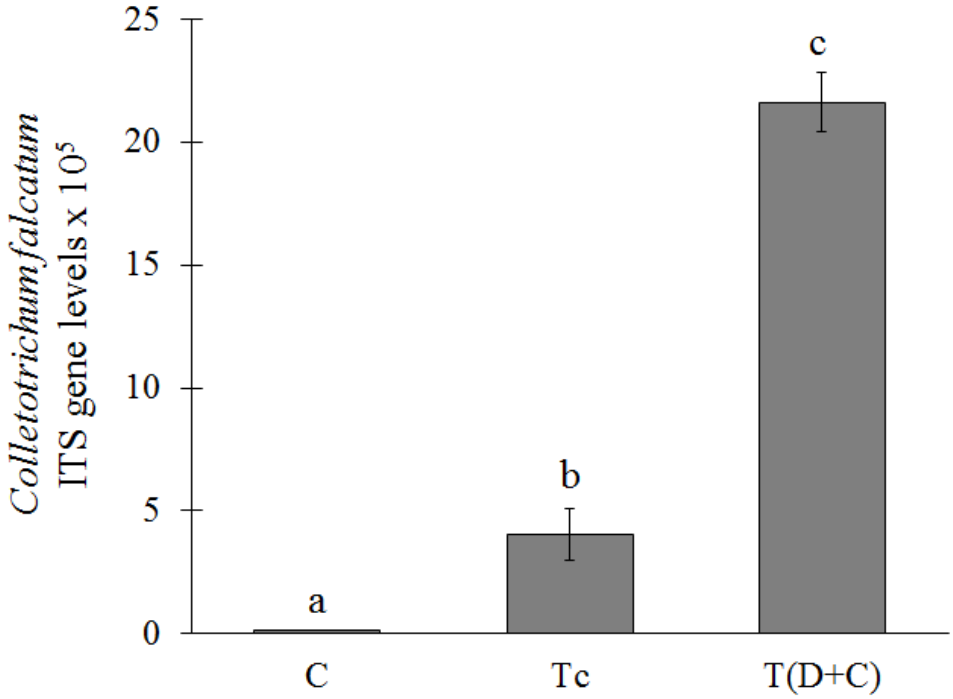


Figure 2. Quantification of *C. falcatum* in sugarcane (SP80-3280). The quantification of *C. falcatum* was performed by qRT-PCR using the standard curve method and the ITS region in response to 10 days of different treatments. C (negative control); Tc (*C. falcatum* treatment); T(D+C) (*D. saccharalis* plus *C. falcatum* treatment). Different letters indicates significant difference between treatments by *t*-test ($P \leq 0.01$)

3.3.2. *C. falcatum* and *F. verticillioides* positively influence *D. saccharalis* feeding

Caterpillars gained less weigh when fed on the control diet and *A. nidulans*-colonized than in *F. verticillioides* and *C. falcatum*-colonized diets. The results showed that the fungi *F. verticillioides* and *C. falcatum* positively influence caterpillar feeding, while the presence of *A. nidulans* negatively influences caterpillars feeding (Fig. 3 and 4). In the control diets, without the presence of any contaminant, and in the *A. nidulans*-colonized diet, caterpillars showed loss of weight. The fructose diet as well as sucrose diet *C. falcatum*-colonized and *F. verticillioides*-colonized showed a significant difference when compared to the negative control ($P \leq 0.05$). The ball-milled sugarcane bagasse *F. verticillioides*-colonized diet showed a significant difference when compared to the negative control at ($P \leq 0.05$).

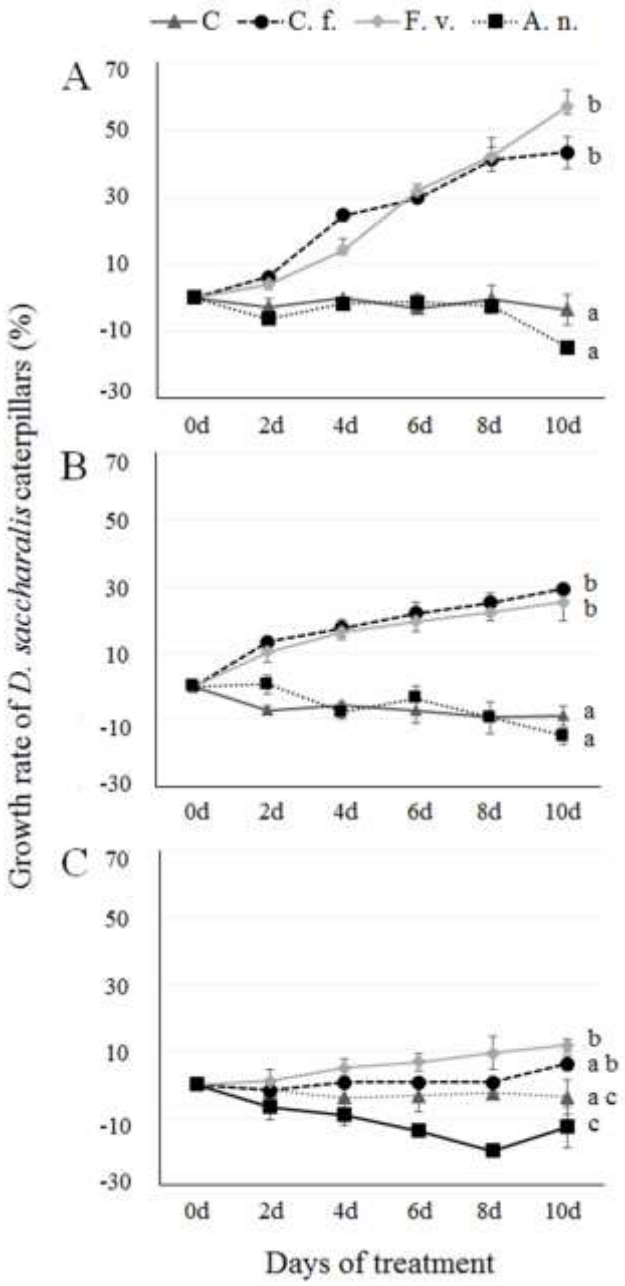


Figure 3. Effect of fungi on *Diatraea saccharalis* feeding. Fifth instar *D. saccharalis* caterpillars were treated in three different diet, Fructose (A), Sucrose (B) and ball-milled Sugarcane Bagasse (C), in different conditions: C (only the diet); C. f. (*Colletotrichum falcatum*-colonized diet); F. v. (*Fusarium verticillioides*-colonized diet) and A. n. (*Aspergillus nidulans*-colonized diet). The weight of caterpillars was evaluated every two days for ten days. Values are the means (± standard error) of three replicates. The assay was repeated twice. Different letters indicates significant difference between treatments by *t*-test ($P \leq 0.05$)

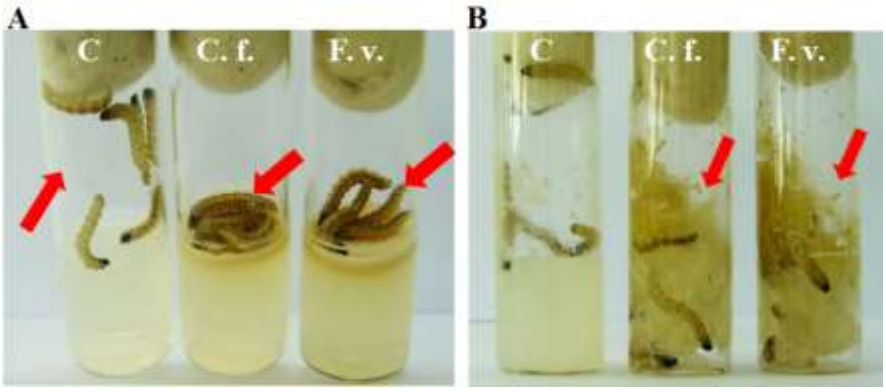


Figure 4. Influence of *Fusarium verticillioides* and *Colletotrichum falcatum* in *Diatraea saccharalis* feeding. *D. saccharalis* feeding after 15 minutes of treatment (A) and after two days of treatment (B). C (only diet); C. f. (*C. falcatum*-colonized diet); F. v. (*F. verticillioides*-colonized diet). The arrows are highlighting the insect interest in the diet.

3.3.3. *D. saccharalis* is attracted by volatiles emitted by *C. falcatum* and *F. verticillioides*

Third instar *D. saccharalis* caterpillar preferentially oriented to *F. verticillioides*-colonized diet over the control, the similar result was observed to *C. falcatum*-colonized diet (Fig. 5A and 6). However, the *A. nidulans*-colonized diet showed repelling, or do not attract the caterpillar, in this case, the caterpillars showed preference for the control diet (Fig. 5A). We also performed the olfactory choice assay using *S. frugiperda* caterpillars, however, this insect showed do not be attracted by any tested fungi (Fig. 5B), indicating a specificity between *D. saccharalis* and fungi interaction. The assay was also performed with fifth instar *D. saccharalis,* to know if the insect attraction to fungi VOCs can depend of larvae stage. Fifth instar *D. saccharalis* caterpillar also preferentially oriented to *F. verticillioides*-colonized diet over the control (Fig. 7), indicating a similar behavior during insect immature stage.

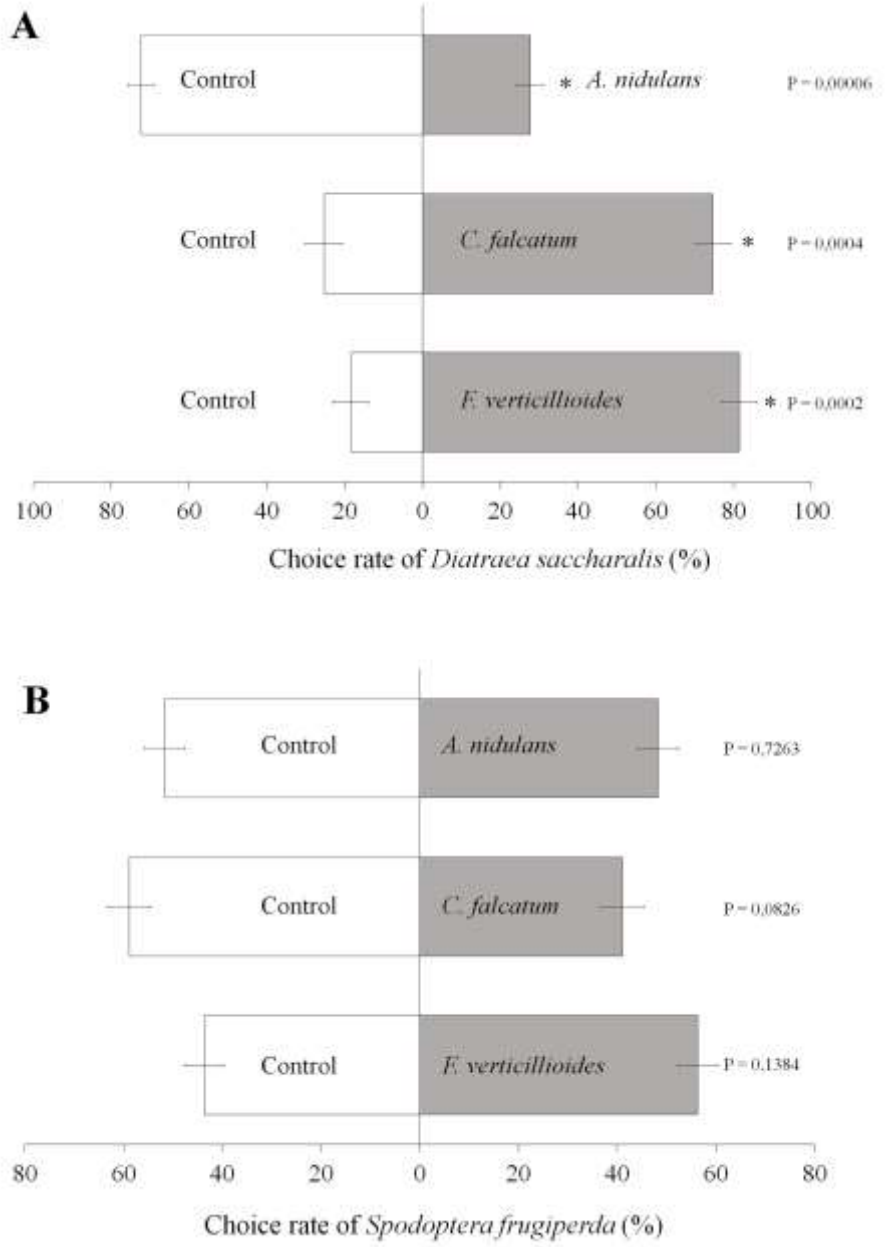


Figure 5. Olfactory choice assay with caterpillars of *Diatraea saccharalis* (3rd instar, N=20) (A) or *Spodoptera frugiperda* (3rd instar, N=20) (B). The caterpillars were added into the central region of a Petri dishes adapted with tubes containing fructose diet in one side and fructose diet *Fusarium verticillioides*, *Colletotrichum falcatum* or *Aspergillus nidulans*-colonized in the opposite side. The dishes were sealed and placed in the dark for 5h at 25°C. Asterisks inside the bars represent significant differences in comparison with the control by *t*-test ($P \leq 0.05$). Values are the means (± standard error) of ten replicates.

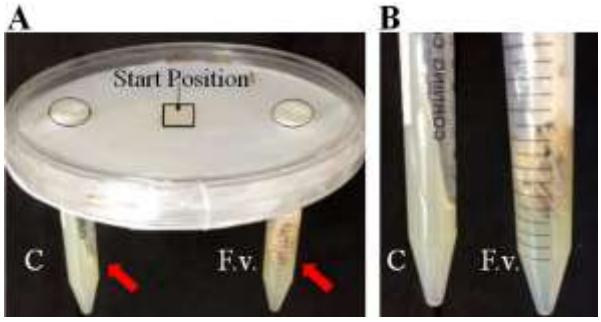


Figure 6. Olfactory choice assay. (A) Result of *Diatraea saccharalis* olfactory choice after 5h. Twenty caterpillars of *D. saccharalis* (3rd instar) were added into the central region of a Petri dishes adapted with tubes containing fructose diet in one side (Control = C), and fructose *Fusarium verticillioides*-colonized diet in the opposite side (F. v.). (B) Magnifications of (A).

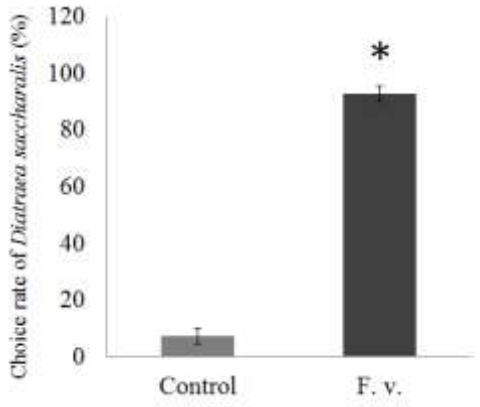


Figure 7. Olfactory choice assay. Ten caterpillars of *Diatraea saccharalis* (5th instar) were added into the central region of a Petri dishes adapted with tubes containing fructose diet in one side and fructose *Fusarium verticillioides*-colonized diet (F. v.) in the opposite side. The dishes were sealed and placed in the dark for 5h at 25°C. Asterisks above the bars represent significant differences in comparison with the control by *t*-test ($P \leq 0.05$). Values are the means (± standard error) of twenty replicates.

3.3.4. *F. verticillioides* and *C. falcatum* emit organic volatiles compounds that can attract *D. saccharalis*

The VOCs emitted by fungi was quantified and identified as 1-octen-3-ol and 3-octanone emitted only by *A. nidulans,* acoradiene and acorenol emitted by *F. verticillioides* and *C. falcatum*, and ß-elemene emitted only by *F. verticillioides*, in fructose diet (Fig. 8). These compounds were not identified in the control (diet without fungi contamination). The *F. verticillioides* VOCs extract were used in an olfactory preference assay, with *D. saccharalis*, to confirm the insect preference by fungi VOCs. *D. saccharalis* caterpillar preferentially oriented to *F. verticillioides* VOCs over the control (Fig. 9).

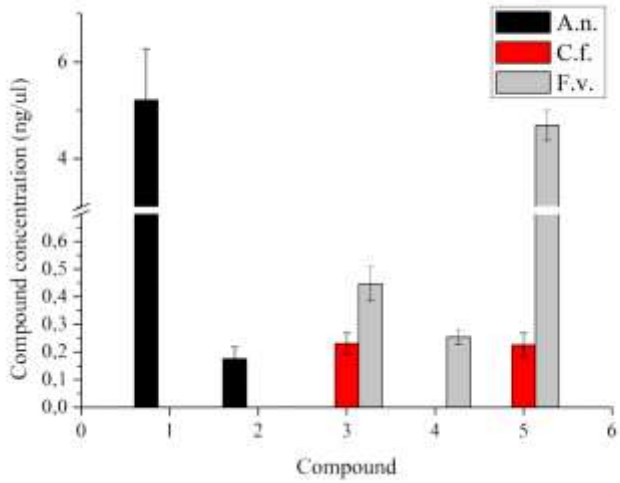


Figure 8. Volatiles emitted by *Fusarium verticillioides, Colletotrichum falcatum and Aspergillus nidulans* in fructose diet. The volatiles compounds were collected using ARS Volatile Collection System (ARS, Gainesville, FLA, USA), identified by GC-MS (Varian gas chromatograph model 3800 coupled to Varian mass spectrometer model 4000 type ion trap) and quantified by GC-Fid Shimadzu, 2010 Plus. The compounds mass spectra data were compared with the equipment library "NIST08", and confirmed by Kovats retention index on pherobase [31] and Adams (2012) [30]. Six replicates were used. (1) 1-octen-3-ol; (2) 3-octanone; (3) acoradiene; (4) ß-elemene; (5) acorenol.

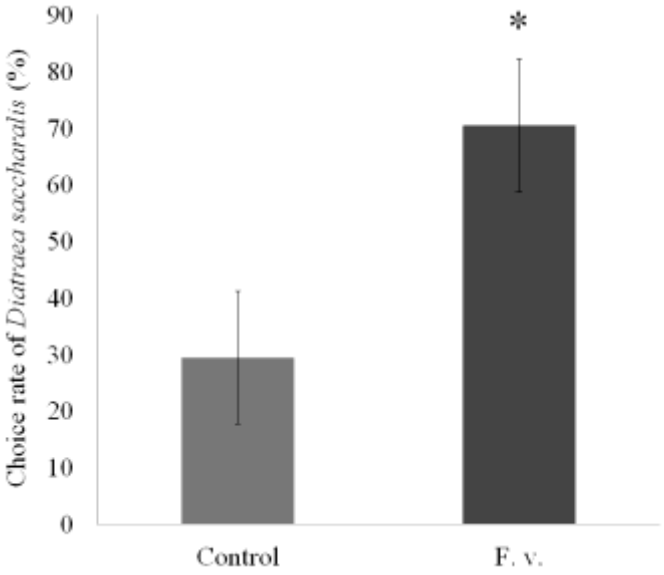


Figure 9. *Diatraea saccharalis* olfactory preference assay using *Fusarium verticillioides* volatiles compounds extract. Ten caterpillars of *D. saccharalis* (5th instar) were added into the central region of a Petri dishes adapted with tubes containing an extract of volatiles organic compounds emitted by *F. verticillioides* in fructose diet in one side, and an extract of volatiles compounds emitted by the fructose diet (negative control) in the opposite side. 30µl of the crude extract was placed on a piece of cotton and added to the tube. The dishes were sealed and placed in the dark for 48h at 25°C. Asterisk above the bar represent significant differences in comparison with the control by *t*-test ($P \leq 0.05$). Values are the means (± standard error) of twenty replicates.

3.4. DISCUSSION

Different studies have shown interaction between fungi and insects in plant colonization [3-8, 34]. In this work, we showed a close interaction between the sugarcane borer *D. saccharalis* and the opportunistic fungi *C. falcatum* and *F. verticillioides*. Our results show that *D. saccharalis* positively influences *C. falcatum* infection on sugarcane (Fig.1). The previously insect attack contributed to the fungus infection, causing a faster fungus growing when compared to infection in the absence of the caterpillar (Fig. 2). Others studies have shown insect influence in fungus infection; in maize, the European Corn Borer (*Ostrinia nubilalis*) attack significantly increase *F. verticillioides* infection [35]. The percentage of maize grain infected by *Aspergillus flavus* increase with increasing earborer *Mussidia nigrivenella* damage [36]. In our study *D. saccharalis* seems to create a favorable microclimate to *C. falcatum* infection due tissue maceration and cell rupture, facilitating the absorption of nutrients by the fungus. However this interaction can show a higher complexity and specificity when we analyzed the effect of fungi in *D. saccharalis* performance. 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 (Fig. 3), nevertheless the *A. nidulans*-colonized diet showed a negative influence in *D. saccharalis* attraction and performance, indicating a specificity in insect-fungi interaction.

The influence of fungi on insect behavior can be result of VOCs emission [7, 9, 10], visual cues [12, 13, 37, 38] or nutritional supplementation, directly due feeding of insect mycelia and spores, or indirectly due nutritional changes [14-17]. The different diets used in this work were important to identify how this insect-fungi interaction may occur. The figure 3 shows the result of *D. saccharalis* caterpillar feed in three different diets, composed by essential nutrients with only carbon sources variation. In the negative control, without any contamination, the caterpillars showed weight loss due to the deficiency on feeding. Similar results were show in the diet colonized by *A. nidulans*, which is not a pathogen of sugarcane; this fungus showed a negative influence on caterpillars feeding (Fig 3). However, *D. saccharalis* showed feed preference to *F. verticillioides* and *C. falcatum*-colonized diet when compared to the control diet (Fig. 3). These results do not exclude a possible influence of gustatory cues; however, the insect weight gain is probably not due the direct mycelium feeding once the insect weight gain was reduced in ball-milled sugarcane bagasse diet, and in *A. nidulans*-colonized diet (Fig. 3C). The ball-milled sugarcane bagasse diet represent a complex carbon source [39] to the insect that may have resulted in slow weight gain when compared to fructose, a simple saccharide, and sucrose, a disaccharide carbon source (Fig. 3).

The insect attraction to the colonized diets (Fig. 4) was interesting and led us to investigate the influence of fungi VOCs on *D. saccharalis* behavior. The olfactory preference assay showed that the *D. saccharalis* caterpillars are highly attracted to volatile compounds emitted by *F. verticillioides* and *C. falcatum* (Fig 5A and 6). While *A. nidulans* appears do not attract or to repel *D. saccharalis* caterpillars (Fig. 5A), what corroborate to the feeding assay (Fig. 3). Interesting, caterpillars of *S. frugiperda* did not show preference to any fungi in study (Fig. 5B), indicating a specific interaction between *F. verticillioides* and *C. falcatum* VOCs with *D. saccharalis*. The positive orientation of thirty and fifth instar *D. saccharalis* to *F. verticillioides*- colonized diet indicates that this attraction is not specific to a larval stage, but occur during larval stage (Fig 5 and 7).

Previous studies have shown that *F. verticillioides* produces a series of volatile compounds such as alcohols, esters and aldehydes that are attractive to nitidulid beetles [10]. In the same way, *F. verticillioides* was shown to attract and decrease the mortality of insects as *Eldana saccharina*, *Cryptophlebia leucotreta*, *Mussidia nigrivenella* and *Sesamia calamistis* in maize [6]. Other interactions involving volatile compounds in insect-fungi interactions have been described by McFarlane et al. (2009), suggesting that volatiles emitted by *Fusarium pseudonygamai* can be attractive to *Eldana saccharina*. In addition, the beetle *Tribolium castaneum* was attracted by volatiles emitted by fungus-infected cotton seed [40]. In this study, we identified and quantified VOCs emitted by *F. verticillioides* and *C. facatum* in fructose diet, such as acoradiene and acorenol (Fig. 8). Probably these two VOCs are the attractants to *D. saccharalis*, once both fungi that can attract the insect emit them. Acorenol have been studied in fungi such as *Trichoderma harzianum, Trichoderma koningii* and *Fusidium coccineum* [41-43], while acoradiene was previously identified in *Fusarium oxysporum* [44], and *Fusarium sambucinum*. However, the insect response to these compounds has not yet been elucidated. The VOCs extract collected from Fructose *F. verticillioides*-colonized diet were used in an olfactory preference assay, that showed *D. saccharalis* preference when compared to the control (Fig. 9), confirming the role of VOCs in *D. saccharalis* behavior.

Interestingly, the fungus *A. nidulans*, which showed negative influence to *D. saccharalis* feeding and attraction, emitted different VOCs when compared to *F. verticillioides* and *C. falcatum*, such as 1-octen-3-ol and 3-octanone. The production of 1-octen-3-ol and 3-octanone have been previously identified in *A. nidulans* [45] and several other fungi, such as *Aspergillus flavus* [46], *Aspergillus fumigatus* [47], *Isaria fumosorosea* [48], *Paenibacillus polymyxa* [49]. These VOCs showed repellence of insects, such as *Sitophilus zeamais* [50], *Coptotermes formosanus* [48], *Tribolium castaneum* [49], that corroborates with our data. However, the same compounds can be attractive to *Malthodes fuscus*, *Anaspis marginicollis, Anaspis rufilabris*, *Epinotia tedella* and *Lordithon lunulatus* [51]. Plants of *Cucurbita moschata* infected by powdery mildew *Podosphaera* sp. showed a different profile

of VOCs when compared to health plants, including 3-octanone and 1-octen-3-ol, which showed to be highly attractive to *Psyllobora vigintimaculata* beetles [52]. The fungi VOCs extraction and quantification in this work reinforce the differences between fungi that can explain *D. saccharalis* behavior.

Our results showed a strong synergistic interaction between *F. verticillioides* and *C. falcatum* with *D. saccharalis* in sugarcane colonization, incluing a role of fungal VOCs in this association. These knowledge enhance our understanding of plant-insect-fungal interation and may influence the use of integrated pest and disease management. Studies envolving adults of *D. saccharalis* need to be performed to understand the influence of these compounds in adults attraction and oviposition, the identification of insect olfactory receptor to these compounds in *D. saccharalis* adults and caterpillars are also important to a global undertandy of this important and complex ecological interaction.

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