Industrial Crops & Products 140 (2019) 111651

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Short communication

^{1}H NMR metabolomic approach reveals chlorogenic acid as a response of sugarcane induced by exposure to *Diatraea saccharalis*

Adilson R. Sabino[a,*], Sheila S. Tavares[a], Alessandro Riffel[c], Jia V. Li[b], Demetrios J.A. Oliveira[a], Chrystian I.M.A. Feres[f], Lucas Henrique[a], Jaim S. Oliveira[e], Gonçalo D.S. Correia[b], Anderson R. Sabino[g], Ticiano G. Nascimento[a], Geofrey Hawkes[b,d], Antônio E.G. Santana[a], Elaine Holmes[b], Edson S. Bento[a]

[a] Institute of Chemistry and Biotechnology, Federal University of Alagoas, Maceio, Brazil
[b] National Phenome Centre and Imperial Clinical Phenotyping Centre, Department of Surgery & Cancer, Imperial College London, W12 0NN, UK
[c] Embrapa Tabuleiro Costeiros, Rio Largo, Alagoas, Brazil
[d] Department of Chemistry, Queen Mary College, London, UK
[e] Tiradentes University Center (UNIT), Maceio, Brazil
[f] Faculdades Integradas do Norte de Minas, Campus JK, Brazil
[g] Centro de Ciências Agrárias (CECA), Federal University of Alagoas, Maceio, Brazil

ARTICLE INFO

Keywords:
Sugarcane
Diatraea saccharalis
Metabolomic
NMR
Herbivory
Chlorogenic acid

ABSTRACT

Sugarcane *(Saccharum officinarum)* has been considered one of the most efficient energy crops, but its production yield is sensitive to outbreaks of pest insects, especially the sugarcane borer *Diatraea saccharalis*. Genetic breeding programs and biotechnology projects have been developed to decode the defense mechanisms of sugarcane against herbivorous insect attacks, and the develop plague-resistant plants. We performed metabolic profile analysis of the SP791011 sugarcane variety's response to *Diatraea saccharalis* herbivory, using Nuclear Magnetic Resonance (NMR) spectroscopy of organic leaf extracts. The leaf response of SP791011 to *D. saccharalis* resulted in depletion of choline, alanine, sucrose, glutamate, trigonelline, and isomers (*E*)-aconitate, (*Z*)-aconitate, and higher expression of chlorogenic acid and other caffeic acid conjugates in sugarcane leaves. The increase in chlorogenic acid suggests the shikimic acid pathway was induced by *D. saccharalis* herbivory, increasing the biosynthesis of phenylpropanoids such as chlorogenic acid in the sugarcane leaves. In addition to the herbivory test, we performed an in vivo biological assay by adding chlorogenic acid to an artificial diet to *D. saccharalis* caterpillars. This assay demonstrated a decrease in the development time of the pupae compared with pupae from caterpillars raised under normal diet. However, deformations in moth wings fed with chlorogenic acid were observed for three concentrations tested (0.05 mg/mL, 0.5 mg/mL and 5 mg/mL) during the in vivo bioassay. Chlorogenic acid may be considered a natural biopesticide and its production could be induced to develop more resistant sugarcane varieties against *D. saccharalis*.

1. Introduction

Domestic sugarcane varieties originated in China and New Guinea 2000 years ago, and have been cultivated in the New World for more than 500 years since their introduction there. It is a gramineous plant belonging to the family Poaceae and originated mainly from the species *Saccharum officinarum*. Currently the production of this crop is one of the largest in the world and stands out in countries with a tropical climate, such as Brazil, India and China, respectively, the largest producers. Sugar and ethanol are the main sugarcane products manufactured by the mills and are mainly used in the food industry and as fuel for automobiles (Henry and Kole, 2010; Aitken et al., 2006).

Sugarcane can be attacked by several insect pests, among which *Diatraea saccharalis* stands out. Popularly known as the sugarcane borer, *D. saccharalis* (Fabricius 1794, Lepidoptera, Crambidae) is sugarcane's main pest. The moth oviposits on the dorsal blade of sugarcane leaves and, within 10 days, the eggs hatch generating caterpillars. The caterpillars will then feed on leaves up to the stalks, and develop until the moth stage, causing damage inside the plant (Sandoval and Senô, 2010; Nakano et al., 2001). Despite the deployment of chemical and

* Corresponding author.
E-mail address: adilson.sabino@iqb.ufal.br (A.R. Sabino).

https://doi.org/10.1016/j.indcrop.2019.111651
Received 13 January 2019; Received in revised form 4 August 2019; Accepted 6 August 2019

biological control strategies, *Diatraea saccharalis* still causes financial losses of about millions per year. It has been estimated that a damage of 1 percent of internodes by sugarcane borer causes 0.2–1.1% loss in yield. A typical damage rating of 3% in Brazil's Central South region represents a loss of about 300 Kg of total sugars recovered (TSR)/ha to sugarcane borer under current best management practices (Cristofoletti et al., 2018).

Metabolomics aims to study qualitatively and quantitatively the set of metabolites produced or modified by a given organism. The metabolome is defined as the partial or complete identification of molecules present in an organism. Primary and secondary metabolites may be biomarkers of a certain characteristic of the organism involved in the insect-plant interaction (Hall, 2006; Saito et al., 2006). The metabolomics application in the insect-plant interaction studies is an effective approach for the identification and quantification of secondary metabolites and their variability in response to herbivory (Kim et al., 2010). Modulation of secondary metabolism is a potential strategy to generate pest-resistant plants, and it is likely that some secondary metabolites are biopesticides, whose identification and characterization will be invaluable in the fight against agricultural pests (Hong et al., 2016). Our research group used NMR-based metabolomics technology to identify metabolites differentially synthesized in the leaves of the SP791011 sugarcane varietu sugarcane in response to herbivory by *Diatraea saccharalis*. Our results will contribute to the elucidation of metabolic pathways and the identification of secondary metabolites involved in defense mechanisms of the sugarcane plant. This information could be used to develop sugarcane plants more resistant against herbivorous insects.

2. Material and methods

2.1. Plant material

Sugarcane plants (cv. SP79-1011) were propagated by cuttings of the stalk containing one bud. Stalk cuttings were grown in 500 mL plastic cups containing a commercial substrate (Bioplant) and cultivated for 40 days under greenhouse conditions. Plants were watered daily.

2.2. Experimental design and bioassay

A total of 17 sugarcane plants, (SP791011 variety), were used in the herbivory bioassays. Before collecting leaves, the attack of caterpillars on plant leaves was verified. Then, 10 plants were infested with 5 *D. saccharalis* larvae and 7 plants remained uninfested (control plants) for a period of 72 h. The leaves were immediately frozen under liquid nitrogen to quench plant metabolism and stored at −80 °C until the extraction of the metabolites.

2.3. Insects

The *D. saccharalis* larvae were raised on an artificial diet (Hensley and Hammond, 1968) and maintained at environmental conditions (25 °C, 1 atm). Young larvae (7 days posthatch) and 3rd instar larvae were used for the metabolomics and toxicity bioassay respectively.

2.4. Extraction of plant materials

For each biological replicate, one gram of sugarcane leaves were lyophilized and then 30 mL of ethyl ether were added, before stirring for 15 min at room temperature, followed by vacuum filtration in order to remove fat and grease. The sugarcane extract was obtained adding 30 mL of a solvent system (chloroform: methanol:distilled water, 12:5:3 V/V/V) on the defatted extract, then incubated in an ultrasound bath for 30 min and filtered under vacuum. The resulting filtrate was evaporated in a rotary evaporator under stirring and reduced pressure at 40 °C. The resulting extract was stored in desiccators until NMR analysis proceeded.

2.5. Nuclear magnetic ressonance analisys (NMR)

The NMR analysis was performed with 10 mg of dried extract dissolved in 1 mL of deuterated solution containing 500 µL of methanol-d4 and 500 µL of phosphate buffer (90 mM, pH = 6) in deuterated water containing 0.01% sodium salt of trimethylsilyl propionic acid (TSP), and transferred to a 5 mm NMR tube. The NMR spectra were recorded at 20 °C on a 600 MHz Bruker AVANCE spectrometer (Bruker, Germany) operating at a proton ^{1}H frequency of 600.13 MHz. Methanol-d4 was used as the internal lock. The ^{1}H NMR spectra were carried out with 128 scans (10 min e 26 s), acquisition time of 2.72 s, spectral width of 16 ppm, FID size of 32 K, pulse width (p1) of 30 (10.5 µs) and relaxation delay (d1) of 2 s. The J-resolved spectrum was acquired with 32 scans by 8192 × 128 points, spectral width of 5.208 KHz and 60 Hz spin-spin coupling and relaxation delay of 1.5 s. The COSY ^{1}H-^{1}H spectrum was carried out with 32 scans by 2048 × 512 points, spectral width of 13 ppm on both dimensions with relaxation delay of 1.5 s. The spectra were processed using TopSpin 2.1 software from Bruker® with a line broadening of 0.3 Hz and phase correction, correct baseline and calibration by the internal standard trimethylsilyl propionic acid sodium salt (TSP), signal at 0.0 ppm. the quantification of chlorogenic acid (CGA) was done using the methodology the relative determination, in which the molar ratio of CGA and TSP.

2.6. Data analysis

The ^{1}H NMR spectra were optimized, reduced (δ 0.7–9.5 ppm) and divided into wide standard regions (bucket = 0.0005 ppm), aligned and normalized by total area using the MATLAB software version 2014A. The regions of δ 4.7–5.0 and δ 3.24–3.33 were excluded from the analysis because of the residual signals of methanol and water. The orthogonal partial least square discriminants analysis (O-PLS-DA) was performed with SIMCA-P software (version 14.0, Umetrics, Umeå, Sweden) with scaling based on the method of unit variance (UV). Differences between abundances of chlorogenic acid and other continuous variables (life cycle in days) between groups or concentration dosages were assessed using a two-tailed Welch *t*-test. Statistical differences in proportions of caterpillars were analyzed using a two-tailed 2-sample chi-squared *test* for equality of proportions with Yates continuity correction, with the function *prop.test* on R. Box-and-whiskers plots, scatterplots and bar plots were generated using the R package *ggplot2*.

2.7. D. saccharalis toxicity in vitro bioassay

To evaluate the defensive importance of CGA against *D. saccharalis*, we fed individual young larvae (7 days posthatch) with an artificial diet (Hensley and Hammond, 1968) containing dissolved CGA at final concentration of 0 mg/mL, 0.05 mg/mL, 0.5 mg/mL and 5 mg/mL, on petri dishes. CGA was dissolved in distillated water and added to melted artificial diet in water bath at 70 °C. We evaluated behavior, pupae mass, longevity and morphological changes during the life cycle. The concentrations of CGA were chosen so that the maximum concentration was similar to the maximum average level measured in the attacked leaves.

3. Results

3.1. Metabolic profile in sugarcane leaves induced by D. saccharalis

NMR is a powerful tool for metabolomics studies, and it is a universal detector for all types of molecules, requiring minimum sample preparation, good capability to reproduce data even in spectrometers

with different frequencies and to be very versatile having several types of 1D, 2D and pseudo-2D experiments (pulse sequences) for structural elucidation. Another advantage of NMR is the ease of data digitization for application of chemometric methods (Leiss et al., 2009). NMR metabolomics technology has been applied in plants on several applications including chemotaxonomic classification, determination of geographic origin, growth monitoring, and plant response to abiotic and biotic factors in agriculture (Mahrous and Farag, 2015). The biomarkers were assigned by comparison of results obtained by 2D NMR spectra, such as, J-resolved, COSY, and TOCSY with chemical shifts and coupling constants from previous literature data (Human Metabolome Data Base). A chemical standard and Statistical Total Correlation Spectroscopy – STOCSY (Cloarec et al., 2005a, 2005b) were used for chlorogenic acid identification, which demonstrated the correlations of driven peak at *δ 7.59 ppm* with signals from the aromatic ring *δ 6.34 ppm* (*H-8′*, doublet, $J = 16$ Hz); *δ 6.88 ppm* (*H-5′*, doublet, $J = 8$ Hz); *δ 7.05 ppm* (*H-6′*, multiplet); *δ 7.14 ppm* (*H-2′*, doublet, $J = 2$ Hz) and *δ 7.59 ppm* (*H-7′*, doublet, $J = 16$ Hz), and also correlated with the multiplets from the quinic acid moiety *δ 5.33 ppm (H-5)*; *δ 4.23 ppm (H-3)*; *δ 3.80 ppm (H-4)*; *δ 2.17 ppm (H-2)* and *δ 2.03 ppm (H-6)* confirming the metabolite (Supplementary Fig. S1).

3.2. Multivariate analysis of the SP791011 sugarcane variety induced by D. saccharalis

In order to identify the discriminant metabolites involved in the response to sugarcane herbivory by *D. saccharalis,* we performed a supervised O-PLS-DA analysis to compare control and herbivory plants. We identified robust differences in the metabolic composition of sugarcane leaves in induced plants compared to control plants. The observed goodness of fit (R^2Y) and goodness of prediction (Q^2Y) values of the generated model were 0.89 and 0.69 respectively. The validation of the O-PLS-DA model was also performed by the CV-ANOVA (Erickson et al., 2008) method (p value = 0.004).

The O-PLS-DA score (Fig. 1a) showed good separation between control and herbivory plants. Interpretation of the loading “S-line” plot highlighted a depletion of alanine, glutamic acid, sucrose, trigonelline, and the isomers *E* and *Z*-aconitate, and a rise of chlorogenic acid and other phenylpropanoids in sugarcane leaves after 72 h infested by *D. saccharalis*. The Fig. 1b shows identified biomarkers obtained by O-PLS-DA model and its respective S-line plot. Metabolites were considered “discriminant” if their correlation value to the Y variable was higher than 0.6. Furthermore, we applied Welch’s *t*-test to verify the significance of the discriminatory metabolites screened in with the O-PLS-DA) “S-line” visualization. The Welch’s *t*-test results were generally consistent with those derived from the O-PLS-DA model metabolites with significant mean differences ($p < 0.05$) between control and herbivory samples (Table 1).

4. Discussion

Plants have evolved defense mechanisms against insects very often involving the production of chemical compounds. Metabolic profiling platforms can detect and identify a large number of compounds and have thus emerged as a useful tools in the search for new sources of plant resistance (Macel and van Dam, 2018). ^{1}H NMR approach was carried out to identify metabolites involved in response to herbivory by the sugarcane borer, *D. saccharalis*. A supervised O-PLS-DA model was performed to compare healthy plants with plants after herbivory. The loading s-line plot have shown a depletion of sucrose, alanine, glutamic acid, trigonelline and the isomers *E* and *Z*-aconitate in induced plants meanwhile an increasing of chlorogenic acid and other caffeic acid conjugates in sugarcane leaves tissues after 72 h of *D. saccharalis* herbivory (Fig. 1b).

Plant response to herbivores begins with plants perceiving damage patterns and herbivore-associated elicitors (HAEs) prevalent in secretions and fluids from herbivores. After different signaling mechanisms, Jasmonic Acid (JA) induces most herbivore resistant traits and expressive changes in primary and secondary metabolism (Schuman and Baldwin, 2016). Plant defense compete with growth and reproduction, the efficiency of photosynthesis, growth rate and remobilization of carbon and nitrogen resources may be often altered, among the most common changes in primary metabolism. The costs and benefits are not well established, depending on the host species, the infested tissue, the specificity of herbivory and the period of insect feeding.

The sugarcane anatomy and architecture, as other members of the Poacea family, is very particular. Sugarcane is a C4 plant with a complex arrangement of vascular bundles, which facilitates rapid transport of photosynthates and other metabolites to the sap in sieve tubes (Colbert and Evert, 1982). aminoin sugarcane leaves during the herbivory. In sugarcane, the sucrose is the main storage and transport sugar reaching up to 0.7 M in the internodes. It is produced in the leaf, translocated in the phloem, and stored in the stem (Moore, 1995). When the plant tissue is attacked, a source of carbon is demanded for defense and production of defensive compounds. For example, in the biosynthesis of defensive plant phenolics, via shikimate and phenylpropanoid pathways, the carbohydrates are used as C source (Schultz et al., 2013). In rice plants, JA application caused a reduction in the photosynthesis, via decreasing in chlorophyll and photosynthesis associated proteins, suggesting a possible limitation of carbon and a demand for C skeletons. The increasing demands for C sources can also be supplied by Glu Dehydrogenases (GDH) activity that catalyzes the amino acid breakdown (Wu et al., 2018).

Amino acids play an important role in plant-herbivore interaction, and either its production or degradation has been observed during herbivory. Amino acids serve as biosynthesis precursors for defense compounds and as key nutrients for most herbivores. (Zhou et al., 2015). We detected a decreasing in both alanine (Ala) and Glutamic acid (Glu) content in leaf tissues from plants upon herbivory. Glutamate is one of the main amino acids responsible for nitrogen assimilation and transport. *D. saccharalis* is a borer that attacks the stem tissue and, in our study, we have analyzed the systemic response in leaf tissue from sugarcane, corresponding to a significant distance between the attack and analysis sites. Wu et al. (2018) also showed that after the exogenous application of MeJA in rice (*Oryza sativa L.*), a member of the Poacea family, there was a remobilization of endogenous N from leaves to roots, reducing the N accumulation in leaves from treated plants, as part of a tolerance-based defensive strategy. Moreover, the amino acid unbalance may reduce the nutritional value of the tissues and hamper the herbivore performance (Zhou et al., 2015).

Trigonelline and *N*-methylnicotinamide, were also found in significant lower amounts in sugarcane plants under herbivory. Trigonelline has long been reported as a compound with multiple regulatory functions in plant metabolism, acting as a cell cycle regulator, playing a role in biological methylation, as a nutrient during the nodulation process and protection against oxidative and salt stresses (Minorsky, 2002). A series of NMR metabolomic studies have described trigonelline as discriminant metabolite with different sort of alterations (Mirnezhad et al., 2010; Sun et al., 2014; Sekiyama et al., 2017). A genotype of the perennial C4 grass Miscanthus, from the same subtribe as sugarcane Saccharine, also showed a decreasing in trigonelline levels during cold acclimation (Le Gall et al., 2017). However, a little is known about its direct involvement in the insect herbivory. In a metabolomic approach to study host plant resistance to thrips in wild and cultivated tomatoes, resistant plants showed lower levels of trigonelline (Mirnezhad et al., 2010). Controversially, Nakayama and Honda (2004) reported that trigonelline exhibits oviposition deterrence to females of a swallowtail butterfly, *Papilio polytes* (Lepidoptera, Papilionidae). Surely trigonelline plays an important role in sugarcane response to herbivory, however further investigation is required.

Phenolic compounds have long been demonstrated to be involved in

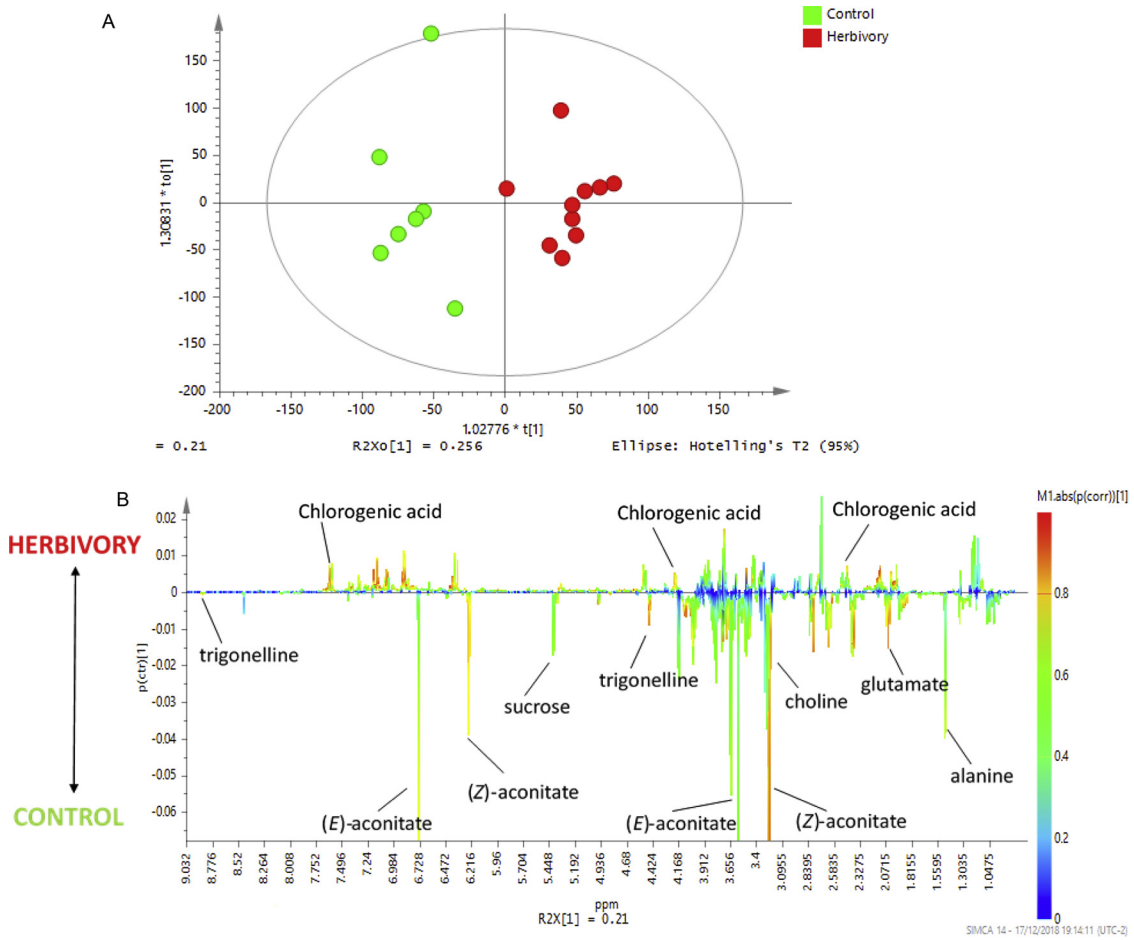

Fig. 1. (a) O-PLS-DA score plot for the herbivory group (n = 10) versus control group (n = 7) discriminant analysis based on ^{1}H NMR spectra of crude organic extracts from sugarcane leaves. OPLS-DA model with 2 components (1 predictive + 1 orthogonal component), $R^2Y = 0.89$, $Q^2Y = 0.69$; CV-ANOVA, *p* value = 0.004. (b) Orthogonal Projections to Latent Structures-Discriminant Analysis (O-PLS-DA) loading S-line plot. Sugarcane leave metabolites with higher abundance in plants exposed to herbivory by *D. saccharalis* (up) compared to control plants (down). The colorbar indicates the Pearson correlation coefficient (*r*) between the NMR spectral variables and the Y vector encoding class. OPLS-DA model with 2 components (1 predictive + 1 orthogonal component), $R^2Y = 0.89$, $Q^2Y = 0.69$; CV-ANOVA, *p* value = 0.004.

plant defense (Appel, 1993). We identified the phenolic compound 5-O-caffeoyl quinic acid (chlorogenic acid, CGA) and other caffeic acid conjugates, as discriminant metabolites, showing higher accumulation in sugarcane plants under herbivory. Chlorogenic acid is widely distributed in different parts of many plants and usually occurs in easily detectable amounts in tissues and it may also function in plants as a metabolic intermediate in the formation of insoluble phenolic compounds (eg, lignin) associated with plant resistance to stress (Friend, 1981).

NMR metabolomics allows to quantify the metabolites based on the signal intensity relative to internal standard. The concentrations of CGA found in leaf tissues of both control and attacked plants were 0.1 mg/g (˜0.02 mg/g fresh weight) and 0.6 mg/g dry weight (˜0.15 mg/g fresh weight), respectively ($p = 0.004$, Supplementary Fig. S2a). Green sugarcane leaves consist of 75–80% water (Beeharry, 1996). During herbivory, higher accumulations of CGA has been reported and it has

Table 1
Sugarcane metabolites associated with response to herbivory plants compared to control plants (Correlation > 0.6, Welch *t*-test *p-value* < 0.05).

Metabolite	Chemical Shift (ppm)	Correlated with	Coefficient of determination (r^2)	*t test* (*p* value)
Chlorogenic Acid	*7.59 (H-7'*, d, *J = 16 Hz), 6.39 (H-8'*,d, *J = 16 Hz), 7.14 (H-2'*, d, *J = 2 Hz), 7.05 (H-6'*,m), *5.33 (H-5*, m), *2.03 (H-6*, m), *3.80 (H-4*, m) and *4.23 (H-3*, m)	Herbivory	0.77	0.003
Alanine	*1.48 (H-3*, d, *J = 7.4 Hz), 3.8 (H-2*, q, *J = 7.4 Hz)*	Control	0.69	0.049
Glutamate	*2.07 (H-3*, m), *2.36 (H-4*, m)	Control	0.86	0.0001
Choline	*3.21 (s)*	Control	0.81	0.0001
Sucrose	*5.40 (H-1*, d, *J = 4 Hz), 4,17 (H-3*,d, *J = 7.7 Hz), 4.04 (H-4*, t, *J = 8.6 Hz), 3.89 (H-2*, m), *3.87 (H-5*, m), *3.82 (H-10,H-11*,m), *3.75 (H-6*, t, *J = 9.6 Hz, 3.67 (H-9*, s), *3.55 (H-7*, m) and *3.46 (H-8*, t, *J = 9.4 Hz)*	Control	0.61	0.035
(*Z*)-Aconitate	*3.56 (H-3*, s) and *6.24 (H-2'*, s)	Control	0.73	0.0003
(*E*)-Aconitate	*3.57 (H-3*, s) and *6.72 (H-2'*, s)	Control	0.71	0.013
Trigonelline	*9.14 (H-1*, s), *8.86 (H-3, H-5*, m), *8.07 (H-4*, m) and *4.45 (H-7*, s)	Control	0.81	4.47E-06

d, doublet; m, multiplet; s, singlet; t, triplet.

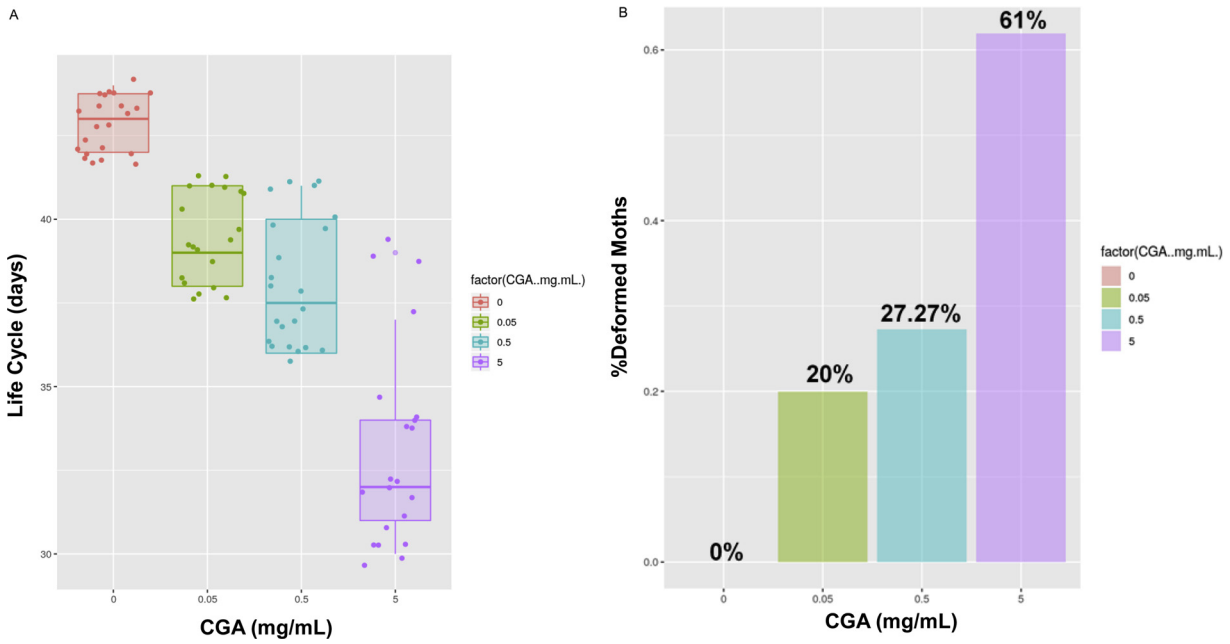

Fig. 2. (a) Dose-dependence of chlorogenic acid on life cycle of *D. saccharalis* from larvae to adult stage. Welch *t*-test was applied to check the significance (p value < 0.001) between caterpillars fed with free CGA diet (0 mg/mL, n = 22) and caterpillars fed with CGA diet containing 0.05 (n = 20, $p = 2.13 \times 10^{-11}$), 0.5 (n = 22, $p = 2.31 \times 10^{-11}$), and 5 mg/mL (n = 21, $p = 1.23 \times 10^{-12}$). (b) Effects of chlorogenic acid on morphology of *Diatreaea saccharalis.* The graph shows the total number of deformed (green, blue and purple bars) and no deformed (orange bar) moths of *D. saccharalis* as a function of artificial diet containing chlorogenic acid (CGA) at concentrations of 0 (n = 22, 0% deformed moths), 0.05 (n = 20, 20% deformed moths), 0.5 (n = 22,3% deformed moths) and 5 mg/mL (n = 21, 61% deformed moths). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

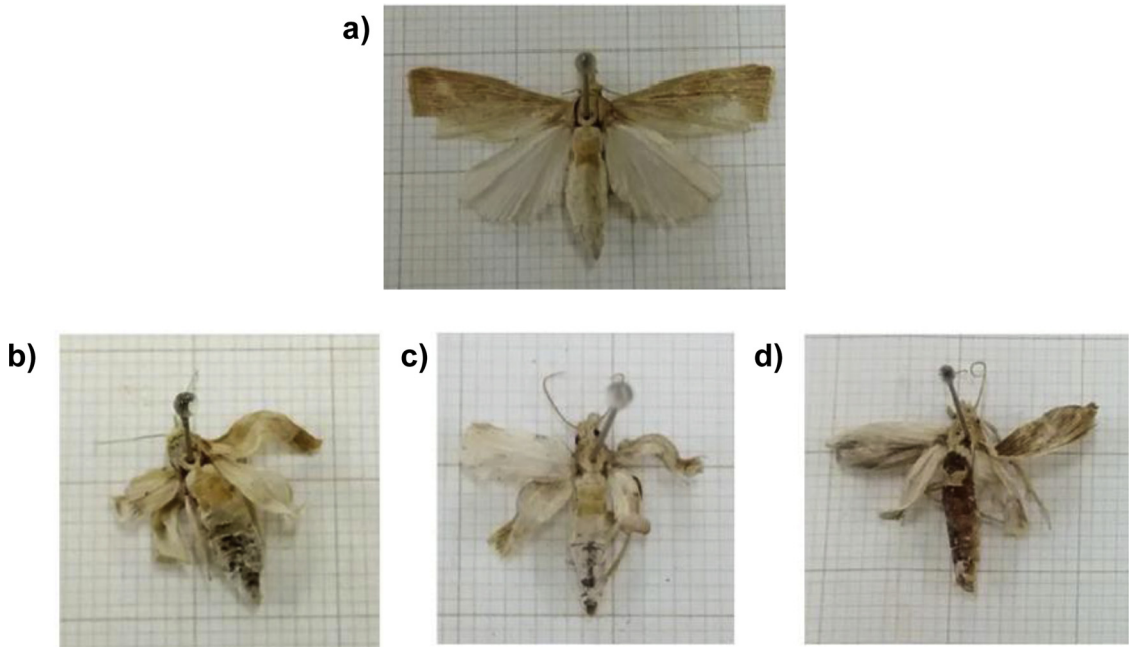

Fig. 3. Deformation of *Diatraea saccharalis* moths caused by chlorogenic acid exposure. a) Non-deformed moth fed with diet free from chlorogenic acid (CGA); b, c, and d) Deformed moths fed with diet containing CGA at 0.05, 0.5, and 5 mg/mL, respectively.

been described as an important defense metabolite in plants, providing defense against plenty of pathogens and a wide range of anti-herbivore activities (Kundu and Vadassery, 2019).

We added different concentrations of CGA to artificial diet, based on the amount existing in attacked tissues, in order to examine the effect on growth and survival of *D. saccharalis*. Regarding pupal weight, there was no difference between the caterpillars fed with different doses of chlorogenic acid when compared with caterpillars fed without chlorogenic acid (control) in the artificial diet. On the other hand, the results showed an expressive reduction on the life cycle period from caterpillar to adult, correlated with increasing doses of CGA in the diet, from 42 days in the artificial diet only to 39, 37 and 32 days in the diet with 0.05, 0.5 and 5 mg/mL CGA, respectively (Fig. 2a). The percentage of adult deformity also increased with the crescent doses in 20%, 27% and 61%, respectively (Figs. 2b and 3). Interestingly, CGA amounts similar to the biological concentrations (0.5 mg/mL) found in leaf tissues were enough to show anti-herbivore activity. Despite of the strong anti-herbivore activity of CGA, the results can differ depending on the plant-

insect interaction (Kundu and Vadassery, 2019). For example, *Nicotiana attenuata* attacked by the stem borer *Trichobaris mucorea* elicited an increase of 1000 fold the CGA levels. *T. mucorea* larvae feeding on artificial diet containing CGA (8.46 mM) gained significant less mass than feeding on control artificial diet. However, silenced *N. attenuata* plants for CGA biosynthesis, did not lead to any increased herbivory for mirids, grasshopper, noctuidae and flea beetles in the field (Lee et al., 2017).

5. Conclusion

The mechanism of response of sugarcane to insect herbivores is still poor understood. The current study shows that one of the mechanisms of sugarcane defense against one of the main sugarcane pests, *D. saccharalis,* involves a reduction of the primary metabolism and the activation of the phenylpropanoids pathway and a consequent accumulation of CGA in sugarcane leaf tissues. CGA showed a strong anti-herbivore activity affecting negatively the biology and development *D. saccharalis* thus playing a crucial role in chemical defense of sugarcane against herbivory. It is also worth pointing out our metabolomics approach used only one sugarcane clone, the commercial hybrid SP 79-1011. Sugarcane cultivars have an extreme complex genome and genetic diversity derived from hybridizations between different species: *S. officinarum* and the wild species *S. spontaneum* (Henry and Kole, 2010). Therefore, CGA could be used as a marker and support sugarcane breeders to search for more resistant cultivars producing higher contents of CGA. Moreover, the sugarcane variability and the wild relatives could be explored to find new metabolites involved in plant defense against herbivory. We therefore conclude that our findings proved that NMR metabolomics can be a successful tool to explore and identify metabolites involved in plant defense against herbivores.

Acknowledgments

To the technicians of the NMR labs Harold Toms from Queen Mary College; and Peter Haycock and Michael Kyriakides from Imperial College London. The authors thank the CNPq and MCTI, Fapeal and Capes for the schorlaship with grant number PVE/CSF/CAPES, A021-2013.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111651.

References

Aitken, K.S., Li, J.-C., Jackson, P., Piperidis, G., McIntyre, C.L., 2006. AFLP analysis of genetic diversity within Saccharum officinarum and comparison with sugarcane cultivars. Aust. J. Agric. Res. 57, 1167–1184. https://doi.org/10.1071/AR05391.

Appel, H.M., 1993. Phenolics in ecological interactions: the importance of oxidation. J. Chem. Ecol. 19 (7), 1521–1552. https://link.springer.com/article/10.1007%2FBF00984895.

Beeharry, R.P., 1996. Extended sugarcane biomass utilisation for exportable electricity production in mauritius. Biomass Bioenergy 11 (6), 441–449. https://doi.org/10.1016/S0961-9534(96)00050-5.

Cloarec, O., et al., 2005a. Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic ^{1}H NMR data sets. Anal. Chem. 77, 1282–1289. https://doi.org/10.1021/ac048630x.

Cloarec, O., et al., 2005b. Limitations caused by chemical shift variability and improved visualization of biomarker changes in ^{1}H NMR spectroscopic metabonomic studies. Anal. Chem. 77 (2), 517–526. https://doi.org/10.1021/ac048803i.

Colbert, J.T., Evert, R.F., 1982. Leaf vasculature in sugarcane (*Saccharum officinarum* L.). Planta 156 (2), 136–151. https://doi.org/10.1007/BF00395428.

Cristofoletti, P.T., Kemper, E.L., Capella, A.N., Carmago, S.R., Cazoto, J.L., Ferrari, F., Santos, N.Z., 2018. Development of transgenic sugarcane resistant to sugarcane borer. Trop. Plant Biol. 11, 17–30. https://doi.org/10.1007/s12042-018-9198-y.

Erickson, L., Trygg, J., Wold, S., 2008. CV-ANOVA for significance testing of PLS and OPLS® models. J. Chemom. 22, 594–600. https://doi.org/10.1002/cem.1187.

Friend, J., 1981. Recent Advances in the Biochemistry of Fruits and Vegetables. Academic Press, Cambridge, UK.

Hall, R.D., 2006. Plant metabolomics: from holistic hope, to hype, to hot topic. New Phytol. 169, 453–468. https://doi.org/10.1111/j.1469-8137.2005.01632.x.

Henry, R., Kole, C., 2010. Genetics, Genomics and Breeding of Sugarcane 2010. Science Publishers, pp. 276.

Hensley, S.D., Hammond, A.H., 1968. Laboratory techniques for rearing the sugar cane borer on an artificial diet. J. Econ. Entomol. 61, 1742–1743. https://doi.org/10.1093/jee/61.6.1742.

Hong, J., et al., 2016. Plant Metabolomics: an indispensable system biology tool for plant science. Int. J. Mol. Sci. 767, 1–16. https://www.mdpi.com/1422-0067/17/6/767.

Kim, H.K., Choi, Y.H., Verpoorte, R., 2010. NMR-based metabolomic analysis of plants. Nat. Protoc. 3, 536–549. https://doi.org/10.1038/nprot.2009.237.

Kundu, A., Vadassery, J., 2019. Chlorogenic acid-mediated chemical defence of plants against insect herbivores. Plant Biol. 21 (2), 185–189. https://doi.org/10.1111/plb.12947.

Le Gall, H., Fontaine, J.X., Molinié, R., Pelloux, J., Mesnard, F., Gillet, F., Fliniaux, O., 2017. NMR-based metabolomics to study the cold-acclimation strategy of two miscanthus genotypes. Phytochem. Anal. 28 (1), 58–67. https://doi.org/10.1002/pca.2649.

Leiss, K.A., et al., 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in seneciohybrids. J. Chem. Ecol. 2, 219–229. https://doi.org/10.1007/s10886-008-9586-0.

Minorsky, P.V., 2002. The hot and the classic. Plant Physiol. 130, 1077–1078.

Macel, M., van Dam, Nicole M., 2018. Metabolomics of plant resistance to insects. The Biology of Plant-Insect Interactions. CRC Press, pp. 129–149.

Mirnezhad, M., Romero-González, R.R., Leiss, K.A., Choi, Y.H., Verpoorte, R., Klinkhamer, P.G., 2010. Metabolomic analysis of host plant resistance to thrips in wild and cultivated tomatoes. Phytochem. Anal. 21 (1), 110–117. https://doi.org/10.1002/pca.1182.

Moore, Paul H., 1995. Temporal and spatial regulation of sucrose accumulation in the sugarcane stem. Funct. Plant Biol. 22 (4), 661–679. https://doi.org/10.1071/PP9950661.

Nakano, O., Romano, F.C.B., Pessini, M.M.O., 2001. Broca do rizoma (*migdolus* spp.). In: Nakano, O., Romano, F.C.B., Pessini, M.M.O. (Eds.), Pragas De Solo. ESALQ/USP, Piracicaba, pp. 25–35.

Nakayama, T., Honda, K., 2004. Evolutionary, mechanistic and environmental approaches to chemically-mediated interactions. Chemoecology 14, 199. https://doi.org/10.1007/s00049-004-0272-6.

Saito, K., Dixon, R.A., Willmitzer, L., 2006. Biotecnology in Agriculture and Forestry: Plant Metabolomics, 57 ed. Springer, Hamburg.

Sandoval, S.S., Senô, K.C.A., 2010. Comportamento e controle da *Diatraea saccharalis* na cultura da cana-de-açúcar. Nucleus 7, 243–257. https://doi.org/10.3738/1982.2278-311.

Schultz, J.C., Appel, H.M., Ferrieri, A.P., Arnold, T.M., 2013. Flexible resource allocation during plant defense responses. Front. Recent Dev. Plant Sci. 4, 324. https://doi.org/10.3389/fpls.2013.00324.

Schuman, M.C., Baldwin, I.T., 2016. The layers of plant responses to insect herbivores. Annu. Rev. Entomol. 61, 373–394. https://doi.org/10.1146/annurev-ento-010715-023851.

Sekiyama, Y., Okazaki, K., Kikuchi, J., Ikeda, S., 2017. NMR-based metabolic profiling of field-grown leaves from sugar beet plants harbouring different levels of resistance to cercospora leaf spot disease. Metabolites 7 (1), 4. https://www.mdpi.com/2218-1989/7/1/4.

Zhou, S., Lou, Y.-R., Tzin, V., Jander, G., 2015. Alteration of plant primary metabolism in response to insect herbivory. Plant Physiol. 169 (3), 1488–1498. https://doi.org/10.1104/pp.15.01405.

Sun, J., Zhang, X., Cao, C., Mei, X., Wang, N., Yan, 2014. Similar metabolic changes induced by HIPVS exposure as herbivore in *Ammopiptanthus mongolicus*. PLoS One 9 (4), e95474. https://doi.org/10.1371/journal.pone.0095474.

Wu, X., Ding, Chaohui, Lian, Fazhuo, Lin, Xianhui, Zhang, Liqin, Wu, Choufei, Hwang, Shaw-Yhi, Zeng, Rensen, Song, Yuanyuan, 2018. The roles of jasmonate signalling in nitrogen uptake and allocation in rice (*Oryza sativa L.*). Plant Cell Environ. 42 (2), 659–672. https://doi.org/10.1111/pce.13451.