Metabolomic study of maize leaves infested by two-spotted spider mite
*Tetranychus urticae*

_estudo metabolômico de folhas de milho infestadas pelo ácaro _Tetranychus urticae_

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ABSTRACT

Maize (Zea mays) is a Poaceae of great global importance, which requires great technological investment, such as the Bt events, for herbivores populational control which can cause low productivity. This study aims to create a parallel between the metabolomics behavior of a maize variety and its isoline under the influence provoked by two-spotted spider mite damage. Differences in identified ions concentration and distribution were observed as coming from the monosaccharides, jasmonic acid and methyl jasmonate ionizations, metabolites that would be directly related to the induced defense, which showed higher concentration in the Bt maize leaves, clean and infested, and in the conventional infested maize leaves. Furthermore, other two ions, m/z: 391.018 and 217.012, were observed in higher concentrations in Bt maize leaves, clean and infested, and in the conventional infested maize leaves. With the obtained data, It was possible to conclude that the maize plants, both Bt and conventional, are capable of respond to damage provoked by two-spotted spider mite infestation, and the maize Bt plants it was an expressive response in comparison to its isoline.

Keywords: Zea mays, Tetranychus urticae, metabolites, DESI-MS.
1 INTRODUCTION

Maize (*Zea mays*) is a Poaceae originated from Central America of great global importance in economics (MIRANDA *et al*., 2021). The brazilian production of maize reached the 155.662 tons mark, distributed in an area of 21.667 thousand ha, in 2021/2022 harvest (CONAB, 2022).

Due to its high demand, the productive system that normally is adopted to maize farming is monoculture, which prioritizes the cultivated area padronization. The simplification of the agroecosystem is beneficial to the producer, as it guarantees more commercially desirable characteristics and facilitates cultural practices. This practice leads to biotic stress losses, such as herbivory. It is necessary the use of technologies with the goal to contain the population growth of herbivores, such as Bt transgenic events (ZIMMERMANN, 2009; PAULO *et al*., 2015).

Many species of arthropods can easily achieve a pest status for maize crop, amidst the Insecta class organisms some can be highlighted, such as the Spodoptera frugiperda species. Other class that presents plague organism status is the Arachnida, with phytophagous species from the acari order, such as the two-spotted spider mite (*Tetranychus urticae*) (PAULO *et al*., 2015).

The two-spotted spider mite is a cosmopolitan organism, that strikes crops with great economic importance. Its feeding is done through little punctures in plants leaves, through which cellular content is extravasated, resulting in the death of vegetal cellular tissue (BUENO *et al*., 2009). Despite not being deemed maize crop key-plague, there are efforts to measure the real impacts from the species, due to its endemic presence and biological unbalance provided by the indiscriminate use of populational control technologies for agricultural pests (PAULO *et al*., 2015).

Plants, to ensure its development and survivability, synthetize metabolites that can be classified between primary or secondary metabolites. This classification bases itself on their function in the organism, with the primary metabolites being those that act in key processes to the plant, such fatty acids, amino acids, proteins, lipids, carbohydrates, and nucleic acids (GOMES, 2017; FREITAS, 2018).

Secondary metabolites, however, are those synthesized to ensure the plant survivability. There are countless secondary compounds present in the vegetal body, making an arsenal of mechanisms with high degree of specificity within a wide variety of taxons, which can be
grouped as phytochemicals, such as alkaloids, terpenoids, flavonoids, phenolic compounds, tannins and lignins (KREIS et al., 2017; VON POSER, 2017).

The synthesis of compounds related to the plant induced defenses can vary according to the perceived stimulus, and the concentration of such compounds varies with the phenological state of the plant and the environmental conditions (GOBBO-NETO & LOPES, 2007). In the case of the biological agents, such as herbivores, the induction occurs by the accumulation of oxygen reactive species (ORS) on affected tissue. Resulting in the activation of metabolic pathways capable of synthesizing compounds that affect the consumer directly or indirectly (PINTO-ZEVALLOS et al., 2013; TRINDADE, 2021).

One of the means of direct defense is associated to the compound production with antinutritional properties, which decreases the ability of herbivores to digest food, affecting the primary consumer biological survival parameters. Indirect defenses are related with the attraction of primary consumers natural enemies or the repellent effects over herbivores, through the production of volatile compounds, such as terpenoids (PINTO-ZEVALLOS et al., DICKE et al., 2009).

Metabolomics corresponds to the “omic sciences” part that has the objective of study cell metabolic processes, emerging with the arrival of technology capable of measuring the concentration of metabolites amidst intracellular content, such as Secondary Ion Mass Spectrometry (SIMS) and Matrix-Assisted Laser Desorption Ionization (MALDI) which are ambient ionization techniques (FREITAS, 2018).

The electrospray ionization and desorption (DESI) is one of the ambient ionization techniques, that, when used with mass spectrometry, allows - with little processing of the sample - the identification of ions present on the analyte, as well as its concentration and 2D space distribution (FREITAS, 2018; VASCONCELOS).

The present study aims to compare the metabolomic behavior of a Bt maize variety and its isoline, when infested by two-spotted spider mite.

2 MATERIALS AND METHODS

The experiment was carried out at the Federal University of São João del Rei- Campus SeteLagoas. The structures of the Organic Chemistry and Photochemistry and Agricultural and Florestal Entomology Laboratories were utilized for the mite creation and infestation and maize
The mites were obtained from Empresa Brasileira de Pesquisa Agropecuária Milho e Sorgo (Embrapa) and the Chemistry Institute of the Estadual University of Campinas (UNICAMP), and were the desorption and electrospray ionization analysis was carried out, respectively.

2.1 TWO-SPOTTED SPIDER MITE GROWTH

The two-spotted spider mite, utilized in maize infestation, were collected from sorghum (Sorghum bicolor) plants cultivated at the vegetation house from Embrapa, Sete Lagoas - MG. The mites were moved with the help of a brush for Canaliaensiformis when they reached 15 days after germination. The Canaliaensiformis growth was carried out in 6.3L plastic pots, and Terral Solo® was used as substrate, under screened cages, in vegetation house, with room temperature and irrigated when needed, using the contact humidity as parameter. Non-infested plants were added at the side of infested plants every seven days, as food for the mites. After the migration to the new plants, old plants were discarded.

2.2 CULTIVATION OF THE MAIZE PLANTS

Hybrid 30F35 Hx (Herculex, Pioneer®) maize plants were chosen due its Cry1F protein expression (one of the proteins expressed by plants that possesses the Bt transgenic event), and its conventional analog 30F35. The sowing was carried out in 1L plastic pots, and Terral Solo® was used as substrate. Three seeds were sowed in each pot so that there were two pots with Bt maize and two pots with conventional maize plants. The pots were kept in anti-aphids screened cages, in a vegetation house and, at the end of two weeks, thinning was done, leaving only one plant per pot, irrigated when needed, using the contact humidity of the substrate. 0.2g of ammonium sulfate was added to the substrate, to supply nutritional demand, every 15 days after the thinning.

2.3 MAIZE INFESTATION

Two plants of each variety were used, one infested and the other used as control. Infestation occurred 40 days after sowing, using screened cages to separate the infested plants from the control group. For the infestation, in both Bt and conventional maize, 10 female two-spotted spider mites were used, kept inside an entomological glue barrier (Biocontrole©) to prevent escape from the abaxial leaf parts.
2.4 DESI-MSI analysis

For sample preparation, the removed leaf fragments were subjected to the imprint process to obtain a plain surface. To this end, the fragments were washed with HPLC-grade toluene to remove the cuticle, exposing the epidermal surface. Afterwards, the fragments were pressed for 60 seconds between two porous polytetrafluoroethylene (PTFE) membranes in a manual press, which the pore size was 0.45µm and 47mm in diameter (Allcrom). The membrane of the abaxial side was chosen to be submitted to DESI-MSI analysis because its less resistance to photo-assimilates transfer. The fragments were arranged on the same membrane to facilitate the comparison of treatments.

The following parameters were used to operate the mass spectrometer and the ionization source: capilar voltage of 5.0kV, capilar temperature of 300°C, 70.000FWHM resolution; the solvent used for spray formation was HPLC-grade methanol, under a 10µL min⁻¹ flux, 100 to 1000m/z mass range for both positive and negative ionization modes. A step size of 200µm was determined on the moving platform, pixel size of 200µm x 200µm and scanning rate of 7400µm sec⁻¹.

The mass spectra analyses were made in Xcalibur (Thermo Fisher Scientific Inc.) software. Images were converted to the Firefly (version 2.1.05) software and BioMAP (version 3.8.04) for visualization. Some compounds were identified through comparison between the m/z found in the spectra and the m/z of compounds similar to phytochemical groups, such: carotenoids, flavonoids and phenolic compounds; found on the PubChem platform.

3 RESULTS AND DISCUSSION

3.1 ENERGY PRODUCTION AND CONSUMPTION

The 179.056 m/z ion was identified, in negative ionization mode, as a product of ionization of monosaccharides (Figure 1). The color scale refers to the ion concentration along the surface of the leaf, red areas are the regions of highest monosaccharide concentration, and black areas indicate their absence.
Similarity in monosaccharide distribution between Bt and conventional maize fragments was expected, considering the gene similarity between the varieties, as they were in the same phenological stage and exposed to the same environmental and nutritional conditions. However, it was possible to observe a higher concentration of monosaccharides in the Bt maize leaf than in the conventional maize leaf, which may be the result of a higher production of monosaccharides or a low translocation of them to other parts of the plant. The higher production of sugars in variety B studied, either due to a higher content of chlorophyll present in the leaf or to the low rate of translocation to other parts of the plant, could affect sensory characteristics of fresh grains and their derivatives.

In the fragments from conventional maize, it was possible to observe a similar metabolic behavior, presenting the monosaccharides more located in the margins of the fragment, which makes sense if we think about the biosynthetic routes of polysaccharides and lignins that would be produced aiming at the prevention against pathogenic infections, at the site of leaf fragmentation (VON POSER, 2017). However, the infested leaves had regions of higher concentration in the middle of the fragment, which is an important point considering that monosaccharides serve as a basis for the biosynthesis of other compounds that would be associated with defense against herbivores and oxidative damage associated with mesophyll
exposure to ROS (PEREIRA & ANDRADE, 2014). The way in which sugars are distributed in the plant may be useful in observing an induced response to herbivory stress, as suggested by PAULO et al. (2015).

It is possible to notice a difference in the concentration of sugars between clean and infested Bt maize leaves, where the infested leaves present a lower concentration of monosaccharides. This suggests a higher metabolic activity in infested Bt maize leaves, considering that both the conversion of monosaccharides into the energy required for metabolic processes and the increase of carbon skeletons that act as substrates in such metabolic processes, would consume the sugars present regardless of the type of biosynthetic route activated to respond to the stimulus (PEREIRA & ANDRADE, 2014).

3.2 DEFENSE INDUCTION

The ion of m/z 209.118 was identified in the negative ionization mode as the ionization product of jasmonic acid (Figure 2). Throughout the fragments, the areas in red are those with the highest concentration of the ion and, in contrast, the areas in black indicate its absence.

Figure 2. Jasmonic acid concentration (m/z 209.118) in the fragments, where “a” corresponds to Bt maize leaf infested by spider mites, “b” clean Bt maize leaf, “c” conventional maize leaf infested by spider mites and “d” clean conventional maize leaf.

Source: Authors (2023)
The jasmonic acid (JA) is one of the compounds synthesized from the octadecanoid route, in which hydroperoxide-octadecatrienoic acid (13-HPOT) is converted to oxo-phytodienoic acid (OPDA). Thus, OPDA can remain in the cytosol - exerting a signaling function - or be converted to oxo-pentenyl- cyclopentanes, in the peroxisomes - where they are metabolized to JA (SOARES & MACHADO, 2007). Its function, together with ethylene and salicylic acid, is linked to the expression of stress response genes, such as in the activation of the terpenoid biosynthetic pathway, acting both locally and systemically (PINTO-ZEVALLOS et al., 2013). Despite the detection of such a compound, it was not possible to identify terpenoids as they are not susceptible to ionization using the DESI-MS technique.

In the clean Bt maize leaf fragment, a higher concentration of JA was found. In the fragment of the Bt maize leaf infested by mites, it was possible to observe a lower concentration of JA, when compared to the fragment of the clean Bt maize leaf, something that could explain this fact would be the activation of metabolic pathways that are antagonistic to the jasmonate route, such as the biosynthetic route of salicylates (PINTO-ZEVALLOS et al., 2013), or the consumption of JA in the production of its derivatives, such as methyl jasmonate. In the leaf fragment of the conventional maize infested by spider mite, a higher distribution of JA was observed when compared to the leaf fragment of the clean conventional maize. This supports the idea that the conventional maize plant would be able to respond to the mechanical damage caused by the mite, which does not mean that it would be able to contain the present population of spider mites. BOTTI et al. (2019) observed that predatory mites, Neoseiuluscalifornicus, were unable to distinguish between volatiles released by mite-infested and clean Bt and conventional maize plants. Therefore, the non-preference for infested plants may be an indication of a low adaptability of the plant-herbivore-predator system, in the co-evolutionary process, thinking that the volatiles released do not signal the presence of the herbivore to the predator, or that the release of volatiles stimulated by the presence of the herbivore was not expressive enough to trigger a response to the predators N. californicus.

In the clean Bt maize leaf, the high concentration of JA could be indicative of a greater susceptibility of Bt maize plants to infectious agents, due to the antagonism between the metabolic pathways of jasmonates, linked to defense mechanisms against herbivores; and the salicylate pathway, linked to responses to biotic stress caused by the presence of pathogens (PINTO-ZEVALLOS et al., 2013). In addition, in clean conventional maize leaves it was
possible to observe a higher concentration of JA bordering the fragment, which can be explained by the accumulation of ROS caused by leaf fragmentation in the sample preparation.

Another ion observed in the negative ionization mode was the $m/z$ 223.109, identified as the ionization product of methyl jasmonate (MeJA). Figure 3 shows the distribution of MeJA in the studied fragments. It can be observed through the color scale ranging from red (areas of highest concentration) to black (areas of lowest concentration), patterns very similar to the distribution of JA (Figure 2).

Synthesized from the methylation of JA in peroxisomes, the function of MeJA in the plant is associated with both positive and negative regulation of protease activity, which would reduce the digestibility of plant material in herbivores that consume them (HILDER et al., 1987). Its protease regulatory function, a hypothesis that could explain the abundance of MeJA in the fragment from the Bt plant, would be the regulation of Cry1F protein biosynthesis.

Other two compounds that are possibly linked to maize defense are the ionized compounds with $m/z$: 391.018 and the 217.012, observed in the negative ionization mode. In Figures 4 and 5 it is possible to observe the presence of both compounds following the same color pattern in which the areas in red are the areas of higher concentration and the areas in black
are those of lower concentration. In the leaf fragment of attacked conventional maize and the low concentration in clean conventional maize, suggest that the appearance of both was due to the presence of the spider mite. In addition, it is possible to observe that both compounds were present in the leaf fragments of Bt maize before and after infestation, suggesting that both compounds were synthesized constitutively or that the Bt variety evaluated was under a stress condition before infestation to displace energy resources and allocate them to the pathways that would synthesize both compounds (DIAS et al., 2015).

Figure 4. Concentration of the ion of m/z 391.018 in the fragments, where “a” corresponds to Bt maize leaf infested by spider mites, “b” clean Bt maize leaf, “c” conventional maize leaf infested by spider mites and “d” clean conventional maize leaf.

Source: Authors (2023)
Figure 5. Concentration of the ion of 217.012 in the fragments, where “a” corresponds to Bt maize leaf infested by spider mites, “b” clean Bt maize leaf, “c” conventional maize leaf infested by spider mites and “d” clean conventional maize leaf.

Source: Authors (2023)

FRANZIN et al. 2020 report the absence of the expression of a direct induced defense and explain the fact with the low intensity of the injury and the short time of exposure of Bt and conventional maize leaves to T. urticae. However, the higher intensity of the ions of m/z: 391,018 and 217,012 in the infested conventional maize, when compared to the clean conventional maize leaf fragment, and the intensity of the ion that was identified as being the product of the ionization of MeJA in the treatments submitted to infestation, suggest the expression of defense induced by T. urticae herbivory, which would corroborate the data obtained by PAULO et al. (2015), regarding the presence of a direct induced response, against T. urticae herbivory.

The presence of the compounds that ionize resulting in ions of m/z: 391.018 and 217.012, in the leaf fragment of clean Bt maize could also be associated with the results of non-preference for feeding, as pointed out in the studies carried out by COSTA et al. (2009). When using Spodoptera frugiperda, he observed that the Spodoptera frugiperda rejected the Bt maize leaves at the first contact with them, while they fed on the offered conventional maize leaves and stopped feeding over time. The expression of such compounds in Bt maize may be just a marker between the Bt variety and its isoline or a response of a hypersensitivity reaction provided by the insertion of the gene encoding the Cry1F protein. However, further studies need to be developed to elucidate the chemical structure of such compounds and their respective functions in the plant.
4 CONCLUSIONS

DESI-MSI has shown to be a promising technology for the phytosanitary area, as it allows the identification of the metabolomic behavior of plants in the face of stresses, and can be a strong ally in plant breeding programs.

Regarding the compounds identified, monosaccharides were important to raise the hypothesis of a higher production of sugars or a low translocation of these, but more detailed studies regarding the parameter are necessary for the adaptation to the management techniques of maize crops.

The concentrations of jasmonic acid and methyl jasmonate were important indicators of plant health status. Jasmonic acid was an important indicator of greater susceptibility of Bt maize to pathogens due to the intensity with which it is expressed in clean Bt maize and the antagonistic relationship of the jasmonate and salicylate pathways. It also provides information on the maize's ability to respond to minor spider mite damage in infested conventional maize.

Regarding the concentration of methyl jasmonate, it was an important parameter to raise the hypothesis of a hypersensitivity reaction of maize to the Cry1F protein synthesized in the variety carrying the Bt technology evaluated, studies in the field of proteomics could be able to answer such a hypothesis.

The identification of the compounds m/z: 391,018 and 217,012 in the leaves carrying the Bt technology and in the conventional leaves were important indicative of the induction of defense in maize by the spider mite. In addition, the intensity which the compounds were expressed in clean Bt maize suggests a metabolic imbalance.
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