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Resistance elicitors and defense response enhancers of maize to *Spodoptera frugiperda* (J.E.Smith) (Lepidoptera: Noctuidae)

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Abstract

The objective of this research was to assess the potential of abiotic compounds as defense response enhancers in maize using enzymatic quantification and their efficiency in reduction of damages caused by *S. frugiperda*. The experiment was laid out in a randomized complete block design with six treatments and five replications. The treatments were: T1: positive control (distilled water + infestation); T2: negative control (without product application, uninfested); T3: biofertilizer; T4: acibenzolar-S-methyl (ASM); T5: potassium silicate; T6: potassium silicate + ASM. The treatments were applied in V6 stage of maize plants. After five days, the plants were artificially infested with 2nd instar caterpillars of *S. frugiperda*. Damage assessments were carried out at 4, 8, 16 and 22 days after infestation using a scale of notes. To evaluate the enzymatic activity, one leaf of each plant was removed at 2, 4, 8, 16 and 22 days after infestation. The largest peroxidase activities were observed at two DAI (days after infestation) using the potassium silicate + ASM (2,344.12 UAE.mg^{-1} of proteína.min^{-1}) treatment, while the larger polyphenol oxidase activity peaks were observed after treatment with ASM at 22 DAI. The application of potassium silicate + ASM in corn plants can promote peroxidase activity. The polyphenol oxidase activity was increased after application of ASM, whereas the presence of the pest (on positive control) could potentially increase the activity of phenylalanine ammonia-lyase. The applications of ASM alone and potassium silicate + ASM contributed to reduction of the foliar damage level caused by *S. frugiperda* in corn plants.

Keywords: Fall armyworm; Phenylalanine ammonia-lyase; Polyphenol oxidase.
Abbreviations: ASM_ Acibenzolar-S-Methy; DAI_days after infestation.

Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith, 1797) is distributed throughout Latin America, the Caribbean and the southern United States. It is a polyphagous insect that, feeds on corn and other grasses such as wheat, sorghum and rice. It also feeds on cotton (Bueno et al., 2011). Due to the high *S. frugiperda* frequency and distribution, it occurs during all seasons of the year and can destroy the corn crop leaves and cartridges, causing vascular damages on foliar tissues and affect the grains, resulting in a yield decrease up to 38% (Brewer et al., 2014).

The control of *S. frugiperda* by conventional methods is limited because of its feeding habit on the leaves and cartridges, which makes it protected by the plant. The insect completes its larval development, causing large damages (Moraes et al., 2015). Thus, the use of external products or agents (inductors) with the objective of inducing resistance in plants is an alternative to the use of insecticides.

Increased enzyme activities including accumulation of phenolic compounds, phytoalexins and pathogenesis-related proteins (such as β-1.3 glucanase, chitinase, peroxidase, phenylalanine ammonia-lyase and polyphenol oxidase), are among induced resistance-related events in plants (Barros et al., 2010).

Peroxidase is an enzyme that participates in several physiological processes in plants such as lignification, suberization, formation of cell wall components, protection against attack of pathogens, insects and abiotic stressors (Almagro et al., 2009). The polyphenol oxidases catalyze reactions responsible for producing toxic compounds to pathogens and insects and can prompt to plant resistance (Webb et al., 2013).

The phenylalanine ammonia lyase enzyme plays a fundamental role catalyzing the conversion of L-phenylalanine to trans-cinnamic acid, a deamination reaction. This reaction is considered an essential step in the phenylpropanoid pathway producing many products, including lignin, which is involved in plant defense reactions (André et al., 2009).

The use of resistance elicitors has shown satisfactory results in insect pest control. War et al. (2011) evaluated the induced resistance in groundnut plants by jasmonic acid (JA) on *Spodoptera litura.* The authors found that this product induced significant activity increases of the enzymes peroxidase, polyphenol oxidase and amounts of total phenol and protein after application of JA and infestation of S. *litura.* The authors suggest that pretreatment with elicitors such as JA provides more opportunity for plant defense against herbivores.

Induced resistance of plants to control insect is an important tool to reduce the impact caused by pesticides over the years. It is a low cost and an easy alternative for pest management that can be associated with chemical control of insect pests. Thus, this study aimed to evaluate the potential of abiotic compounds as defense response elicitors through enzymatic quantification and their efficiency to reduce damage caused by *S. frugiperda* in corn.

Results and Discussion

Effects of resistance elicitors on enzymatic activity

Both the positive and negative controls were adjusted to the quadratic regression model, with higher peroxidase activity at the initial and final evaluation periods at two and 22 days after infestation (DAI), with 846.4 and 866.3 UAE.mg^{-1} of protein.min^{-1} for the positive and 1,100.6 and 655.7 UAE.mg^{-1} of protein.min^{-1} for negative control, respectively. In the intermediate periods (at 11.3 and 13.1 days), minor activities of these enzymes were recorded to these treatments, 452.4 and 431.4 UAE.mg^{-1} of protein.min^{-1}, respectively (Fig. 1A and 1B).

The biofertilizer and ASM treatments did not adjust to linear or quadratic regression models and presented means of 839.9 peroxidase activity and 882.8 UAE.mg^{-1} of protein.min^{-1}, respectively, regardless of the assessment period (Fig. 1C and 1D). The potassium silicate treatment was adjusted to the linear regression model with decreasing peroxidase activity over time, with higher rates in the first assessment period, averaging 1,342.1 UAE.mg^{-1} of protein.min^{-1} (Fig. 1E). The potassium silicate + ASM treatment was adjusted to the cubic regression model, recording two peaks of peroxidase activity, the first at 2 DAI (2,344.12 UAE.mg-1 of protein.min-1) and the second at 16 DAI (1,034.4 UAE.mg -1 of protein.min-1) (Fig 1F). Apparently, the treatments with higher peroxidase activity cause a rapid but non-durable response, followed by a decrease, which also occurs in control and in the potassium silicate, where although they presented less activity upon applying potassium silicate + ASM, they also recorded the peak production of this enzyme at 2 DAI.

Despite the fact that peroxidase enzyme is related to events involving the resistance induction, there is no set pattern to their behavior, depending on type of inducer or elicitor, their concentration. In addition, there is no pattern on time of application in the plant and patho-systems (Peiter-Beninca et al., 2008). Therefore, this is an indication of variation in behavior of this enzyme upon different treatments in this study.

According to Gulsen et al. (2010), increase in peroxidase activity may result in lignifications, suberisation, wound healing, secondary metabolites production, increased phytoalexin production, suggesting that they are key enzymes involved in the last steps of lignification, promoting plant defense against insects and pathogens.

The increase in peroxidase activity in the potassium silicate + ASM treatment was probably due to a progressive incorporation of phenolic compounds in the cell wall affecting the *S. frugiperda* feeding, therefore, the plant-insect interaction. The reduced activity of this enzyme upon application of some treatments is likely indicates requiring extra application of this enzyme to keep it capable to activate various mechanisms during the resistance induction process.

All treatments influenced the polyphenol oxidase activity in maize leaves. The relative control treatment and potassium silicate + ASM was adjusted to the linear regression model, with increasing growth in relation to time (Fig 2A and 2F). The remaining treatments were adjusted to the quadratic model with a decrease in the polyphenol oxidase activity in the initial period and a subsequent increase in the following periods, with the lowest activities recorded at 8.8 days (0.58 UAE.mg-1 of protein.min-1) in the absolute control. In addition, null activities were observed for ASM at 8.5, 6.4 and 5.7 days, with averages of 0.28 and 0.27 UAE.mg-1 of protein.min-1 for the biofertilizer and potassium silicate treatments, respectively (Fig 2B, 2C, 2D and 2E). In general, all treatments were marked with the highest activity in the last assessment period (22 DAI), with ASM treatment showing the highest peak activity (3,347 UAE.mg -1 of protein.min-1).

In the present study, polyphenol oxidase showed an increasing activity in relation to time, even though it showed a decrease in activity in the initial periods in some treatments, contrary to what observed for peroxidase activity. The results corroborate the studies conducted by Vieira et al. (2016) that evaluated the potential of potassium silicate in the activity of enzymes involved in the defense system of *Citrus reticulata* in the growth and development of *Aleurocanthus woglumi*. They found that the greatest enzymatic activities of polyphenol oxidase is occurred between 31-59 days after pest infestation.

The polyphenol oxidase has been related to defense reactions against herbivory in several plant species. The defensive activity is based on the ability of this enzyme to rapidly oxidize *o*-dihydroxy phenols to the corresponding *o*-quinone when tissue is damaged. Quinones covalently bind to alquilation of the protein amino acids - lysine, histidine, cysteine and methionine, enabling them to become unavailable for insects,

Table 1. Average damage (Mean ± SE) caused by *Spodoptera frugiperda* fed on maize leaves treated with different resistance elicitor products. Temp.: 25 ± 1 °C, RH: 70% and photophase: 14 hs.

Treatments	Damages[1]			
	4 days	8 days	16 days	22 days
Positive control	3.055 ± 0.326 a	3.550 ± 0.526 a	3.685 ± 0.402 a	3.759 ± 0.360 a
Biofertilizer	2.647 ± 0.403 ab	3.142 ± 0.474 ab	3.405 ± 0.272 a	3.536 ± 0.307 a
Acibenzolar-S-methyl (ASM)	2.401 ± 0.738 ab	2.788 ± 0.671 ab	2.709 ± 0.339 b	2.691 ± 0.427 bc
Potassium silicate	2.629 ± 0.525 ab	3.467 ± 0.525 a	3.343 ± 0.283 a	3.281 ± 0.299 ab
Potassium silicate + ASM	1.759 ± 0.380 b	2.130 ± 0.608 b	2.484 ± 0.470 b	2.480 ± 0.344 c
CV(%)	19.92	19.10	10.35	10.85

[1]Means followed by the same letter in columns are not statistically different by the Tukey Test at 5% probability.

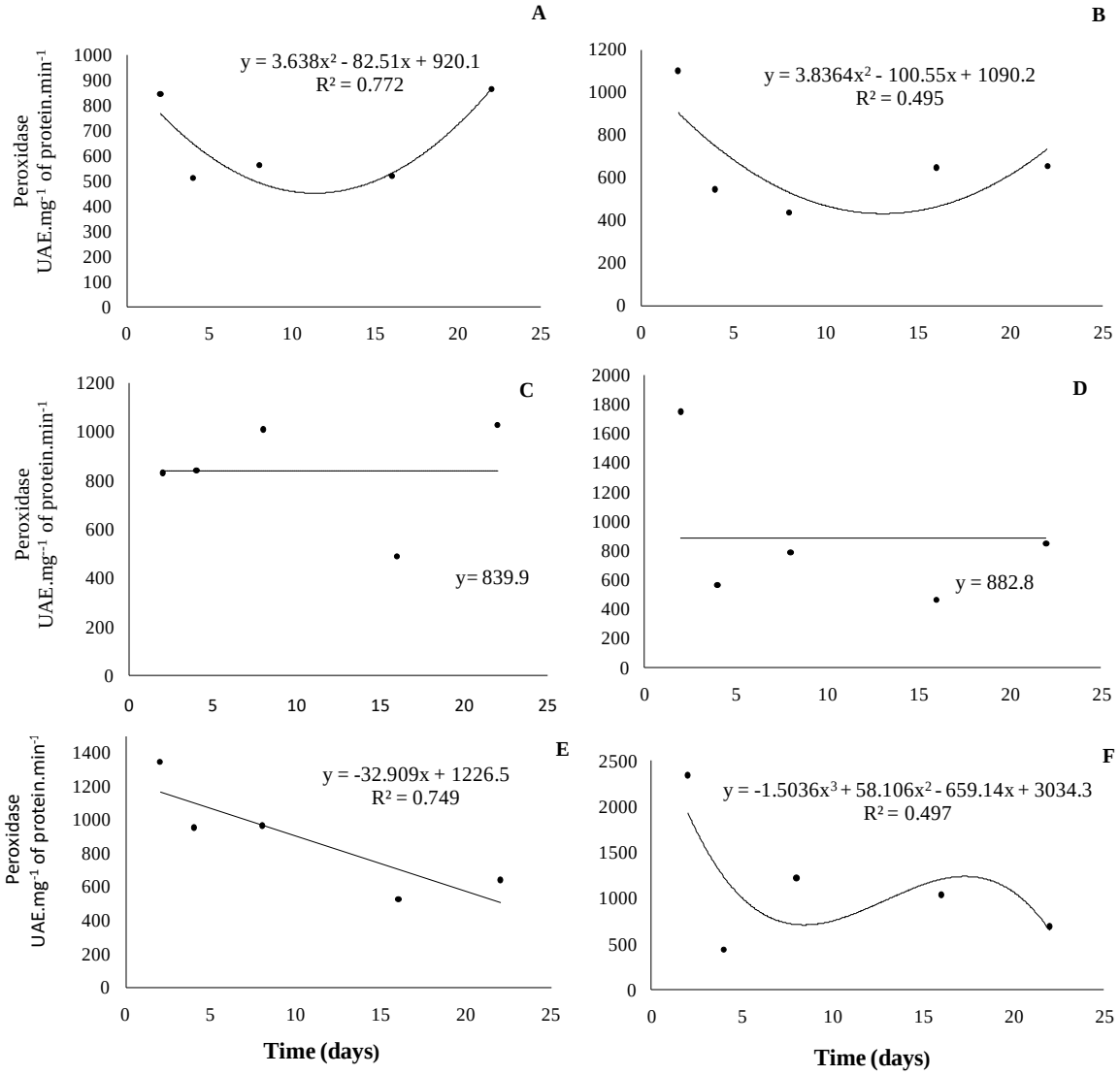

Fig 1. Peroxidase activity in response to the time after treatment application: A = positive control; B = negative control; C = biofertilizer; D = Acibenzolar-S-methyl (ASM); E = potassium silicate; F = potassium silicate + ASM.

Table 2. Damage visual scale to *Spodoptera frugiperda*

Score	Description
0	No leaf damage
1	Plants with scraped leaves
2	Plants with perforated leaves
3	Plants with perforated and injured leaves
4	Plants with leaves and whorl injured
5	Plants with many leaves and whorl totally destroyed with the presence of excrements

Source: Wiseman et al. (1968), with adaptations.

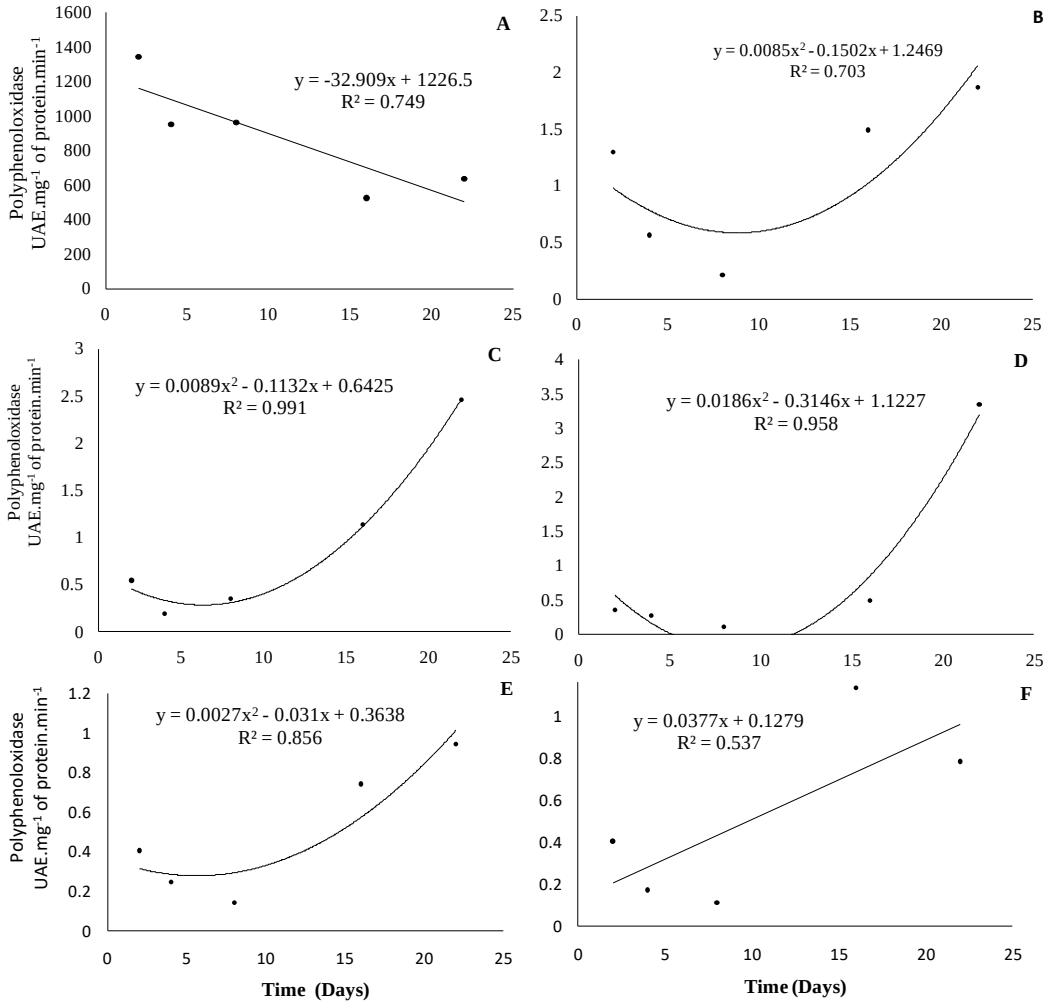

Fig 2. Polyphenol oxidase activity in response to the time after treatment application: A = positive control; B = negative control; C = biofertilizer; D = Acibenzolar-S-methyl (ASM); E = potassium silicate; F = potassium silicate + ASM.

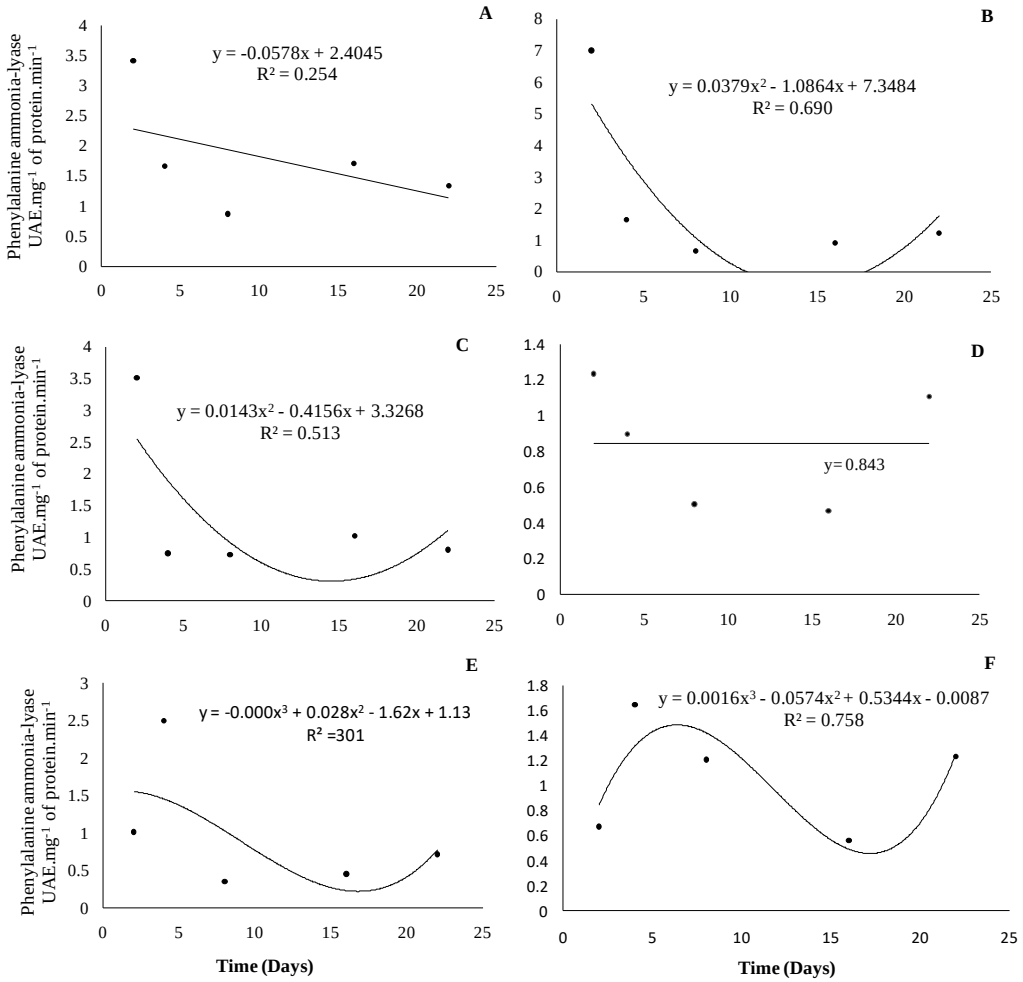

Fig 3. Phenylalanine ammonia-lyase activity in response to the time after treatment application: A = positive control; B = negative control; C = biofertilizer; D = Acibenzolar-S-methyl (ASM); E = potassium silicate; F = potassium silicate + ASM

Besides, reducing the plant tissue nutritional quality (Chen and Buntin, 2009; Bhonwong et al., 2009).

Thus, the increase in the polyphenol oxidase activity observed in corn plants probably caused a reduction in the quality of this protein in leaf tissue, due to the toxicity of quinones to insects, affecting digestibility and; therefore, minor damage caused by *S. frugiperda* as observed in this study.

With respect to the phenylalanine ammonia-lyase (PAL) activity, only the positive control was adjusted to the linear model, and then decreased over the assessment time (Fig.3A). The negative control and biofertilizer treatments adjusted to the quadratic model, where the maximum enzymatic activity was observed at two DAI for both treatments, with 5.32 and 2.55 UAE.mg^{-1} of protein.min^{-1}, respectively. However, the lower values for these treatments were recorded at 14.3 DAI with no activity for the negative control treatment and 14.5 DAI with activity of 0.31 UAE.mg^{-1} of protein.min^{-1} for biofertilizer (Fig.3B and 3C). None of the regression models were significant for ASM, showing an average activity for this enzyme at 0.81 UAE.mg^{-1} of protein.min^{-1} (Fig.3D). The other treatments adjusted to the cubic model and; therefore, presented two peaks in phenylalanine ammonia-lyase activity. The first was recorded at 4.07 DAI in potassium silicate and in the interaction of potassium silicate + ASM, respectively. The second peak was observed at 22 DAI for both treatments. In general, the values did not exceed 1.2 UAE.mg^{-1} of protein.min^{-1} (Fig.3E and 3F).

The phenylalanine ammonia lyase enzyme activity may be affected by several factors such as injury, UV radiation, heavy metal, low nitrogen, phosphate and iron concentrations, as well as due to the attack of insects or pathogens (Dai et al., 2006). Thus, it is a highly sensitive enzyme, which possibly explains the variation of this enzyme in negative control treatment, showing high activity in the first days of assessment.

The increase in the phenylalanine ammonia-lyase activity in the treatments may be attributed to defensive pathways in response to damage caused by *S. frugiperda*. These pathways are responsible for the production of several secondary metabolites in plant, which formed various defense compounds during the oxidation process. Furthermore, the phenylpropanoid precursor, to which phenylalanine ammonia-

lyase is a key enzyme, is responsible for lignin synthesis (Mao et al., 2007; Zhao et al., 2009).

War et al. (2015) assessed the induced resistance of peanut plants (*Arachis hypogaea* L.) to *Helicoverpa armigera* and defense enzymes activity by exogenous application of jasmonic acid (JA) and salicylic acid (AS). The authors reported that both JA and AS induced antioxidant responses promoting increased peroxidase and polyphenol oxidase activities, which reduced growth and development of *H. armigera* in the treated plants.

Damages of S. frugiperda in maize plants

The scores assigned to leaf damage due to feeding of *S. frugiperda* showed significant effect (Table 1). The greatest damage was observed in control corn plants, for all assessed periods. However, the leaves treated with ASM + potassium silicate were significantly different from the positive control at four and eight DAI having minor damage. At 16 and 22 DAI, potassium silicate + ASM and the plants treated only with ASM were not statistically different from each other, showing minor damage.

The results from this study are in accordance with those obtained by Pinto et al. (2014) who reported a 62% reduction in the number of infested leaves and 20 and 35% in the level of leaf damage in the CCN 51 and Congo cocoa genotypes respectively, after application of 6 mL L^{-1} of potassium silicate.

Acibenzolar-S-methyl is an inducer of resistance, which interferes with physiological and biochemical processes of the plants, such as rapid absorption by the foliar tissues and activation and accumulation of PR proteins, regulating secondary metabolites as phytoalexins or structural defense compounds (Furtado et al., 2010).

The interaction of potassium silicate + ASM, constituted as defense barriers in the corn plant to *S. frugiperda*, which possibly contributed significantly to the increased resistance; and therefore, to the minor damage caused by the pest. This result corroborates Assis et al. (2015) who evaluated the induction of resistance in sunflower plants (*Helianthus annuus* L.) against *Chlosyne lacinia sauderssii* (Lepidoptera: Nymphalidae) using Silicon and ASM. The authors found that combined application of ASM + Silicon may inhibit *C. lacinia* growth conferring resistance due to accumulation of silicon and lignin; thus, promoting better protection and less damage caused by the insect pest.

Materials and Methods

This research was carried out in the Entomology Laboratory and in a greenhouse covered with plastic and the lateral walls covered with 60% black shade cloths in the Agronomy Biotechnology Nucleous of the Maranhão State University, Campus São Luis, Brazil.

Mass rearing of Spodoptera frugiperda

Insects were collected in corn fields located in São Luis and placed in plastic cups with acrylic lid arranged in polystyrene supports and transported to the Entomology Laboratory to perform mass rearing of *S. frugiperda*. The caterpillars coming from the field were individualized in glass tubes (8.5 cm in height and 2.5 cm in diameter) containing artificial diet (Greene, 1976), packed in climatic chambers at 25 ± 1ºC, with relative humidity of 70 ± 10% and photophase of 14 hs.

The caterpillars remained in glass tubes with artificial diet until pupal stage, separated by gender and placed in cages made with PVC tubes (10 cm in diameter and 21 cm in height), closed at both ends by Petri dishes. The PVC tubes were coated inside with Sulphite paper to facilitate egg removal. A 10% honey solution was supplied to adults in order to remain nourished.

The eggs were placed in Petri dishes (100 mm in diameter and 20 mm in height), sealed with PVC plastic film and transferred to the climatic chamber until hatching of larvae. After hatching, two caterpillars were transferred to tubes containing the diet; thus, restarting the cycle.

Experimental set up

The experiment was laid out in a randomized complete block design with six treatments and five replications. The experimental plot comprised of 4 vessels, each with two plants.

The treatments were applied at 35 days (V6 stage). They were: T_1: positive control (distilled water + infestation); $T_{2:}$ negative control (without products application and without infestation): $T_{3:}$ biofertilizer (25 mL/L), T_4: Acibenzolar-S-methyl - ASM (2 g/L), T_5: potassium silicate (10 mL/L), T_6: potassium silicate (10 mL/L) + ASM (2 g/L).

The biofertilizer was produced by anaerobic fermentation in PVC box of 500 L having the following constituents: cattle manure (50 kg) cow's milk (10 L), crushed sugarcane (2 kg) phosphate rock (1 kg), wood ash (1 kg) and boric acid (1 kg). The solution volume was supplemented with water to 500 L and presented in its mineral composition the nitrogen sources ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$), N_{total} (12.7), phosphorus (18 g kg^{-1}), potassium (1.5 g kg^{-1}) and pH 6.6.

Treatment application was performed foliar with 20 mL of the solution per plant using a spray bottle with adjustable nozzle. Each plant was protected at the base with a polyethylene bag to prevent solution deposition on the soil to avoid overdoses. Five days after treatment application, each plant was artificially infested with 10 caterpillars at 2nd instar stage, placed on the fourth leaf using a brush. Vessels were placed in cages (95 cm in width and 95 in depth and 1m in height) and covered with “voile” tissue in order to avoid the attack of other pests.

Assessment of S. frugiperda damage in corn plants

The damage assessment was performed at four, eight, 16 and 22 days after caterpillar infestation using a rating scale from zero to five, proposed by Wiseman et al. (1968), with adaptations, and the average score obtained from damages was attributed by four evaluators (Table 2).

Effect of resistance elicitor on enzymatic activity

To perform this analysis, one leaf of each corn plant was excised at 2, 4, 8, 16 and 22 days (DAI) after infestation with *S. frugirperda* caterpillar. The leaf samples were taken to the Entomology Laboratory, individually wrapped in aluminum foil, frozen in liquid nitrogen (N2) and then stored at -20 °C in a Ultra-freezer for experimental analysis. Enzyme assays were performed in triplicate for each leaf extract.

Protein extraction

In order to determine the protein extracts, an amount of 0.25 g of corn leaves was weighed, mechanically homogenized in 4 mL of sodium acetate buffer 100 mM (pH 5.0) using a mortar. The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was considered as enzymatic extract for determination of the peroxidase, polyphenol oxidase and phenylalanine ammonia lyase enzyme activities.

Peroxidase activity

The peroxidadse activity was determined by the direct spectrophotometric method described by Lusso and Pascholati (1999) with modifications by the guaiacol conversion measurement as the tetraguaiacol 470 nm. The reaction mixture contained 0.02 mL of protein extract with 0.5 mL of guaiacol and 0.5 mL of hydrogen peroxide in 1.5 mL of 0.1 M phosphate buffer (pH 6.0). The peroxidase activity was expressed as specific activity (absorbance unit mg^{1} of protein.min^{-1}).

Polyphenol oxidase activity

The polyphenol oxidase was determined according to the methodology described by Duangmal and Apenten (1999) with modifications. The test consisted in measuring the oxidation of catechol converted into quinine. This reaction mediated by the enzyme under study. The substrate was composed of catechol at a concentration of 20 mM dissolved in 100 mM sodium phosphate buffer (pH 6.8). The reaction consisted in the mixture of 0.5 mL of substrate, 0.5 mL of the enzyme extract and 1.5 mL of reaction buffer. The reaction temperature was 30 °C for 15 min and stopped after this time by the addition of 0.05 mL of 5N HCl and reading in a spectrophotometer at 420 nm. The results were expressed in absorbance unit mg^{-1} of protein.min^{1}.

Phenylalanine ammonia lyase activity

The phenylalanine ammonia lyase activity was determined by colorimetric quantification of the trans-cinnamic acid released from the substrate phenylalanine (Umesha, 2006). The reaction mixture was incubated at 40 °C for 2 h, containing 0.10 mL of the protein extract, 1.5 mL of 25 mM Tris HCl buffer (pH 8.8) and 0.5 mL of the substrate. The sample absorbance was determined at 290 nm versus extraction buffer, and subtracted from each sample the value of control (this control corresponded to a mixture of 0.10 mL of the protein extract to 1.5 mL of Tris HCl buffer). The reaction stopped by adding 0.05 mL of 5N HCl. The enzymatic activity was expressed in absorbance units mg^{-1} min^{-1} protein.

Soluble protein by the Bradford method

The patterns 50 uL of sample solutions or extracts, 50 uL of distilled water and 1 mL of Bradford, were pipetted into cuvette separately, stirred and put at rest for 10 minutes. The readings were made with a spectrophotometer at 595 nm wavelength. In the pH of the reaction, the interaction between the high molecular weight protein and the dye reagent Coomassie Brilliant Blue (G-250) present on the Bradford reagent caused the shift of the dye equilibrium to the anionic form, which absorbs at 595 nm.

Statistical analysis

Data of the damage caused by *S. frugiperda* were analyzed for normality by the Shapiro Wilk test and the Bartlett test to verify the variance homogeneity. After this procedure, the data were subjected to Analysis of Variance 9ANOVA) and the means compared by Tukey test at 5% significance. The enzymatic activity data were subjected to Lilliefors normality test and the means subjected to regression analysis. The statistical analyses were carried out by the Assistat software, version 7.7 (Silva and Azevedo, 2016).

Conclusion

The application of potassium silicate + ASM in corn plants is able to promote an increase in peroxidase activity with a rapid and short-lived response. The polyphenol oxidade activity is increased after application of ASM, whereas the presence of the pest (on positive control) potentiates the activity of phenylalanine ammonia-lyase. The applications of ASM and of potassium silicate + ASM contribute to reduce the level of foliar damage caused by *S. frugiperda*.

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