EFFECT OF THE A*nticarsia gemmatalis* INJURY ON THE LIPOXYGENASES ACTIVITY FROM SOYBEAN LEAVES

EFEITO DA INJÚRIA CAUSADA POR Anticarsia gemmatalis NA ATIVIDADE DE LIPOXIGENASES FOLIARES DE SOJA

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ABSTRACT: Lipoxygenases (LOX) from soybean leaves submitted to the attack of *Anticarsia gemmatalis* (Lepdoptera:Noctuidae) were characterized by biochemical and kinetic analyses. A commercial cultivar CAC-1 and three lines derived from this cultivar, which differ for the presence and absence of lipoxygenases and the protease inhibitor KTI, were used for those analyses. The leaf LOX pool showed two pronounced peaks of activity at pH 4.5 and 6.5 and optimum temperature at 25 °C. Plants from all genotypes, submitted to the attack of the insect, showed values of lipoxygenase specific activity higher than the controls in the different pHs and temperatures. Also, LOX pool obtained from leaves exposed to the insect at 6, 12 and 24 h, and at 6, 12 and 48 h, after removal of the insect, had "apparent" K_M values lower than those obtained in the respective controls. Our results suggest that the soybean plants respond to the attack of *A. gemmatalis* by increasing lipoxygenase activity, and that the genetic removal of LOX and KTI from soybean seeds does not interfere with the plant's ability to respond to wound via the LOX pathway.

UNITERMS: Plant-insect interaction, Lipoxygenases pathway, Soybean, A*nticarsia gemmatalis*.

INTRODUCTION

Brazil is responsible for 26% of the total world production of soybean grains and is the second largest world exporter of soybean in grain, bran and also oil. In Brazil soybean crops represent a considerable source of hard currency and there is a great extension of soybean planted area. Consequently, it is expected that area losses in grain crops caused by insect attack tend to increase. The larvae of *Anticarsia gemmatalis*, a chewing insect, is an important pest in Brazilian soybean agriculture. Even at low populational density, this insect can cause great damage to soybean crops, ranging from the defoliation up to total lost of the plant. The defoliation compromises the pod filling with consequent reduction of the grain yield (EMPRESA BRASILEIRA DE PESQUISA AGROPECUÁRIA, EMBRAPA, 2000).

Lipoxygenases (LOX) are a class of enzymes widely distributed in nature that have been detected in a large number of plant species (SIEDOW, 1991). In higher plants, these enzymes catalyze the hydroperoxidation of unsaturated fatty acids containing the group cis-cis 1,4 pentadiene, such as linoleic and linolenic acids (SANZ et al., 1992). Three LOX isozymes have been extensively characterized by Axelrod et al. (1981) in soybean seeds, where they correspond to approximately 1% of the total proteins present in dry soybean seeds.

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Several studies have indicated that LOX can be considered an important component of the plant response to pathogen and insect attack. LOX activity was found to increase in response to mechanical wounding, for a number of plant/pathogen systems and induced by treatment of cell cultures or plants with elicitors (KATO et al., 1992; KONDO et al., 1993; OHTA et al., 1991). In soybean leaves, it has been observed that LOX expression increases considerably as a result of pod removal (KATO et al., 1993), reproductive sink removal (BUNKER et al., 1995) and mechanical wounding (SARAVITZ; SIEDOW, 1996; VIEIRA et al., 2001). Also, increased LOX expression has been detected in plants during or following a variety of stress, including insect feeding (HILDEBRAND et al., 1989) and infection by both bacterial and fungal pathogens (MELAN et al., 1993).

In tomato leaves, it is proposed that linolenic acid hydroperoxides derived from LOX activity are precursors of jasmonic acid which would in turn activate transcription of genes encoding for protease inhibitors which would play a role against insect attack (FARMER; RYAN, 1992). In addition, Hilder et al. (1987) suggest the involvement of protease inhibitors on a protection of plants against Lepidoptera larvae. These inhibitors that corresponds to about 6% of the total protein present in soybean seeds are represented by the Kunitz trypsin inhibitor (KTI) and by the trypsin and chymotrypsin inhibitor Boward-Birk (BBI) (BRANDON et al., 1993).

Both LOX and KTI are considered undesirable factors in soybean seeds (ANDERSON-HAFERMANN et al., 1992; RACKIS et al., 1979). The hydroperoxidation of unsaturated fatty acids catalyzed by LOX lead to compounds responsible for the characteristic undesirable grassy-beany flavors in soybean-derived products (HILDEBRAND, 1989). KTI is responsible for approximately 80% of the tryptic inhibition activity present in the seed. The presence of active KTI in soybean-derived products has been implicated in the development of nodular pancreatic hyperplasia and cellular adenomes in rats (BRANDON et al., 1993).

The genetic elimination of seed LOX and KTI by introduction of both null LOX and KTI constitutes an approach to reduce undesirable flavors associated with soybean products and thereby to increase their consumer acceptability. However, such elimination should not change the composition, and, consequently, the physiological functions of LOX and its products present on soybean leaves, since a role on the plant defense against insects and pathogens has been assigned to them.

In this study the biochemical characterization of the LOX pool present in leaves of soybean plants submitted to the attack of *A. gemmatalis* was conducted in order to gain insigths regarding the involvement of the LOX pathway in the plant physiology, mainly in the insect defense mechanism of soybean plants. A wild type genotype and mutant lines lacking seed LOX and KTI were used to determine if these genetic materials respond to this stress by activating the LOX pathway. In a short, the main goal of this work was to evaluate if the genetic removal of these proteins, aiming a better acceptance of the soybean product, compromises the soybean defense response.

MATERIALS AND METHODS

Plant material

Soybean (*Glycine max* L.) seeds from commercial cultivar CAC-1 were obtained from the Germplasm Bank of the Department of Plant Sciences of the Federal University of Viçosa (UFV), MG Brazil. Lines derived from CAC-1 were developed by conventional breeding after five cycles of backcrossing in an Breeding Program conducted at the UFV Biotechnology Institute (BIOAGRO). The genotype of soybean lacking KTI in their seeds (BRM 92 5262) were kindly supplied by EMBRAPA/soybean (Londrina, PR, Brazil). These genotypes were denominated as follows: CAC-1 (LOX+/KTI+) commercial cultivar, having LOX and KTI on their seeds; CAC-1 (LOX+/KTI-) genotype with genetic removal of KTI from their seeds; CAC-1 TN (LOX-/KTI+) genotype with absence of seed LOX, but presence of KTI in their seeds, and CAC-1 TN (LOX-/KTI-) genotype with absence of LOX and KTI in their seeds. The plants were germinated at 27 °C for 48 h, transfered to 4 Kg pots containing three plants and cultivated in the greenhouse. It was utilized 40 pots for each genotype to complete a total of 120 plants.

Insect attack

To examine the effects of the presence of *A. gemmatalis*, the soybean plants at the V3 development stage were exposed to the larval insect. It was utilized one insect for each trifoliolate. Three pots were randomly selected and used to obtain three enzymatic extract from which LOX activity were determined. After 6, 12 and 24 h of the attack the three leaflets of the first trifoliolate leaf were collected, immediately frozen in liquid nitrogen and kept at –80 °C for the biochemical analyses. Thereafter, the insect was removed from the plants and the same procedure was conducted at 6, 12, 24 and 48 h after removal of the insect. Leaves from control plants, not exposed to the insect, were also collected at the same time points.

Preparation of leaves extract

The frozen leaflets were powdered in a mortar and pestle, and a crude extract was obtained as described by Otha et al. (1986), with some modifications. Essentially, the leaflets were homogenized in 0.05 M sodium phosphate, pH 6.5 with no Triton X-100, and the homogenate centrifuged at 17,200 x g for 60 min at 4 °C. The resulting supernatant was used for determining the protein content and LOX activity. Protein concentration was determined by the bicinchoninic acid method (SMITH et al., 1985), using bovine serum albumin as standard.

Lipoxygenase assay

LOX activity was measured spectrophotometrically using linoleic acid as substrate (AXELROD et al., 1981). For this purpose, 1.0 µL of leaf extract was added to a mixture containing 4.0 µL of a 10 mM linoleic acid stock solution and 1.0 mL of 0.05 M sodium phosphate buffer, pH 6.5, at 25 °C. These reaction conditions were used in all lipoxygenase activity tests, unless otherwise indicated. The absorbance at 234 nm was determined at 30-s intervals during 2.5 min period. The same procedure was used with the blank reaction mixtures which contained no leaf extract. All incubations were done in triplicate. The initial velocities of product development in the reaction mixtures were calculated using the A_{234} data and the extinction coefficient of 25,000 M^{-1} cm^{-1} for linoleic acid hydroperoxides. The specific activities were expressed in molar concentration of linoleic acid hydroperoxydes formed per second per miligram of protein.

Effect of temperature and pH

The effect of temperature upon the initial velocities of formation of hydroperoxides were determined at 20, 25, 30, 35, 40, 45 and 50 °C. The temperature of the work solutions were adjusted in a water bath and the spectrophotometer cell was calibrated to the temperature values described above. For optimum pH determination different buffer systems at a final concentration of 50 mM were used covering the range of pH 2 to 10.

Determination of kinetic parameters

LOX activity was determined using linoleic acid at the following concentrations: 10, 20, 40, 80, 160, 320, and 640 µM. The kinetic parameters, apparent K_M and V_{max}, in the steady state, were obtained by non-linear regression analyses using the program Enzifitter (LEATHERBARROW, 1987). All determinations were done in triplicate.

RESULTS

Figure 1 shows the pH profiles of enzyme activities, analyzed in crude extracts from two cultivars and their respective controls at 24 h after removal of *A. gemmatalis*. Two prominent activity peaks are seen around pHs 4.5 and 6.5, indicating the existence of at least two LOX isozymes in the pool of leaf extract. The activity profiles for all time points analyzed for the other cultivars, followed the same trend observed in this time point (data not shown).

In the Figure 2, temperature profiles are represented for two cultivars and their respective controls at 24 h after removal of the insect. The highest leaf LOX activity was observed at 25 °C, for all genotypes analyzed and their respective controls in this time point (data not shown).

As a whole, our data suggest that LOX specific activities in the different pHs and temperatures tended to be higher in the plants submitted to the attack of the insect compared to their respective controls. This indicates that the four genotypes respond to this type of the stress by increasing LOX activity in their leaves.

The leaf LOX pool activities from the soybean genotypes followed the Michaelis-Menten kinetics, within the substrate concentration range tested (Figures 3A and 3B). It can be observed in Table 1 that there were no significant differences between the K_{Mapp} values for leaf LOX extracted from all cultivars, indicating that the genetic removal of LOX and KTI from the soybean seeds, did not affect the expression of leaf lipoxygenases. However, these values determined after exposure of the plants to the insect were reduced for all genotypes, which suggest an alteration of the leaf LOX pool, indicating that the plants submitted to the attack of *A. gemmatalis* respond to this stress by activating the LOX pathway. Table 1 also shows that, after removal of the insect, K_{Mapp} values for LOX are continuing decreasing for all genotypes, which suggest that the soybean plant responded to the attack of the insect, up to 48 h after its removal.

The decrease on K_{Mapp} values for the control plants (not exposed to the insect), by the length of time (Tables 1 and 2), suggests that the expression of leaf LOX changes during leaf development, i.e., it depends on the physiological state of the cell. Even though these variations in LOX expression could mask injury-related changes, a comparision of K_{Mapp} values between each genotype and its respective control in all time points clearly shows that those values are smaller for the plants submitted to the attack of the insect.

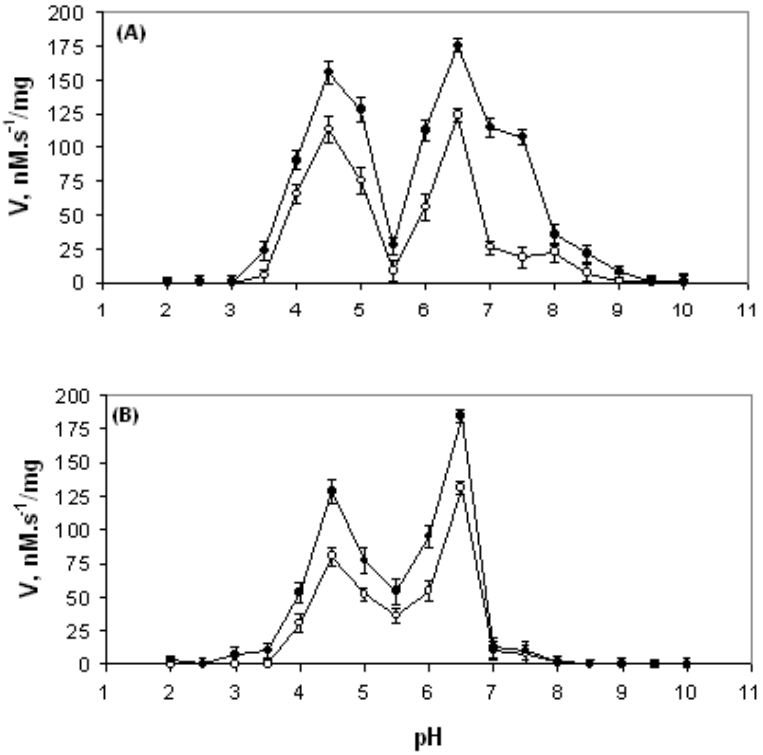

Figure 1. Effect of pH on the specific activity of soybean leaf lipoxygenases collected at 24 h after removal of the insect. **(A)** CAC-1 (LOX+/KTI+) and **(B)** CAC-1 TN (LOX-/KTI+) genotypes. (o—o) Local response (first trifoliolate leaf) of control plants. (•—•) Local response (first trifoliolate leaf) of plants submitted to the larval insect attack.

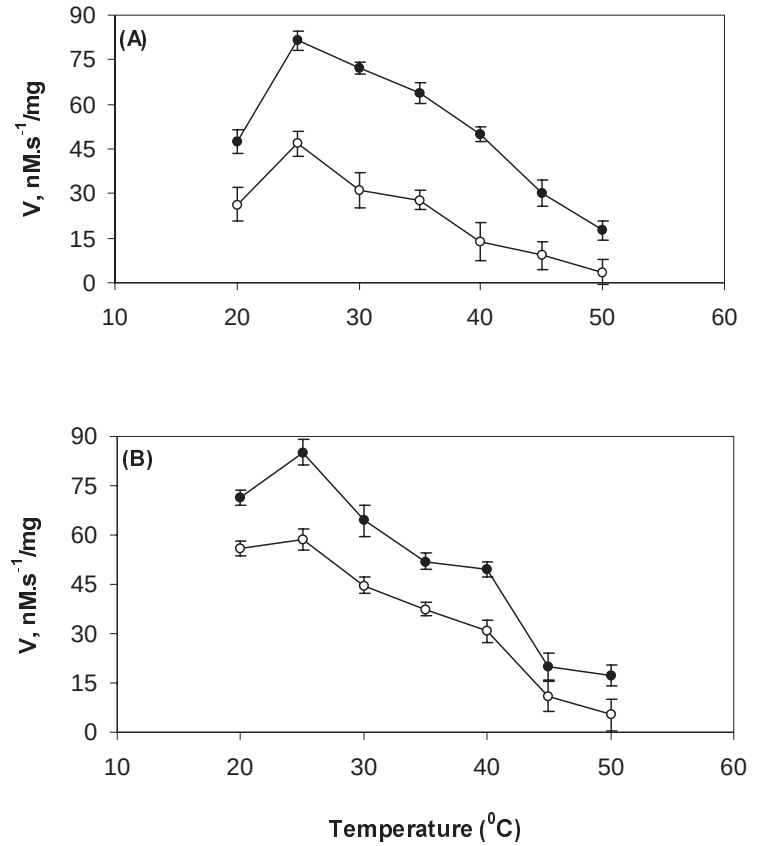

Figure 2. Effect of temperature on the specific activity of soybean leaf lipoxygenases collected at 24 h after removal of the insect . **(A)** CAC-1 (LOX+/KTI+) and **(B)** CAC-1 TN (LOX-/KTI+) genotypes. (o—o) Local response (first trifoliolate leaf) of control plants. (•—•) Local response (first trifoliolate leaf) of plants submitted to the larval insect attack.

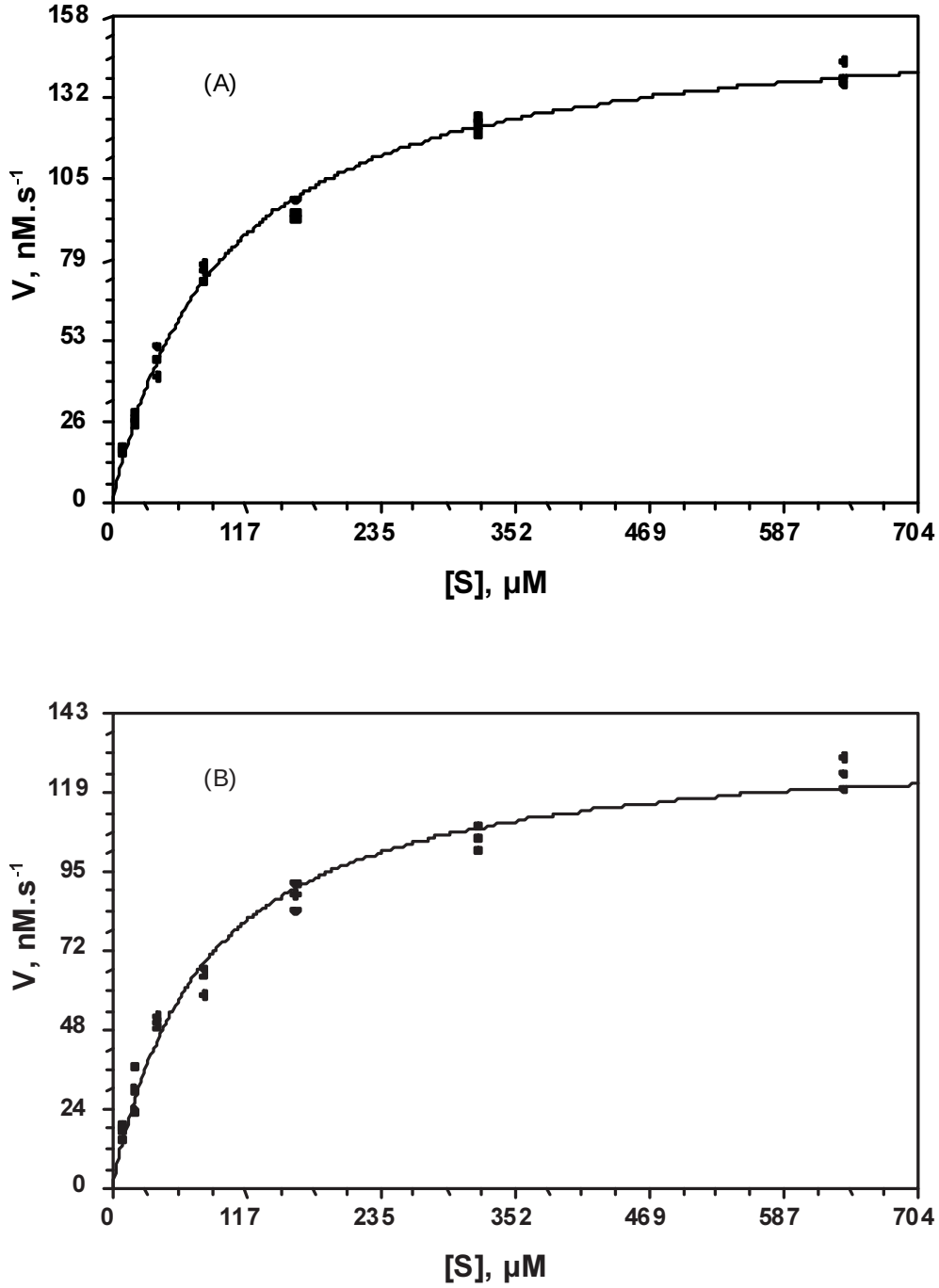

Figure 3. Michaelis-Menten plot of soybean leaf lipoxygenases activity. Leaves were collected at 24 h after removal of the insect. (A) CAC-1 (LOX+/KTI+) and (B) CAC-1 TN (LOX-/KTI+) genotypes. The continuous line were traced over the experimental data points, using the Michaelis-Menten equation in order to obtain K_{Mapp} and V_{maxapp} values

Table 1. K_{Mapp} and V_{maxapp} values of soybean leaf lipoxygenases upon linoleic acid.

Time (h)	CAC-1(LOX+/KTI+)				CAC-1 (LOX+/KTI-)			
	Control		*A. gemmatalis*		Control		*A. gemmatalis*	
	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)
Presence of the insect								
06 h	239.29	58.26	186.92	194.05	253.24	92.25	164.66	68.42
12 h	230.03	61.95	168.95	89.31	175.74	71.10	140.38	100.51
24 h	169.48	36.92	114.01	49.07	150.91	108.60	106.64	107.08
Removal of the insect								
06 h	167.20	51.23	113.60	153.33	148.96	67.98	101.85	153.10
12 h	160.86	42.63	101.95	97.43	129.65	157.59	101.12	96.98
24 h	131.60	92.84	96.28	159.17	125.36	77.73	70.45	82.85
48 h	122.54	178.01	94.18	116.27	115.59	64.77	69.49	141.67

Table 2. K_{Mapp} and $V_{max\ app}$ values of soybean leaf lipoxygenases upon linoleic acid

Time (h)	CAC-1 TN (LOX-/KTI+)				CAC-1 TN (LOX-/KTI-)			
	Control		*A. gemmatalis*		Control		*A. gemmatalis*	
	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)	$K_{M\ app}$ (µM)	$V_{max\ app}$ (nM/s)
Presence of the insect								
06 h	241.80	87.14	173.13	76.89	227.51	62.08	225.63	63.01
12 h	152.27	138.84	139.53	66.41	203.64	89.33	196.29	83.34
24 h	167.29	144.57	114.48	66.25	192.10	62.30	187.29	129.11
Removal of the insect								
06 h	145.31	107.02	93.78	206.37	181.90	98.52	137.39	268.48
12 h	118.88	123.78	95.44	118.44	129.46	164.84	128.79	157.45
24 h	105.24	111.08	80.59	136.04	136.39	106.81	114.69	170.64
48 h	101.33	85.29	67.07	124.21	125.36	144.72	113.61	123.77

DISCUSSION

To biochemically characterize the LOX pool present in soybean leaves submitted to the attack of *A. gemmatalis*, it was first determined pH and temperature effects on the activity of these enzymes. The pH and temperature profiles found at this work were similar to those obtained by other authors for soybean plants (DUBBS; GRIMES, 2000; LANNA et al., 1996; VIEIRA et al., 2001).

The existence of more than one peak for pH activity can be related to the fact that LOX enzymes in soybean plants are organized as a great multigene family (BUNKER et al., 1995). It results in a certain tissue in the presence of isozymes that differ from each other regarding their activity as a function of pH, pIs, the K_M values, and the specificity with distinct substrates (GARDNER, 1991). In fact, it has been suggested that 10 to 12 genes encoding LOX are present in the soybean genome (KOETJE; GRIMES, 1992). In addition, it can be related to the fact that the LOX expression and activity levels present in a certain tissue can vary substantially as a physiological plant response to different type of stress (ROSAHL, 1996).

In soybean, increases in LOX transcripts, protein, and activity occur in leaves after wounding (BELL; MULLET, 1993; HILDEBRAND et al., 1989). Kato et al. (1993) verified an increase of gene expression and accumulation of soybean lipoxygenase L-4 in leaves induced by pod removal. In response to a variety of sink limitations, the involvement of several members from the LOX multigene family (vlx) was reported in soybean leaves (BUNKER et al., 1995; SARAVITZ; SIEDOW, 1996).

The kinetic behaviour of the leaf LOX pool were assessed using linoleic acid (Tables 1 and 2). These data can only give average values for the isozymes within the LOX pool. Thus, the "apparent" K_M values reported in this study correspond to the isozyme(s) that is(are) predominantly being expressed as a response of the soybean plant to the attack of the *A. gemmatalis*.

The K_M values reported for leaf LOX pool of plants submitted to the attack of the insect were lower than their respective control. K_M is a kinetic constant that establishes an approximate value for the physiological level of the substrate. It is unlikely that this level would be significantly greater or lower than K_M so that the decrease on K_M values suggests a better adaptation of the substrate to the enzyme active site (SEGEL, 1993). This probably is a strategy utilized by the foliar tissue to improve the catalytic efficiency of lipoxygenases from soybean leaves submitted to the attack of the pest. By expressing different forms of LOX with a better catalytic efficiency, the soybean plant is involving the LOX pathway to respond to this type of injury.

Our results also show a similar kinetic behaviour for the four genotypes, which indicate that the genetic removal of seed LOX and KTI does not interfere with the plant response to the wounding. Consequently, the expression of seed LOX genes for the genotypes used in this study does not interfere with the expression of genes coding for the LOX pool present in the leaves.

CONCLUSIONS

1. The optimum pH and temperature for leaves LOX isozymes were 6.5 and 25 ^oC, respectively.
2. LOX specific activities in the different pHs and temperatures tended to be higher in the plants submitted to the attack of the insect compared to their respective controls.
3. K_M values were lower for the plants submitted to the attack of *A. gemmatalis*.
4. The soybean plants responded to the attack of *A. gemmatalis* via the LOX pathway.
5. The genetic removal of LOX and KTI from soybean seeds did not affect the plant defense against this insect, i.e., the expression of leaf lipoxygenases was not affected.

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RESUMO: Lipoxigenases (LOX) de folhas de soja submetidas ao ataque de *Anticarsia gemmatalis* (Lepdoptera:Noctuidae), foram caracterizadas através de análises bioquímicas e cinéticas. Foram utilizadas a variedade comercial de soja CAC-1 e três linhagens derivadas dessa variedade, diferindo pela presença e ausência de lipoxigenases e do inibidor de protease KTI em suas sementes. O *pool* de lipoxigenases foliares mostrou dois picos pronunciados de atividade em pHs 4,5 e 6,5 e ótimo de temperatura a 25 ^oC . As plantas de todos os cultivares analizados, submetidas ao ataque do inseto, apresentaram valores de atividade específica de lipoxigenases maiores que os respectivos controles, em diferentes valores de pH e temperatura. Ainda, o *pool* de lipoxigenases obtido das folhas expostas ao inseto por 6,12 e 24 h, e em 6, 12 e 48 h após a remoção do inseto, apresentaram valores de K_M aparente inferiores àqueles

obtidos nos respectivos controles. Esses resultados sugerem que as plantas de soja respondem ao ataque de *A. gemmatalis* através do aumento da atividade de lipoxigenases, e que a remoção genética de LOX e KTI das sementes da soja não interfere na habilidade da planta em responder à essa injúria por meio da via bioquímica das lipoxigenases.

UNITERMOS: Interação planta-inseto, Via das lipoxigenases, Soja, *Anticarsia gemmatalis*.

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