LACK OF SEED LIPOXYGENASES DOES NOT AFFECT SOYBEAN DEFENSE BY REPRODUCTIVE TISSUE REMOVAL

AUSÊNCIA DE LIPOXIGENASES NAS SEMENTES NÃO AFETA A DEFESA DA PLANTA DE SOJA PELA REMOÇÃO DE TECIDO REPRODUTIVO

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ABSTRACT: The genetic elimination of lipoxygenase isozymes from soybean seeds is a way to overcome the problems associated with the undesirable beany flavor of soybean products. Although the role of the lipoxygenases in higher plants has not yet been established conclusively, several studies have indicated the physiological relevance of the lipoxygenase pathway induction in plants under biotic and abiotic stress conditions. To elucidate the effect of seed lipoxygenase elimination on soybean defense against injury, a biochemical assessment of the lipoxygenase pathway in soybean leaves subjected to flower removal was carried out in a normal genotype (IAC-100) and its backcross-derived line lacking seed lipoxygenase (IAC-100 TN). The soybean plants responded to the removal of reproductive tissue via the LOX pathway, by increasing LOX activity and protease inhibition in their leaves. Since jasmonic acid seems to activate transcription of genes encoding for protease inhibitors, the soybean plant responded to sink deprivation via LOX pathway, preferentially to jasmonate and protease inhibitors production. There was no association between the lack of lipoxygenases from the seeds and the leaf defense against injury, indicating that the loss of the genes for lipoxygenases from soybean seeds does not interfere with the plant ability to respond to sink deprivation via this pathway.

UNITERMS: Lipoxygenases, Sink deprivation, Soybean.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] has great social and economical value around the world, since it produces a leading vegetable oil and is a low-cost and high protein food source for human consumption and animal feed. However, the greater use of soybean products in Western countries is impaired by the undesirable flavor of the commercially cultivated soybean seeds, which is attributed to the high proportion of unsaturated fatty acids, and the abundant presence of the enzyme lipoxygenases (LOX) in soybeans (HILDEBRAND, 1989). The primary products of LOX-catalyzed reactions, hydroperoxy derivatives of linoleic and linolenic acids, are further metabolized by two major pathways starting from hydroperoxide lyase and hydroperoxide dehydrase (GARDNER, 1991). The activity of the latter enzyme results in the formation of signal molecules, such as jasmonic acid, which are involved in senescence and in the response of plants to wounding and pathogens (SEMBDNER; PARTHIER, 1993).

The LOX-pathway is also responsible for the production of volatile compounds such as hexanal and other aldehydes by the action of hydroperoxide lyase. Many of these compounds are responsible for the undesirable grassy-beany flavor characteristic of soybean

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products (RACKIS et al., 1979). Besides, several unsaturated aldehydes are produced when soybean plants are wounded mechanically or by pathogens. For instance, trans-2-hexenal, a component of the characteristic flavor and odor of fruits and leaves, has been shown to possess antiprotozoal, antifungal (VAUGHN; GARDNER, 1993), insecticidal and acaricidial activity (CROFT; JÜTTNER; SLUSARENKO 1993).

LOX present in leaves of several plant species have been implicated in defense against pathogens and insects as they are induced by mechanical wounding and by different pathogens (BUNKER et al., 1995; KATO et al., 1993; VIEIRA et al., 2001). Linolenic acid hydroperoxides derived from LOX activity are likely precursors of jasmonic acid in tomato leaves, which seems to activate transcription of genes encoding for protease inhibitors that protect the plant against insect attack (FARMER; RYAN, 1992). In addition, LOX has been proposed to function as a vegetative storage protein (VSP) in both seeds (SIEDOW, 1991) and leaves (TRANBARGER et al., 1991).

The genetic elimination of seed LOX by the introduction of null LOX alleles into elite soybean lines constitutes an approach to reduce the undesirable flavors associated with soybean products and thereby increasing their consumer acceptability. However, such elimination should not change the composition, and, consequently, the physiological functions of LOX and its products present in soybean leaves, what may compromise the plant defense against insects and pathogens. Thus, the effect of seed LOX elimination on soybean defense was assessed here through the involvement of the LOX pathway in plants submitted to the removal of their reproductive tissue and subsequent quantification of LOX activity, levels of the trypsin inhibition and contents of hexanal and total aldehydes in leaves of two genotypes. This biochemical characterization was carried out using a commercial cultivar and a near-isogenic line derived from this cultivar but lacking seed LOX, to determine if these genetic materials respond to sink deprivation by activating the LOX pathway and to evaluate if the genetic removal of the seed LOX compromises the soybean defense response.

MATERIAL AND METHODS

Plant material and preparation of leaf extracts

Soybean [*Glycine max* (L.) Merrill] leaves at the reproductive stage R1 (beginning of anthesis) were obtained from the commercial cultivar IAC-100 and from an advanced line derived from IAC-100 but lacking seed LOX (IAC-100 TN). The plantlets were germinated at 27 °C for 48 h, transfered to 4 Kg pots and greenhouse cultivated. Flowers and flower buds were daily removed for a period of 2 weeks beginning at anthesis (SARAVITZ; SIEDOW, 1995). Floral tissues were not removed from control plants and seed pods were allowed to develop normally. Leaves were harvested at 4, 8, 12 and 16 days after flower removal. The three leaflets of the last fully expanded trifoliolate were collected, weighted and immediately frozen in liquid nitrogen and stored at –80 °C. The frozen leaflets were powdered in a mortar and pestle, and a crude extract was obtained as described by Ohta; Mikami; Morita (1986). The supernatant solutions obtained from the centrifuged homogenate were used to determine the protein content by the bicinchoninic acid method (SMITH et al., 1985), using bovine serum albumin as standard, and for the determination of LOX activity.

Lipoxygenase assay

LOX enzyme activity was measured spectrophotometrically by monitoring the increase in absorbance at 234 nm using linoleic acid as substrate (AXELROD; CHEESBROUGH; LAASKO, 1981). 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.

Determination of trypsin inhibitor activity

The ability of soybean leaf extracts to inhibit trypsin activity was evaluated using N-benzoyl-D,L-arginine-p-nitroanilide (D-L-BApNA) as substrate, according to KAKADE. (1974). Bovine trypsin (type III) and D-L-BApNA were obtained from Sigma Chemical Co. (Saint Louis, MO, USA).

Determination of hexanal and total aldehydes

Quantification of hexanal was performed on a Shimadzu gas chromatograph following Utumi et al. (1998). Total aldehyde content in leaf extracts was determined according to Wilson and McDonald (1986) using 3-methyl-2-benzothiozolinone hydrazone (MBTH).

Statistical analysis

The effect of removal of flower tissue over time on the variables induced LOX activity, increased trypsin inhibition and hexanal content (Y; differences between determinations in wounded and intact plants), measured on both genotypes (A= IAC 100; B= IAC 100TN) was modelled by means of regression analysis, with the use

of two strategies: (i) adjustment of linear ($Y_i = \hat{a}_0 + \hat{a}_1 X + \mathring{a}_i$) and quadratic ($Y_i = \hat{a}_0 + \hat{a}_1 X + \hat{a}_2 X^2 + \mathring{a}_i$) models on days (X) and (ii) orthogonal decomposition of the ANOVA sum of squares due to days into linear, quadratic and cubic effects. A model was ajusted for each genotype and tests for equallity of model intercepts ($\hat{a}_{0A} = \hat{a}_{0B}$) and slopes ($\hat{a}_{1A} = \hat{a}_{1B}$) were performed. The statistical analysis were conducted with the software SAS (STATISTICAL ANALYSIS SYSTEM Institute, 2001).

RESULTS AND DISCUSSION

LOX specific activity for both genotypes were higher in plants submitted to the removal of floral tissue compared to their respective controls (Figures 1A and 1B), suggesting that soybean plants responded to this type of stress by increasing LOX activity. The test of equality of intercepts and slopes from the regressions of Figure 2 were tested at $p < 5\%$, but only the slopes were different. The models were therefore adjusted for common intercept, yielding the following equations: $\hat{y} = 7 + 1.25$ days for IAC-100 and = 7 + 2.5 days for IAC-100 TN, with $r^2 = 0.9034$.

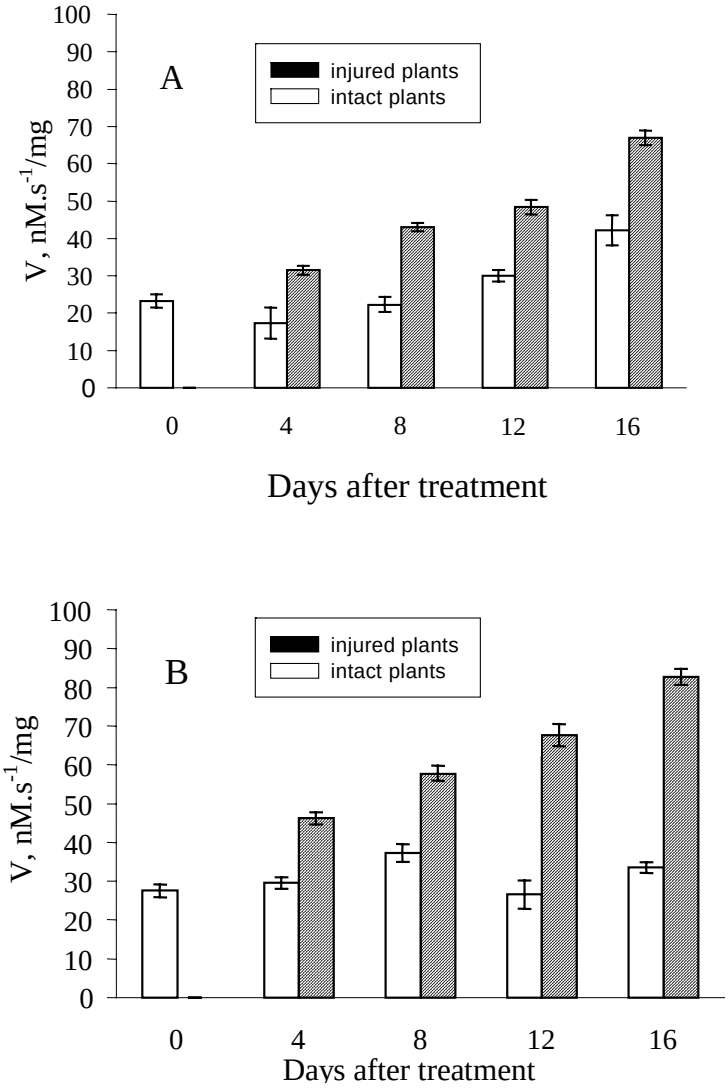

Figure 1. Lipoxygenase specific activity from soybean leaves of IAC-100 (A) and IAC-100 TN (B) genotypes, with intact and removed reproductive sinks. The vertical bars represent the standard error of the means from three separate determinations.

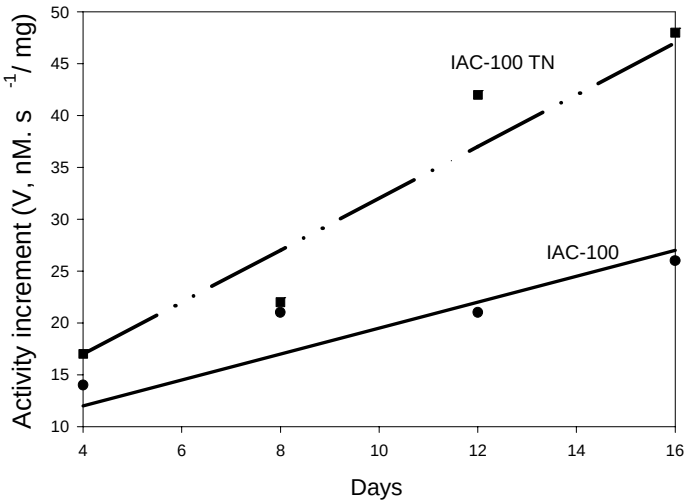

Figure 2. Increase in induction of lipoxygenase activity after injury by sink removal in two soybean genotypes lacking (IAC 100 TN) or not (IAC 100) seed lipoxygenases.

Based on these results the genetic removal of seed LOX seems to favour higher leaf induction of LOX activity through time, suggesting that this genetic modification does not compromise leaf defense to injury. Also, this gradual increase of LOX activity observed following the days after removal of flower tissue (Figures 1A and 1B), suggests that LOX may be able to accumulate in the leaves. In addition of being a response to sink deprivation, the increase of leaf LOX activity may be related to a temporary storage of nitrogen in response to flower removal. Flowering plants have a heavy demand for nitrogen during flower development and seed formation, when the well characterized seed storage proteins are synthesized (BUNKER et al., 1995; TRANBARGER et al., 1991). Our results showed smaller values of specific activity for the control plants where pods and seeds were allowed to develop. Since it is presumed that the storage protein are degraded and the amino acids remobilized to provide nitrogen to developing organs, the low LOX activities levels found for control plants suggest that with reproductive sinks, the lipoxygenases were partially mobilized to supply the amino acid requirement necessary for development and growth of pod and seeds.

There are other reports of increase in LOX transcripts, protein, and activity taking place in soybean leaves after sink deprivation. Kato et al. (1993) verified an increase of gene expression and accumulation of soybean lipoxygenase L-4 in leaves induced by pod removal. The involvement of several members from the LOX multigene family (vlx) was reported as a response to a variety of sink limitations (BUNKER et al., 1995).

Increased trypsin inhibition was observed for injured plants compared to their respective controls, following the removal of flower tissue (Figures 3A and 3B). The highest value was found at 4 and 16 days after the treatment, for IAC-100 and IAC-100 TN respectively, but there was no significant difference in induction between them ($p>0.05$). Participation of LOX in the biosynthetic pathway leading to jasmonates, which activate protease inhibitor genes, has been demonstrated in leaves of several plant species, in response to wounding and elicitors (FARMER; RYAN, 1992). Thus, our results support the involvement of the proposed pathway, since it was observed an increase in the trypsin activity inhibition levels for plants submitted to the wounding.

The levels of total aldehydes were similar for the injured (1.32 ± 0.09 ΔOD) and intact plants (1.33 ± 0.08 ΔOD) for both genotypes. The hexanal levels were also similar for the injured (15.3 ± 3.14 $\mu V.min^{-1}$) and intact plants (14.0 ± 2.8 $\mu V.min^{-1}$) for both genotypes. Therefore, our results involving the determination of the products of the LOX pathway in soybean leaves submitted to sink removal suggest that this pathway is activated during the injury response leading preferentially to jasmonate and protease inhibitors production.

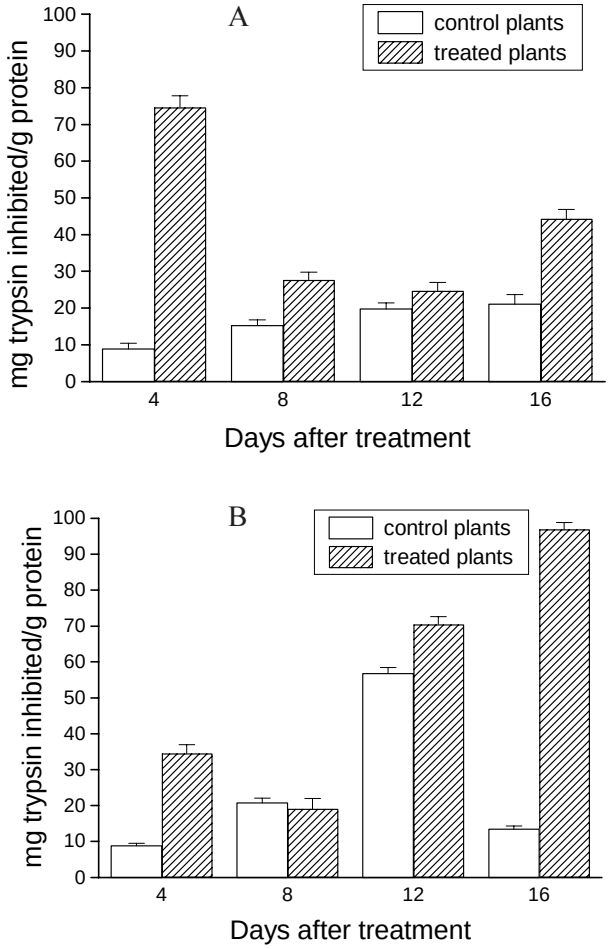

Figure 3. Inhibition of trypsin by soybean leaf extracts of IAC-100 (A) e IAC-100 TN (B) genotypes, with (control plants) and without (treated plants) reproductive sinks. The vertical bars represent the standard error of the means from three separate determinations.

The genetic removal of seed LOX does not seem to interfere with the plant response to sink deprivation based on the results presented here, since the genotypes used in this study presented high levels of LOX and protease inhibition activity in the LOX pool present in the leaves. NARVEL; FEHR; WELKE (1998) reported that there were no significant differences in agronomic and seed traits between the soybean lines lacking seed lipoxygenases (triple-null) and normal lines. Martins et al. (2002) reported that soybean lines lacking seed LOX did maintain the resistance to stem canker, frogeye leaf spot and powdery mildew diseases. Taken as a whole, these results suggest that the varieties that have their seed LOX genetically removed, show a high potential for use as commercial cultivars without compromising their defensive potential against mechanical injury.

CONCLUSIONS

1. The soybean plants responded to the removal of reproductive tissue via the LOX pathway, by increasing LOX activity and protease inhibition in their leaves.
2. The genetic removal of seed LOX from soybean did not affect the plant response to sink deprivation.
3. The soybean plant responded to sink deprivation via LOX pathway, preferentially to jasmonate and protease inhibitors production.

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RESUMO: A eliminação genética de isoenzimas lipoxigenases das sementes de soja é uma maneira de superar os problemas associados com o indesejável *beany flavor* dos produtos de soja. Apesar do papel das lipoxigenases nas plantas superiores não ter sido conclusivamente estabelecido, vários estudos têm indicado a relevância fisiológica da indução da rota das lipoxigenases nas plantas, sob condições de stress abiótico e biótico. Para elucidar o efeito da eliminação de genes de lipoxigenases da semente da soja contra a defesa dessa planta, uma investigação bioquímica da rota das lipoxigenases nas folhas de soja sujeitas à remoção do tecido reprodutivo foi conduzida em um genótipo normal (IAC-100) e em uma linhagem derivada dessa, sem lipoxigenases em suas sementes (IAC-100 TN). As plantas de soja responderam à remoção do tecido reprodutivo através da rota das lipoxigenases, o que foi verificado pelo aumento da atividade das lipoxigenases e também pela inibição de proteases, em suas folhas. Uma vez que o ácido jasmônico é capaz de ativar a transcrição de genes que codificam inibidores de protease, a planta de soja respondeu à essa injúria via rota das lipoxigenases, preferencialmente através da produção de jasmonatos e inibidores de proteases. Não houve associação entre a ausência de lipoxigenases nas sementes e a defesa da planta contra esse tipo de injúria, avaliada através das folhas dos dois genótipos, indicando que a perda de genes para lipoxigenases das sementes de soja não interfere com a habilidade da planta em responder à remoção do dreno reprodutivo através da via das lipoxigenases.

UNITERMOS: Lipoxigenases, Remoção do dreno repodutivo, Soja.

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