Lipoxygenase activities during development of root and nodule of soybean

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Abstract – The objective of this work was to evaluate root and nodule soybean lipoxygenases in Doko cultivar and in a near isogenic line lacking seed lipoxygenases, inoculated and uninoculated with *Bradyrhizobium elkanii*. The lipoxygenase activities from roots collected at 3, 5, 9, 13, 18 and 28 days post-inoculation and from nodules collected at 13, 18 and 28 days post-inoculation were measured. The pH-activity profiles from root and nodules suggested that the lipoxygenases pool expressed in these organs from Doko cultivar and triple-null near isogenic lines are similar. The root lipoxygenase activity of Doko and triple-null lines, inoculated and uninoculated, reduced over time. The highest lipoxygenase activity observed at the beginning of root formation suggests the involvement of this enzyme in growth and development of this organ. However, for nodules an expressive increase of lipoxygenase activity was noticed 28 days post-inoculation. Root and nodule showed, at least, two mobility groups for lipoxygenases in immunoblottings, with approximately 94 and 97 kDa.

Index terms: *Glycine max*, triple-null lines, nodulation.

Atividade de lipoxigenases durante o desenvolvimento da raiz e do nódulo de plantas de soja

Resumo – O objetivo deste trabalho foi avaliar lipoxigenases de raízes e nódulos de plantas de soja da variedade Doko e da linhagem triplo nula derivada, desprovida das lipoxigenases da semente, com e sem inoculação de *Bradyrhizobium elkanii*. A atividade de lipoxigenase foi avaliada em raízes coletadas aos 3, 5, 9, 13, 18 e 28 dias após a inoculação e em nódulos coletados aos 13, 18 e 28 dias após a inoculação. Os perfis de pH-atividade de raiz e nódulo sugerem que o "pool" de lipoxigenases expresso nesses órgãos não difere nos dois genótipos. A atividade de lipoxigenases nas raízes de Doko e Doko triplo nula, com e sem inoculação, declinou com o passar do tempo. A maior atividade de lipoxigenases no início de formação da raiz sugere o envolvimento desta enzima no crescimento e desenvolvimento deste órgão. Nos nódulos houve um aumento acentuado na atividade de lipoxigenase aos 28 dias após a inoculação. Dois grupos de mobilidade com aproximadamente 94 e 97 kDa foram encontrados nos "immunoblottings" para lipoxigenases de raiz e de nódulo.

Termos para indexação: *Glycine max*, linhagem triplo nula, nodulação.

Introduction

Soybean seeds usually contain three lipoxygenases isozymes, denominated LOX 1, 2 and 3 (Axelrod et al., 1981). Those enzymes are responsible for the characteristic beany flavor of soybean, reducing the acceptability of soybean products for the consumer (Nishiba et al., 1995). Triple-null lines (lacking the isozymes LOX 1, 2 and 3 in the seed) derived from elite cultivars have been developed through backcross breeding program to increase the acceptability of soybean products for human feeding.

Lipoxygenases have been related to plant growth and development, senescence, wound responses (Hildebrand, 1989), defense against pest and disease (Croft et al., 1993) and temporary nitrogen storage in vegetative tissues (Tranbarger et al., 1991; Grimes et al., 1993; Bunker et al., 1995). The heterogeneity of physiological roles attributed to plant lipoxygenases is probably due to their involvement in the biosynthesis of signaling molecules such as jasmonates and aldehydes (Hildebrand, 1989; Gardner, 1991; Song & Brash, 1991).

Lipoxygenases have been found in nodules of *Vicia faba* (Perlick et al., 1996), *Pisum sativum* (Gardner

et al., 1996), *Phaseolus vulgaris* (Porta et al., 1999) and *Glycine max* (Mohammadi & Karr, 2003) suggesting that these enzymes would have an important role in the symbiotic interaction between legume plants and rhizobia. However, little has been done to understand lipoxygenase role in nodulation process and nothing has been done to verify if soybean devoid of seed lipoxygenases presents similar performance of lipoxygenase activity in roots and nodules compared to normal soybean lines.

The objective of this work was to evaluate lipoxygenase activity in roots and nodules of soybean lines (with and without lipoxygenases in their seeds) during the nodulation process.

Material and Methods

Soybean near isogenic lines without seed lipoxygenases from the breeding program conducted at the Universidade Federal de Viçosa (UFV), MG, Brazil, were used in this experiment. These lines were derived from the cross between Doko cultivar and a line with absence of seed lipoxygenases isozymes (UFV TN). BC_5F_2 seeds were submitted to non-destructive analyses of lipoxygenases through colorimetric (Suda et al., 1995) and espectrophotometric assays (Axelrod et al., 1981; Oliveira et al., 1998) for selecting dominant and recessive homozygous genotypes for lipoxygenase isozymes in order to select triple-null (DTN) and normal (DN) seeds.

Selected seeds were sterilized with 70% alcohol for one minute and then with 6% hydrogen peroxide for five minutes. Seeds were abundantly washed with sterilized water, and then each seed was placed to germinate in 3 L capacity plastic bags containing autoclaved sand previously washed with water. After a four-day period of being sowed, DN and DTN plants were inoculated with 1 mL of a culture of *Bradyrhizobium elkanii* strain CPAC 15 (10^8 cells mL^{-1}). DN and DTN plants without inoculation were included as control. Soybean plants were cultivated under greenhouse conditions.

Roots were collected at 3, 5, 9, 13, 18 and 28 days post-inoculation (DPI) and nodules at 13, 18 and 28 DPI, from five plants of each line, inoculated and uninoculated. Roots and nodules were frozen in liquid nitrogen and stored at -80ºC, until enzyme extraction.

One hundred mL of complete nutrient solution (Hoagland & Arnon, 1938) and of nutrient solution devoid of nitrogen were supplied twice a week to uninoculated and inoculated plants, respectively, starting at V_1 stage (Fehr et al., 1971). Water was supplied whenever necessary. The inoculated plants received an additional 7.5 μmoles L^{-1} of Co in the form of $CoCl_2$ in the first week of cultivation.

The extract preparation was carried out at 4ºC according to Ohta et al. (1986). Roots and nodules were frozen in liquid N and then triturated using mortar and pestle. The powder obtained was homogenized in sodium phosphate buffer 0.05 mol L^{-1} at pH 8.2, in the proportion of 1:3 (w/v) for roots and 1:12 (w/v) for nodules and then centrifuged at 15,000 *g* for 30 minutes at 4ºC. The supernatant was used for protein and lipoxygenase activity determinations. Soluble protein concentrations were determined according to Smith et al. (1985) using BSA as standard. Lipoxygenase activities were determined by spectrophotometric measurement of the formation of conjugated dienes at 234 nm (Axelrod et al., 1981).

For evaluating lipoxygenase activities in roots and nodules at different pH values, the following buffer systems were used at concentration of 0.05 mol L^{-1}: citric acid/dissodium phosphate (pH 1.5–2.5); citric acid/sodium citrate (pH 3.0–3.5); acetic acid/sodium acetate (pH 4.0–4.5); citric acid/sodium acetate (pH 5.0–5.5); monophosphate/dissodium phosphate (pH 6.0–7.0); Tris-HCl (pH 7.5–8.5); boric acid/ sodium borate (pH 9.0–10.0).

Lipoxygenase pH-activity profile was determined in the following way: 1 μL of crude root or nodule extract and 4 μL of linoleic acid stock solution were mixed in 1 mL of buffer 0.05 mol L^{-1} from each of the pH values described before at 25ºC. The absorbance of the reaction mixture at 234 nm was recorded every 30 seconds for a period of 2.5 minutes.

Protein extracts were prepared from roots at 3, 5, 9, 13, 18 and 28 DPI and from nodules at 13, 18 and 28 DPI. Extracts from roots and nodules were obtained by homogenizing those materials in liquid N using a mortar and pestle with 50 mmoles L^{-1} Tris-HCl at pH 8.2. After centrifugation at 15,000 *g* for 30 minutes, proteins of the supernatant were determined. Extracts from nodules containing 25 μg of protein and from roots containing 30 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE (Laemmli, 1970) using 9% acrylamide mini-gels.

Seed protein extracts of DN and DTN lines were placed in each gel as control. Proteins were electroblotted to nitrocellulose sheets with Mini Trans-Blot Electrophoretic Transfer Cell (BIORAD), according to

the manufacturer's instructions. After the transfer (one hour, 100 V and 4ºC), the nitrocellulose membrane was blocked with TBS-T [0.01 mol L^{-1} Tris-HCl, pH 7.6, 1.5 mmol L^{-1} NaCl, 0.1% Tween-20 (v/v)] for one hour at room temperature and incubated with chicken IgG raised against soybean seed lipoxygenases, diluted 1:500 in TBS-T, for one to two hours under agitation. After the incubation period, the membrane was washed with TBS-T three times, for 10 minutes each and incubated with antibody against IgG conjugated to alkaline phosphatase (SIGMA) in a dilution of 1:5,000 in TBS-T for approximately two hours. The membrane was washed three times for 10 minutes each with TBS-T and incubated with the enzyme buffer (0.1 mol L^{-1} Tris-HCl at pH 9.8, 0.1 mol L^{-1} NaCl, 0.5 mol L^{-1} $MgCl_2$) for five minutes. The activity of alkaline phosphatase was detected using a solution containing 11 µL of NBT (GIBCO/BRL), 8.25 µL of BCIP (GIBCO/BRL) in 10 mL of enzyme buffer. After the staining was completed, the cellulose nitrate membranes were washed to interrupt the reaction.

Results and Discussion

Roots and nodules of DN and DTN have rather similar pH-activity profiles, suggesting the existence of similar lipoxygenase pool in both genotypes (Figure 1). Considering the pH profiles for root and nodule (Figures 1 and 2), these results confirm those found by Peterman & Siedow (1985) and Altschuler et al. (1989) who observed lipoxygenase activity in soybean roots at pH 6.8 and not at pH 9.0. However, Mohammadi & Karr (2003) observed lipoxygenase activity in soybean root and nodule at pH 9.0. The inoculated genotypes presented lipoxygenase activity at pH 3.5. This observation suggested the appearance of new forms of lipoxygenase in the roots after inoculation with *Bradyrhizobium elkanii* (Figure 1). Three-month nodules of common bean plants showed lipoxygenase activity at pH 7.0 (Eiben & Slusarenko, 1994), similar to the results obtained in this study.

Lipoxygenase activity in roots of inoculated lines (DNI and DTNI) declined from 3 to 28 DPI (Figure 3). These

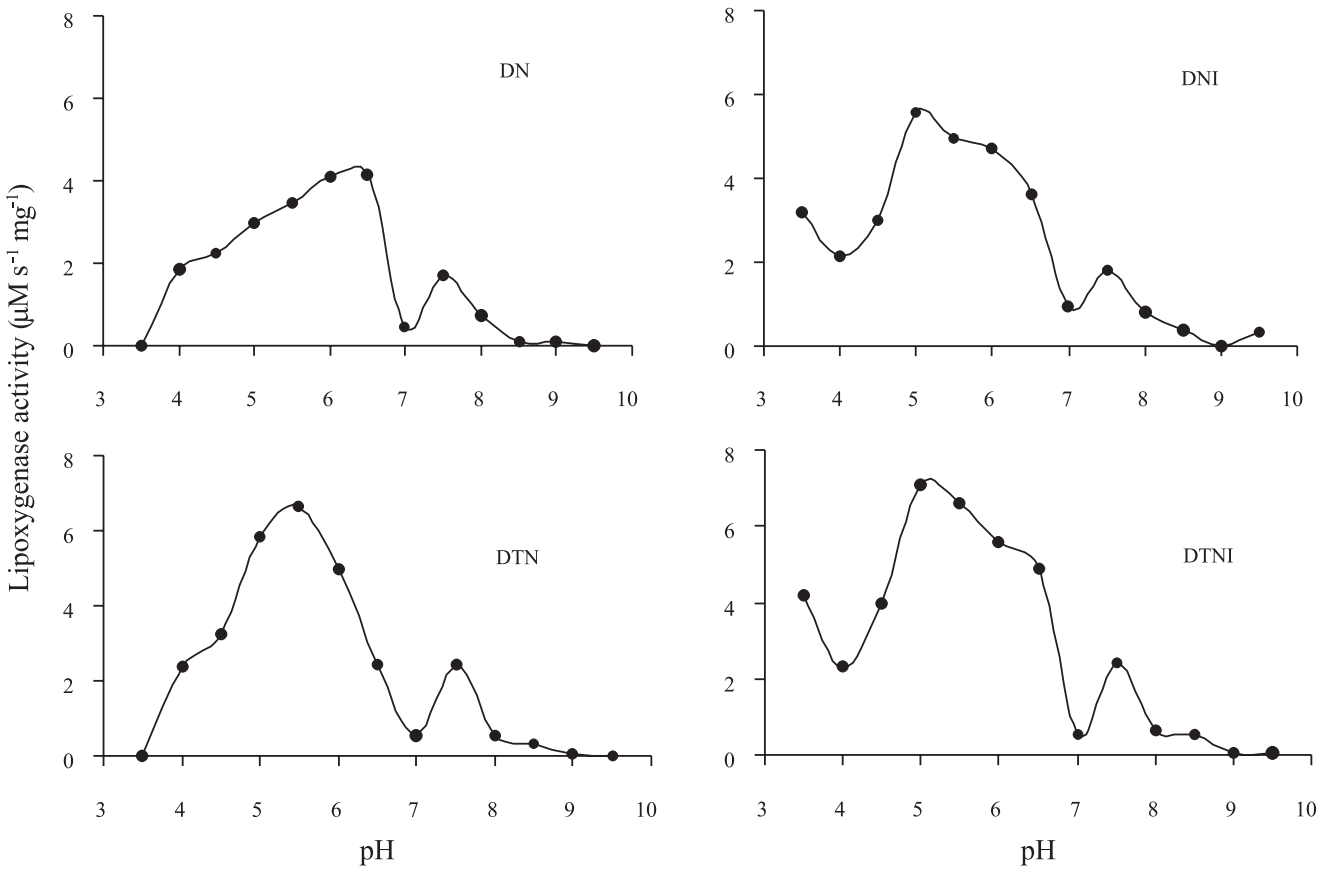

Figure 1. Specific lipoxygenase activity in roots from the Doko (DN), Doko inoculated (DNI), Doko triple-null (DTN) and Doko triple-null inoculated (DTNI) soybean lines 28 days post-inoculation at different pH values.

results were similar to those obtained by Eiben & Slusarenko (1994) in roots of common bean plants; these authors observed that lipoxygenase activity measured at pH 7.0 showed a peak of activity between 5 and 10 days after germination, and then declined to undetectable levels in three-month-old root plants. A higher lipoxygenase activity at the beginning of root formation suggests that the enzyme would be involved in the growth and development of this organ. This hypothesis is reinforced through the observation that root tissue adjacent to the effective nodules had a negligible lipoxygenase activity (Mohammadi & Karr, 2003). The lipoxygenases involvement in growth and development of several plant organs has been suggested by Hildebrand (1989), Siedow (1991) and Koda (1992).

Early root nodules were visible at 13 days post-inoculation. The lipoxygenase activity in the nodules was constant between 13 and 18 DPI and sharply increased between 18 and 28 DPI (Figure 3). Mohammadi & Karr (2003) also observed increased lipoxygenase activities in the nodules during this organ ontogeny. The high lipoxygenase activity at the beginning of nodule formation reinforces the hypothesis that this enzyme is involved in growth and development of that organ. In pea (Gardner et al., 1996) and *Vicia faba* (Perlick et al., 1996), high lipoxygenase activity was found in the nodule apex adjacent to the meristem, or in regions of the nodules which are under rapid division and growth.

Lipoxygenase antibodies were used to detect antigens in western blots of inoculated root extracts, nodules and uninoculated roots of DN and DTN lines with the purpose of analyzing the lipoxygenase dynamic during the biogenesis of this organs. Seeds of DN and DTN lines were used as positive and negative controls, respectively.

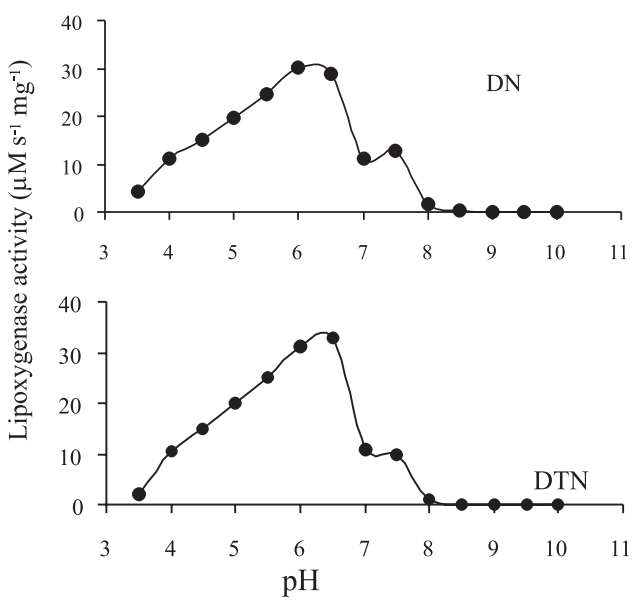

Figure 2. Specific lipoxygenase activity in nodules from Doko (DN) and Doko triple-null (DTN) soybean lines 28 days post-inoculation at different pH values.

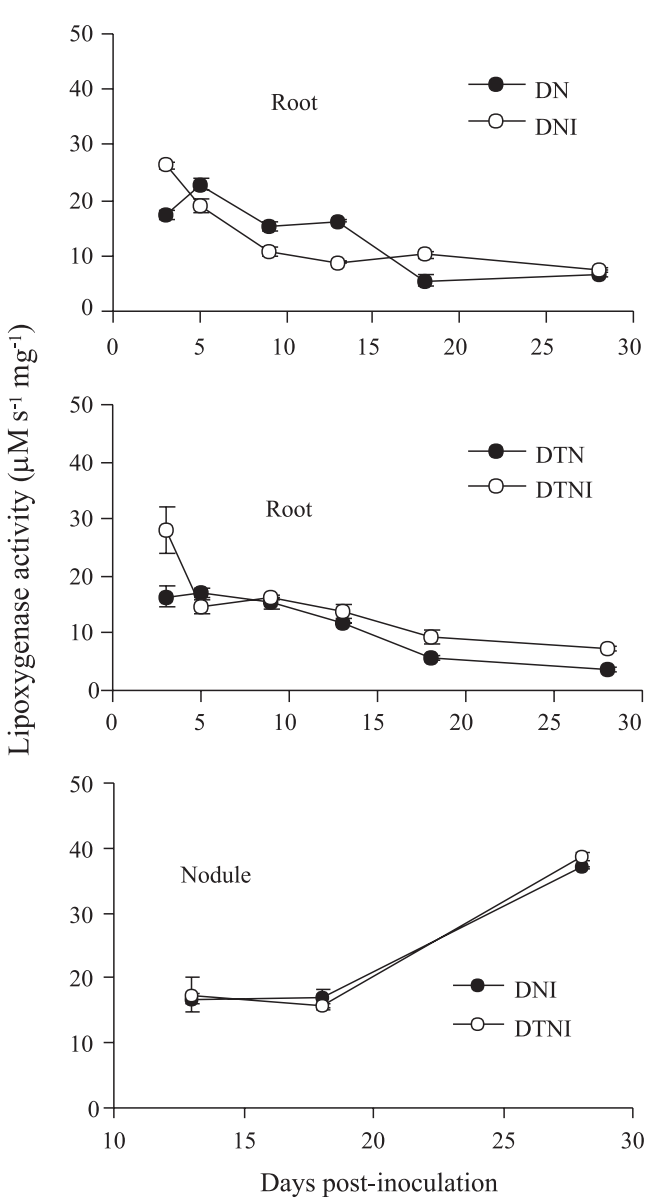

Figure 3. Lipoxygenase activity in roots and nodules from Doko (DN), Doko inoculated (DNI), Doko triple-null (DTN) and Doko triple-null inoculated (DTNI) soybean lines at different developmental stages.

No antigen was detected in seeds of DTN. Multiple immunoreactive bands were visible in seeds of DN, roots and nodules of DN and DTN (Figure 4). Soybean seeds present three isozymes with molecular weights of 94, 97 and 96 kDa, being visualized in western blot in two mobility groups. Root and nodule showed at least two mobility groups that were similar to those of seeds (at approximately 94 and 97 kDa) (Figure 4). Mohammadi & Karr (2003) suggested more than one type of lipoxygenase for nodule and only one for root, probably because they used antibodies raised against seed lipogygenases 2 and 3, while in this work it was used antibody raised against soybean seed lipoxygenases in general. Multiple immunoreactive bands were also detected in pea nodules (Gardner et al., 1996).

The intensity of the immunoreactive bands observed in roots of DN and DTN lines, inoculated and uninoculated, was higher at 5 and 9 DPI and reduced after 13 DPI (Figure 4). This suggests that lipoxygenases in roots could have some metabolic function during root development. In nodules, the intensity of the immunoreactive bands becomes higher at 28 DPI (Figure 4), confirming the results observed with lipoxygenase activity in that organ (Figure 3). This suggests that the variations of lipoxygenase activity in nodules are mainly due to the variations in the concentration of that particular protein in that organ. Mohammadi & Karr (2003) observed that the hybridization band increased in intensity up to 35 days post-inoculation and declined thereafter.

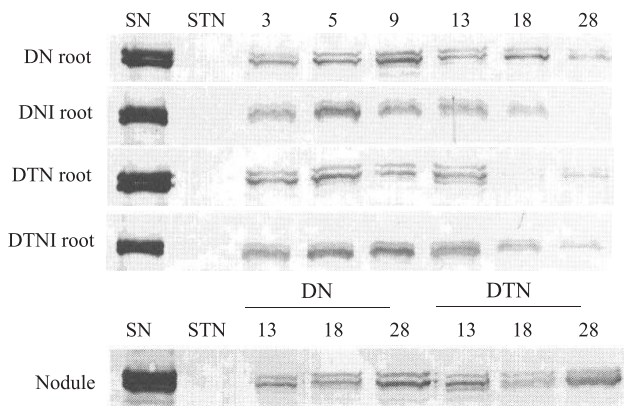

Figure 4. Immunoblotting of lipoxygenases from soybean roots and nodules at different developmental stages. Roots of Doko (DN) and Doko triple-null (DTN) soybean lines, inoculated and uninoculated, 3, 5, 9, 13, 18 and 28 days post-inoculation (DPI) were used. Nodules of the same lines, 13, 18, and 28 DPI were used as controls.

This work characterized the activity of lipoxygenases in the root system of inoculated and uninoculated soybean with *Bradyrhizobium*, subsiding future experiments for understanding lipoxygenase function during the process of nodulation. It also demonstrated that isolines devoid of lipoxygenases in the seeds show a pattern of lipoxygenase activity in their root system similar to the normal genotype from which they derived.

Conclusions

1. The pH-activity profiles of lipoxygenases extracted from root and nodule of soybean lines with and without seed lipoxygenases are similar.

2. The presence of lipoxygenase activity at pH 3.5 in soybean root from inoculated soybean genotypes indicates the appearance of new form of lipoxygenase in symbiotic plants.

3. Lipoxygenase activity of soybean roots declines from day 3 up to 28 days post-inoculation.

4. Nodules present a visible increase in lipoxygenase activity at 28 days post-inoculation.

5. Root and nodules present two lipoxygenase bands in SDS-PAGE with molecular weights of 94 and 97 kDa.

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