www.czasopisma.pan.pl PAN www.journals.pan.pl
ACTA BIOLOGICA CRACOVIENSIA Series Botanica 53/1: 32–37, 2011
DOI: 10.2478/v10182-011-0005-4

RESPONSE OF MAIZE, PEA AND RADISH ROOTS TO ALLELOCHEMICAL STRESS

JOANNA GMEREK AND BARBARA POLITYCKA*

Department of Plant Physiology, Poznan University of Life Sciences, Wołynska 35, 60–637 Poznan, Poland

Received May 26, 2010; revision accepted February 27, 2011

We examined whether allelochemical stress leads to increased lipoxygenase activity in roots of sweet maize (*Zea mays* L. ssp. *saccharata*), pea (*Pisum sativum* L.) and radish (*Raphanus sativum* L. var. *radicula*). The lipoxygenase activity of soluble and membrane-bound fractions was assessed in roots after exposure to ferulic and *p*-coumaric acids. Lipid peroxidation and membrane injury were determined as indicators of stress. Increased lipoxygenase activity of both studied fractions was followed by lipid peroxidation and plasma membrane injury. The results suggest the key role of lipoxygenase in plasma membrane injury during allelochemical stress caused by administration of hydroxycinnamic acids.

Key words: Allelopathic stress, *p*-coumaric acid, ferulic acid, lipid peroxidation, lipoxygenase, pea, plasma membrane injury, radish, roots, sweet maize.

INTRODUCTION

Changes in the structure and permeability of plasma membranes occur in plant species in response to different stress factors. They have been found to occur in response to allelopathic compounds, including phenolics (Glass and Dunlop, 1974; Macri et al., 1986; Vaugham and Ord, 1991; Politycka, 1996; Zeng et al., 2001). Changes in the structure of membrane components lead to an increase of membrane permeability; leakage of cellular substances, most frequently ions, is observed (Baziramakenga et al., 1995; Politycka, 1996; Galindo et al., 1999). One of the reasons for these changes in membrane permeability is peroxidation of lipids, which takes place in fragments of polyunsaturated fatty acids contained in the phospholipid content (Catala, 2009). Hydroxyperoxides are the products of this process. One of them, malonyldialdehyde (MDA), is known to be an indicator of peroxidation (Cruz-Ortega, 2002; Lara-Nuńez et al., 2006). The lipoxygenases (LOXs, linoleate-oxygen oxidoreductases, EC 1.13.11.12) are a large group of enzymes that catalyze oxygenation of polyunsaturated fatty acids (linolic and linoleic acids) to hydroxyperoxides (Vick and Zimmerman, 1987; Siedow, 1991; Feussner and Wasternack, 2002). Biochemical and molecular studies of dicotyledonous species indicate that LOX activity is modulated in response to both biotic and abiotic stresses, such as mechanical wounding, insect feeding and pathogen attack (Blee, 1998).

Our previous work with cucumber seedlings showed that ferulic and *p*-coumaric acids act as allelopathic stressors triggering increased LOX activity (Politycka and Bednarski, 2004). Here we examine whether these phenolic acids induce elevated LOX activity in other plant species as well.

MATERIAL AND METHODS

PLANT MATERIAL

The study used roots of seedlings of three plant species belonging to different families: sweet maize (*Zea mays* ssp. *saccharata* L., Graminae), garden pea (*Pisum sativum* L., Fabaceae) and radish (*Raphanus sativus* L. var. *radicula*, Brassicaceae). Seedlings of maize cv. Waza, pea cv. Bohun and radish cv. Rova were grown in a growth chamber in glass containers on plates covered with wet filter paper at 20°C under fluorescent light (Philips lamps, photon flux density 135 micromoles $m^{-2} \cdot s^{-1}$) and a 14 h photoperiod.

Five-day-old (maize), 6-day-old (radish) and 7-day-old (pea) seedlings were subjected to allelochemical stress by immersing their roots in 0.5 mM

*e-mail: barpolit@yahoo.pl

PL ISSN 0001-5296 © Polish Academy of Sciences and Jagiellonian University, Cracow 2011

(maize) or 1 mM (pea and radish) solutions of ferulic and *p*-coumaric acids. Their age at testing was selected in terms of their different growth dynamics, and the concentrations of the applied phenolic acids depended on the sensitivity of the particular species to their action, which was estimated in preliminary studies. The roots of control seedlings were immersed in water. After 30, 60 and 90 min of stress, root samples were taken and LOX activity was determined in soluble and membrane-bound fractions. For analysis of lipid peroxidation, during stress periods 4 h (pea and radish) and 6 h (maize), samples were successively taken at 1 or 2 h intervals, frozen at –18°C and stored for analysis. At the same 1 or 2 h intervals, measurements were made to determine the index of plasma membrane injury.

LIPOXYGENASE EXTRACTION AND ASSAY

LOX was isolated from soluble and membrane-bound fractions (Baracat-Pereira et al., 2001). Samples of frozen roots (0.25 g) were homogenized using a cooled mortar and pestle at 4°C with 0.025 g PVP in 2.5 ml 50 mM sodium phosphate buffer (pH 6.5) with added 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 18,000 × g for 30 min at 4°C and the supernatant was used as enzymatic extract of the soluble fraction. Before isolation of the membrane-bound fraction of enzyme the pellets were washed twice with sodium phosphate and centrifuged at 18,000 × g. The pellets were extracted with 2.5 ml 50 mM sodium phosphate buffer (pH 6.5) with 1 mM PMSF and 0.25% (v/v) Triton X-100 added. After centrifugation at 20,000 × g for 30 min the supernatant was used as enzymatic extract of the membrane-bound fraction.

The LOX activity of soluble and membrane-bound fractions was determined spectrophotometrically using linoleic acid as substrate (Wang and Hildebrand, 1987). In brief, 100 μl enzymatic extract was added to 1.9 ml substrate mixture containing 0.04 mM emulsified linoleic acid in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was run at 25°C. Absorbance was determined at 234 nm. Lipoxygenase activity was expressed in nanocatales converted to 100 mg protein. For determination of the reaction product a molar absorption coefficient of 23 mM^{-1} cm^{-1} was used (Gibian and Vandenberg, 1987). Determinations were made in 7 replicates. Protein content was determined according to Bradford (1976), with bovine serum albumin as the standard.

LIPID PEROXIDATION

Frozen roots of pea and radish (0.25 g) or maize (0.5 g) were homogenized in a cooled mortar with 5 ml of a mixture of 0.25% thiobarbituric acid in 10% trichloroacetic acid and boiled for 30 min. The reference sample was the same mixture without any root sample. After cooling, the homogenate was centrifuged at 10,000 × g for 10 min and then the absorbance of the obtained supernatant was measured at 532 and 600 nm. Lipid peroxidation was indicated by increased malonyldialdehyde (MDA) content (Heath and Packer, 1968). For estimation of MDA amount a molar absorption coefficient of 155 mM^{-1} cm^{-1} was used. Determinations were made in 5 replicates.

PLASMA MEMBRANE INJURY INDEX

The plasma membrane injury index was determined based on leakage of electrolytes from roots, by measuring the electrical conductivity of the solutions of ferulic acid and *p*-coumaric acid or water (control) in which the seedling roots were immersed. After those measurements, the solutions with roots were boiled for 5 min and cooled down, and after 1 h their conductivity was measured. The membrane injury index (I_i) was measured according to the formula (Kacperska, 1991):

$$I_i = (U_s - U_c/U_t - U_c) \times 100\%$$

where U_s is the conductivity of solution with roots subjected to stress, U_c is the conductivity of solution with control roots, and U_t is the conductivity of boiled solution with roots. Determinations were made in 3 replicates.

STATISTICAL ANALYSIS

The significance of LOX activity results was checked by multiple-range ANOVA, and for MDA content by one-way ANOVA with Duncan's test at $P \leq 0.05$.

RESULTS

LIPOXYGENASE ACTIVITY

In maize roots after 30 min of allelochemical stress, ferulic acid increased the activity of LOX in the soluble fraction by 55% versus the control, and *p*-coumaric acid by 36% (Fig. 1). In roots treated with ferulic acid, LOX activity decreased, reaching the control level after 90 min. On the other hand, in roots treated with *p*-coumaric acid we saw a gradual increase of LOX activity in the soluble fraction: after 60 min it was 58% higher than in the control, and after 90 min it was 70% higher. In the membrane-bound fraction the effect of ferulic acid appeared after 30 min as 37% higher enzyme activity versus the control. In the *p*-coumaric acid treatment the increase of LOX activity occurred at 60 min (36% higher), and at 90 min the increase was 51% versus the control.

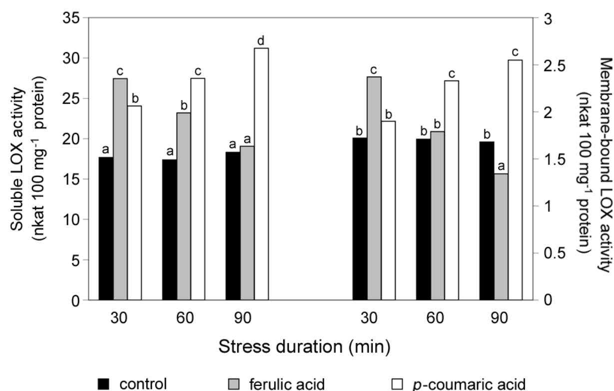

Fig. 1. Activity of soluble and membrane-bound lipoxygenase in maize roots treated with 0.5 mM ferulic and *p*-coumaric acids. Bars with the same letter do not differ significantly at P≤0.05.

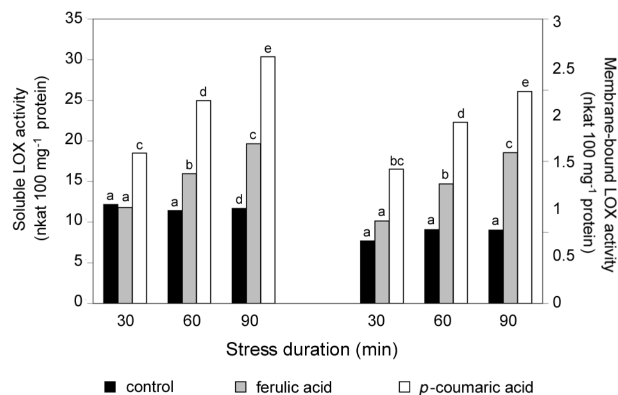

Fig. 2. Activity of soluble and membrane-bound lipoxygenase in pea roots treated with 1 mM ferulic and *p*-coumaric acids. Bars with the same letter do not differ significantly at P≤0.05.

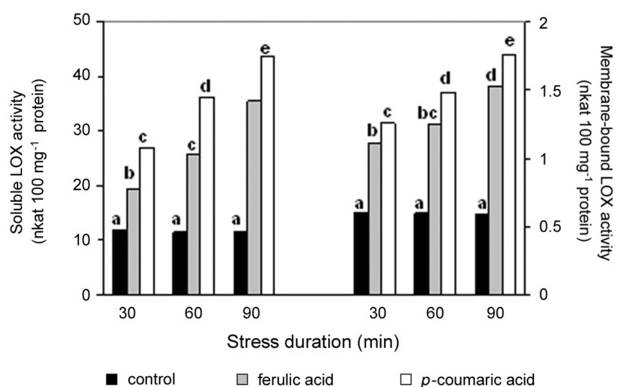

Fig. 3. Activity of soluble and membrane-bound lipoxygenase in radish roots treated with 1 mM ferulic and *p*-coumaric acids. Bars with the same letter do not differ significantly at P≤0.05.

Changes in LOX activity in roots of pea are shown in Figure 2. Treatment with ferulic and *p*-coumaric acids there led to a gradual increase LOX activity in both fractions. The effect of *p*-coumaric acid appeared earlier and was stronger. After 30 min of exposure to ferulic acid there were no significant changes in LOX activity in the soluble or membrane-bound fractions, but in the *p*-coumaric acid treatment it was 52% higher in the soluble fraction and 115% higher in the membrane-bound fraction. LOX activity increased versus the control in roots treated with ferulic acid by 39% (60 min) and 68% (90 min) in the soluble fraction, and 61% and 105% in the membrane-bound fraction. At the same intervals, stimulation of LOX activity by *p*-coumaric acid was stronger: after 60 min the increase was 118% in the soluble fraction and 146% in the membrane-bound fraction; after 90 min the increase was 160% in the soluble fraction and 189% in the membrane-bound fraction.

LOX activity also increased in both fractions of roots of radish treated with ferulic and *p*-coumaric acids (Fig. 3). After 30 min in the ferulic acid treatment it was 62% higher in the soluble fraction versus the control, 124% higher at 60 min, and 209% higher at 90 min. Stimulation of LOX activity in the soluble fraction was strong throughout the whole experiment in roots exposed to *p*-coumaric acid. After 30 min it increased 128%, after 60 min it increased 216% and at 90 minutes it was 280% higher than the control. In the membrane-bound fraction the increase of LOX activity in response to both allelochemical treatments was 60% after 30 min, 127% after 60 min, and 178% after 90 min.

LIPID PEROXIDATION

After 2 h, maize roots treated with 0.5 mM solution of ferulic acid showed a 22% increase of MDA level versus the control, and a 36% increase in response to the same concentration of *p*-coumaric acid (Fig. 4). With time there were further increases. After 6 h the level was 53% (ferulic acid) and 72% (*p*-coumaric acid) higher than the control.

As in maize, lipid peroxidation was altered in treated pea roots (Fig. 5). Already in the first hour there was a significant increase of MDA content, by 22% (ferulic acid) and 8% (*p*-coumaric acid) versus the control. The MDA level gradually increased with time. At 4 h it was 54% (ferulic acid) and 70% (*p*-coumaric acid) higher than the control.

Changes in the degree of lipid peroxidation in radish roots are shown in Figure 6. In the first hour the increase of MDA was significant only in the *p*-coumaric acid treatment, and was 24% versus the control. After 2 h the MDA level increased 93% versus the control in both allelochemical treatments.

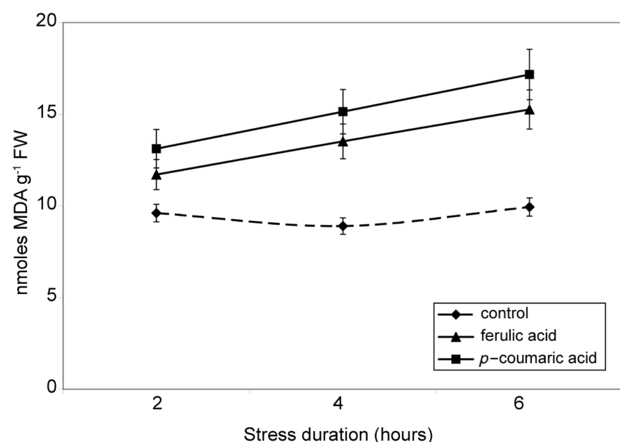

Fig. 4. Lipid peroxidation in maize roots treated with 0.5 mM ferulic and *p*-coumaric acids.

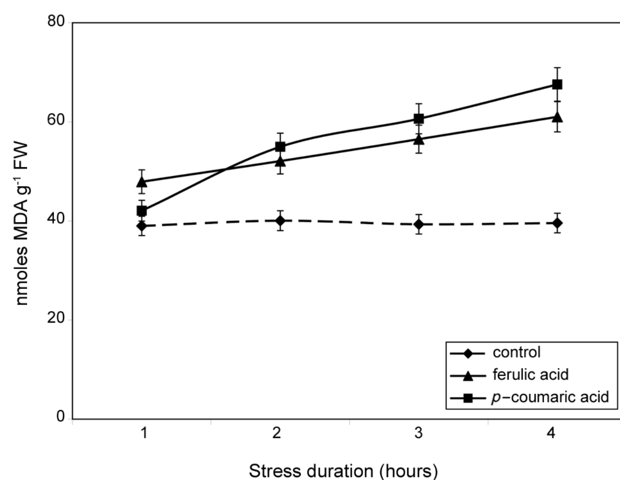

Fig. 5. Lipid peroxidation in pea roots treated with 1 mM ferulic and *p*-coumaric acids.

MDA content decreased to the control level after 3 and 4 h of stress.

PLASMA MEMBRANE INJURY

Membrane injury occurred in maize roots as early as 1 h after the beginning of treatment (Fig. 7). The effect of *p*-coumaric acid was greater than that of ferulic acid, and the degree of injury increased with time. After 6 h the injury index reached 11% in roots treated with ferulic acid and 18% in roots treated with *p*-coumaric acid.

In pea roots, membrane injury followed a similar pattern (Fig. 8): the effect of *p*-coumaric acid was stronger. After 4 h the injury index reached 14% in roots treated with ferulic acid and 18% in roots treated with *p*-coumaric acid.

In radish roots we noted no differences in the action of ferulic and *p*-coumaric acid in terms

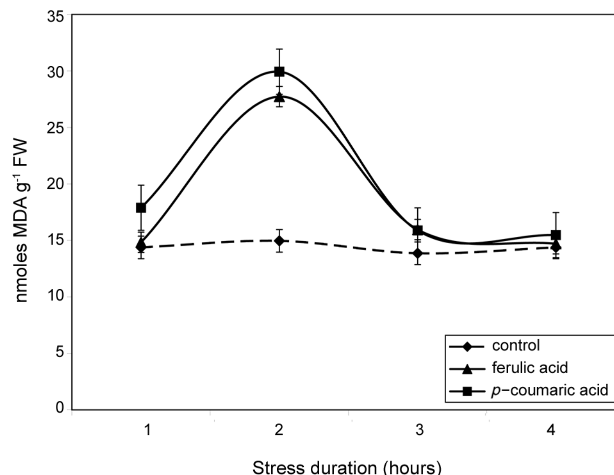

Fig. 6. Lipid peroxidation in radish roots treated with 1 mM ferulic and *p*-coumaric acids.

of plasma membrane injury (Fig. 9). Four h after introduction of stress the index was 18.5% on average.

DISCUSSION

In this work we found that LOX activity was significantly higher in the soluble fraction than in the membrane-bound fraction of the roots of all examined species. LOX activity has been observed in several cell fractions including chloroplasts, mitochondria, vacuoles, lipid bodies and membranes, but the highest activity was associated with soluble cytoplasm fractions (Liavonchanka and Feussner, 2006). The observed induction of LOX activity in the roots of maize, pea and radish treated with ferulic and *p*-coumaric acids may have proceeded in response to accumulation of hydrogen peroxide (H_2O_2), whose generation we observed in previous studies following the same experimental design (Gmerek and Politycka, 2010). H_2O_2 increased at the same time as induction of LOX activity. H_2O_2 is believed to perform a signalling role, and the signal transduction activated by H_2O_2 leads to activation of LOX and to peroxidation of lipids, among other reactions (Eshdat et al., 1997)). The physiological role of LOX in higher plants is not fully recognized, but there many reports indicating increased activity of this enzyme in response to stress factors (Kacperska, 1991; Blee, 1998; Porta and Rocha-Sosa, 2002; Liavonchanka and Feussner, 2006; Eckardt, 2008). LOX carries out peroxidation reactions on plasma membrane lipids, which could increase the level of lipid unsaturation and increase membrane fluidity. Here we showed that ferulic and *p*-coumaric acids evoke peroxidation of lipids in the roots of all three

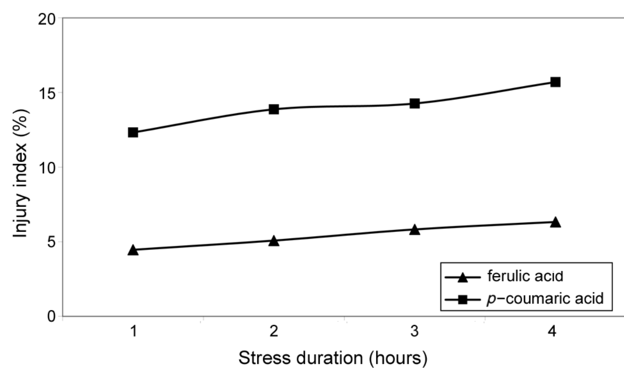

Fig. 7. Injury index of plasma membranes in maize roots treated with 0.5 mM ferulic and *p*-coumaric acids.

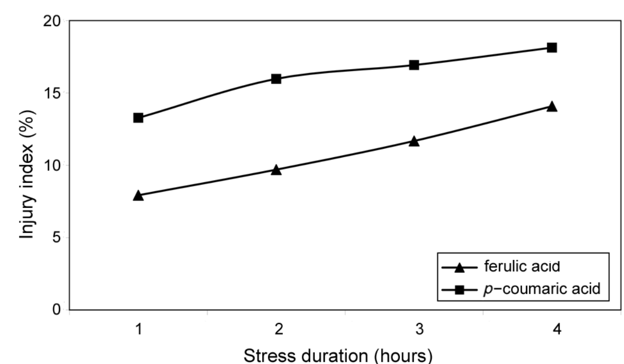

Fig. 8. Injury index of plasma membranes in pea roots treated with 1 mM ferulic and *p*-coumaric acids.

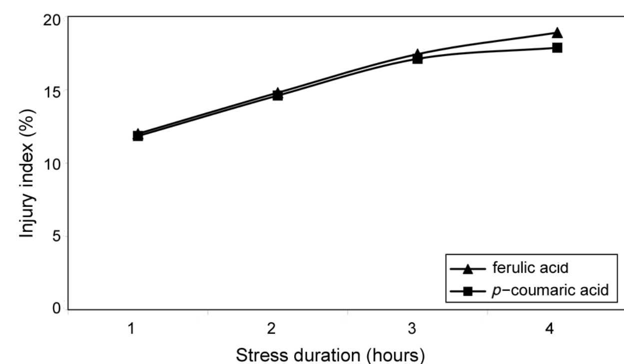

Fig. 9. Injury index of plasma membranes in radish roots treated with 1 mM ferulic and *p*-coumaric acids.

studied plant species. The process of lipid peroxidation consists of three stages: initiation, propagation and termination (Catala, 2006). The initiation phase of lipid peroxidation is the abstraction of hydrogen atoms from lipid molecules. Several free radicals are responsible for this, one being hydroxyl radical (Gutteride, 1988). Earlier we observed a significant increase in the content of this form of reactive oxygen in roots of maize, pea and radish as early as 30 min after treatment with ferulic and *p*-coumaric acids (Gmerek and Politycka, 2010). Peroxidation of lipids is particularly damaging because the products of this process lead to the spread of further free radical reactions (Catala, 2009). Peroxidation of fatty acyl groups occurs mostly in phospholipids of plasma membranes, altering their permeability (Nigam and Schewe, 2000). In our present work we found plasma membrane injury attributable to the action of ferulic and *p*-coumaric acid on roots of the three plant species. Studies on cucumber roots and sorghum showed that injury to membranes under the influence of allelopathic compounds were proportional to lipid peroxidation measured as MDA content (Politycka, 1996; Zeng et al., 2001). In pea and maize roots a gradual increase of MDA level lasted through the whole period of the experiments. In radish roots the changes in MDA level were not great, and were highest level in the second hour of stress. The differences in lipid peroxidation between the species probably were due to differences in the number of the generated reactive oxygen species (ROS), which we documented in earlier work. Under the action of ferulic and *p*-coumaric acids, significantly less ROS developed in radish than in pea and maize roots (Gmerek and Politycka, 2010). A dependence between ROS level and lipid peroxidation was also observed in tomato roots subjected to allelopathic stress by treatment with an extract from *Callicarpa acuminata* (Cruz-Ortega et al., 2002).

The role of LOX in the stress response is complex. One suggested function of LOX in stress conditions is its participation in initiation of protective and acclimatization processes (Blee, 1998; Eckardt, 2008). LOX catalyses the first reaction in the synthesis of compounds derived from polyunsaturated fatty acids, called oxylipins (Feussner and Wasternack, 2002; Eckardt, 2008). The best-characterized oxylipin is the precursor of jasmonic acid, 12-oxy-phytodienoic acid (Block et al., 2005). Numerous biologically active oxylipins are formed nonenzymatically via the action of ROS, which are also accumulated under the influence of different stress factors. They have been shown to activate the expression of stress response genes, leading to enhanced protection against oxidative stress (Eckardt, 2008).

REFERENCES

Baracat-Pereira MC, de Almeida Oliveira MG, de Barros EG, Moreira MA, and Santoro MM. 2001. Biochemical properties of soybean leaf lipoxygenases: Presence of soluble and membrane bound forms. *Plant Physiology and Biochemistry* 39: 91–98.

BAZIRAMAKENGA R, LEROUX GD, and SIMARD RR. 1995. Effects of benzoic and cinnamic acids on membrane permeability of soybean roots. *Journal of Chemical Ecology* 21: 1271–1285.

BLEE E. 1998. Phytooxylipins and plant defence reactions. *Progress in Lipid Research* 37: 33–72.

BLOCK A, SCHMETLZ E, JONES JB, and KLEE HJ. 2005. Coronatine and salicylic acid: The battle between *Arabidopsis* and *Pseudomonas* for phytohormone control. *Molecular Plant Pathology* 6: 79–83.

BRADFORD MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

CATALA A. 2006. An overview of lipid peroxidation with emphasis on outer segments of photoreceptors and the chemiluminescence assay. *International Journal of Biochemistry and Cell Biology* 38: 1482–1495.

CATALA A. 2009. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids* 157: 1–11

CRUZ-ORTEGA R, AYALA-CORDERO G, and ANAYA AL. 2002. Allelochemical stress produced by aqueous leachates of Callicarpa acuminate: effects on roots of bean, maize and tomato. *Physiologia Plantarum* 116: 20–27.

ECKARDT NA. 2008. Oxylipin signalling in plant stress responses. *The Plant Cell* 20: 495–497.

ESHDAT Y, HOLLAND D, FALTIN Z, and BEN-HAYYIM G. 1997. Plant glutathione peroxidases. *Physiologia Plantarum* 100: 234–240.

FEUSSNER I, and WASTERNACK C. 2002. The lipoxygenase pathway. *Annual Review of Plant Biology* 53: 275–297.

GALINDO JCG, HERNANDEZ A, DAYAN FE, TELLEZ MR, MACIAS FA, PAUL RN, and DUKE SO. 1999. Dehydrozaluzanin c, a natural sesquiterpenolide, causes rapid plasma membrane leakage. *Phytochemistry* 52: 805–813.

GIBIAN MJ, and VANDENBERG P. 1987. Product yield in oxygenation of linoleate by soybean lipoxygenase: the value of the molecular extinction coefficient in the spectrophotometric assay. *Analytical Biochemistry* 163: 343–349.

GLASS ADM, and DUNLOP J. 1974. Influence of phenolic acids on ion uptake. IV. Depolarization of membrane potentials. *Plant Physiology* 54: 855–858.

GMEREK J, and POLITYCKA B. 2010. Generation of active oxygen species in roots of maize, pea and radish as response to exogenous ferulic and *p*-coumaric acids. *Allelopathy Journal* 25(2): 475–484.

GUTTERIDE JMC. 1988. Lipid peroxidation: some problems and concepts. In: Halliwell B [ed.], *Oxygen Radicals and Tissue Injury*, 9–19. The Federation of American Societies for Experimental Biology, Bethesda, MD.

HEATH RL, and PACKER L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125: 189–198.

KACPERSKA A. 1991. Plant resistance on environmental abiotic stress factors and the methods of its estimation. *Postępy Nauk Rolniczych* 1(2): 21–32. (In Polish).

LARA-NUŃEZ A, ROMERO-ROMERO T, VENTURA JL, and BLANCAS V. 2006. Allelochemical stress causes inhibition of growth and oxidative damage in *Lycopersicon esculentum* Mill. *Plant, Cell and Environment* 29: 2009–2016.

LIAVONCHANKA A, and FEUSSNER I. 2006. Lipoxygenases: occurrence, functions and catalysis. *Journal of Plant Physiology* 163: 348–357.

MACRI F, VIANELLO A, and PENNAZIO S. 1986. Salicylate-collapsed membrane potential in pea stem mitochondria. *Physiologia Plantarum* 67: 136–140.

NIGAM S, and SCHEWE T. 2000. Phospholipase A2s and lipid peroxidation. *Biochimica et Biophysica Acta* 1488: 167–181.

POLITYCKA B. 1996. Peroxidase activity and lipid peroxidation in roots of cucumber seedlings influenced by derivatives of cinnamic and benzoic acids. *Acta Physiologiae Plantarum* 4: 365–370.

POLITYCKA B, and BEDNARSKI W. 2004. Oxidative burst and lipoxygenase activity induced by hydroxycinnamic acids in cucumber roots. *Allelopathy Journal* 14(2): 197–196.

PORTA H, and ROCHA-SOSA M. 2002. Plant lipoxygenases. Physiological and molecular features. *Physiologia Plantarum* 130: 15–21.

SIEDOW JN. 1991. Plant lipoxygenase: structure and function. *Annual Review of Plant Physiology and Plant Molecular Biology* 42: 145–188.

VAUGHAM D, and ORD BG. 1991. Extraction of potential allelochemicals and their effects on root morphology and nutrient contents. In: Atkinson D [ed.], *Plant Root Growth. An Ecological Perspective*, 399–421. Blackwell Scientific Publications, Oxford, U.K.

VICK BA, and ZIMMERMAN DC. 1987. The lipoxygenase pathway. In: Stumf PK and Nes WD [eds.], *Metabolism, Function and Structure of Plant Lipids*, 383–390. New York, Plenum Press.

WANG XM, and HILDEBRAND DF. 1987. Effect of a substituted pyridazinone on the decrease of lipoxygenase activity in soybean cotyledons. *Plant Science* 51: 29–36.

ZENG RN, LUO SM, SHI YH, SHI MB, and TU CY. 2001. Physiological and biochemical mechanism of allelopathy of secalonic acid F on higher plants. *Agronomy Journal* 93: 72–79.