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Table: 1, Figs : 3

Enhanced lignification and root growth inhibition induced by ferulic and *p*-coumaric acids

J. GMEREK and B. POLITYCKA[*]

Department of Plant Physiology,
Poznan University of Life Sciences, 60-637 Poznan, Wołyńska 35, Poland.
E. Mail: barpolit@yahoo.pl

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ABSTRACT

The hydroxycinnamic acids are well-known compounds responsible for plant growth inhibition in allelopathic relationships. In this study, seedling roots of garden pea, sweet maize and radish were subjected to allelopathic stress by treatment with two derivatives of cinnamic acid: ferulic and *p*-coumaric acids at concentration of 0.5 mM (maize) and 1 mM (pea and radish). In the roots of three test species treated with ferulic and *p*-coumaric acids, syringaldazine peroxidase (POX; EC 1.11.17), phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and lignin content were determined. It was found that hydroxycinnamic acids treatments increased the activities of assayed enzymes, which intensified the lignin synthesis. Lignin production was negatively correlated with root growth. It was suggested that the inhibition of root growth induced by ferulic and *p*-coumaric acids could be due to lignification, which may be a widespread mechanism in plants.

Key words: Ferulic acid, garden pea, hydroxycinnamic acids, lignin, maize, *p*-coumaric acid, phenylalanine ammonia-lyase, *Pisum sativum* L., radish, *Raphanus sativus* L., root growth inhibition, syringaldazine peroxidase, *Zea mays* L.

INTRODUCTION

The inhibitory effects of exogenous ferulic (FA) and *p*-coumaric (*p*-CA) acids on plant growth are well known (6,7,16,34,37,40,41), while the knowledge of their mechanisms of action at the cell level is still unsatisfactory. One of the mechanisms of action of phenols on the growth process involves the metabolism of auxins. The cinnamic acid derivatives - FA and *p*-CA may affect the activity of indole-3-acetic acid (IAA) oxidase (2,19). There is negative correlation between the activity of this enzyme and the intensity of growth (38). An indirect impact on the growth of phenols is their effects on the hydration of tissues, uptake and transport of ions, changes in hormonal balance and intensity of photosynthesis and respiration (17). There is also a view that the lignification process is the cause of stunted growth by phenols (6,14,22,32). It was stated that growth inhibition by FA and *p*-CA was due to their inclusion in the structure of lignin or

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[*] Correspondence author,

formation of bridges linking lignin of cell wall polysaccharides (35,36). This in turn leads to stiffening of the cell walls of growing cells and their growth inhibition.

Our earlier studies conducted with cucumber roots treated with FA and *p*-CA led us to create a hypothetical mode of action of these phenols (27). Subsequently, a proposed mode of inhibitory action of FA on the growth of soybean roots was presented (7). In both these modes, the events following in succession lead to growth inhibition of root *via* enhanced lignification of cell walls. This study aimed to determine whether this mechanism functions in other plant species as well.

MATERIAL AND METHODS

The material of the study included roots of seedlings of three plant species belonging to different families: sweet maize (*Zea mays* ssp. *saccharata* L. from Poaceae), garden pea (*Pisum sativum* L. from Fabaceae) and radish (*Raphanus sativus* L. var. *radicula* from Brassicaceae). Seedlings of maize cv. 'Waza', pea cv.'Bohun' and radish cv.'Rowa' were grown in a growth chamber in glass containers on plates covered with wet filter paper at 20°C under luminescent light (Philips lamps) with photon flux density 135 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a 14/10 day/night 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 (maize) or 0.1 mM (pea and radish) solutions of FA or *p*-CA acids for 6 h. The roots of control seedlings were immersed in water. Differentiated age of seedlings of particular species resulted from the different dynamics of their growth. The various concentrations of applied phenolic acids depended on the sensitivity of particular plant species to their action. Phenolic acids were used at concentrations which inhibited the growth of seedling roots of examined species at a similar level. Separate experiments were conducted for each species. Each experiment was conducted in five replications.

Root samples to determine the syringaldazine peroxidase (syr-POX) activity were taken 1, 3 and 5 h after FA and *p*-CA treatment. At 24, 48 and 72 h the phenylalanine ammonia-lyase (PAL) activity was determined. After 96 h, the determination of lignin content and the measurement of growth indices of plant roots were done. Various times for estimation of particular parameters, i.e. the activity of syr-POX, PAL, lignin contents and growth indices, were adopted based on our earlier experience and preliminary analysis. These ranges of time were chosen, in which the biggest differences between the treated and control roots were found.

Syringaldazine POX assay

POX was extracted at 4°C using 0.05 M Tris-HCl buffer at pH 7.0 (5 ml of buffer per 0.5 g of roots). Samples were ground in a mortar and centrifuged at 3 000 × *g* for 10 min. Then supernatant containing the soluble cytoplasmic fraction was discarded and pellets were used for extraction of wall-bound enzymes (21). To remove the traces of contaminating cytoplasmic fraction, the pellet was treated with buffered 1% Triton X-100 and centrifuged, as above. The supernatant was discarded and the pellet was washed twice with 0.05 M Tris-HCl buffer and centrifuged at 10 000 × *g* for 10 min. The washed pellet was suspended in 2.5 ml of buffered 1 M NaCl, stirred for 1 h at 4°C and centrifuged at 10

000 × *g* for 20 min. The obtained supernatant was filtered using a Microcon centrifugal filter device (Ultracel YM-10) to remove NaCl ions. The supernatant was used as an extract of enzyme ionically bound to the cell wall. For isolation of covalently bound enzymes the pellet was washed twice with Tris-HCl buffer and used for digestion by Macerozyme (5 mg per trial) in 0.05 M phosphate buffer of pH 6.0 for 24 h at 22°C and centrifuged at 16 000 × *g* for 20 min. Syr-POX activity was measured spectrophotometrically at 530 nm using syringaldazine as a substrate (14). The activity was calculated using the absorbance molar coefficient of oxidized syringaldazine, i.e. – 27 $mM^{-1}cm^{-1}$ (23). Measurements were made in five replications.

PAL assay

PAL was extracted at 4°C using 0.1 M Tris-HCl buffer at pH 8.9 (3 ml of buffer for pea and radish and 4 ml for maize per 0.5 g of roots) containing 10 millimoles of mercaptoethanol and 50 mg of Polyclar AT. Samples were ground in a mortar, centrifuged at 12 000 × *g* for 20 min and filtered on Sephadex G 25 columns (0.7 cm x 6.5 cm). Enzyme activity was determined using a method (4) modified and described in detail in the paper (31). The activity was calculated using the absorbance molar coefficient of *trans*-cinnamic acid, i.e. – 17.4 mM^{-1} cm^{-1} (42). Measurements were made in five replications.

Protein content for expression of syr-POX and PAL activities was determined using Bradford reagent (3). Measurements were made in five replications.

Lignin content

Roots of maize, pea and radish were treated for 48 h with twice-changed ethanol, using 10 ml per 1 g of roots. Next the samples were dried in a desiccator for a week and ground in a mortar. Lignin was determined according to the procedure of (8) described in detail in the paper (31). Measurements were made in five replications.

Growth indices

The length and fresh weight of seedling roots were accepted as growth criteria. Biometric measurements were done 48 h after phenolic treatment in 5 replications. Results are means of 50 roots.

Statistical analysis

The results were subjected to analysis of variance, and significant differences were estimated by Duncan’s test at the level $P \leq 0.05$. Regression analysis was conducted to establish the correlation between the lignin content and the length of roots.

RESULTS

The influence FA and *p*-CA on syr-POX activity in the roots of the plants was tested at 1, 3 and 5 h of stress in the fraction ionically and covalently bound with the cell wall (Fig. 1). The ionically bound fraction was characterized by much higher activity of syr-POX compared to the covalently bound fraction in the roots of the examined plants. In the roots of maize a significant increase in the activity of the fraction of ionically bound analyzed enzyme was found under the influence of FA only in the first hour, and it was

27% compared to the control. However, treatment with *p*-CA at the same time increased syr-POX activity by 61% compared with the control. After 3 h of treatment with *p*-CA the activity was 28% higher and after 5 h 18% lower than in the control. Syr-POX activity in the covalently bound fraction remained at a high level at 1 and 3 h after treatment with FA and *p*-CA. At these times, in the roots of maize treated with FA syr-POX activity was 3-fold higher, while in those treated with *p*-CA it was 2.5-fold higher than in the control. After 5 h, the FA caused an increase in the enzyme activity by 74%, whereas in the case of *p*-CA there was no statistically significant difference compared with the control.

In pea roots a significant increase of syr-POX activity in the fraction ionically bound was observed only at 1 h after treatment with *p*-CA, and it was about 42% higher than in the control (Fig. 1). In other cases, there were no significant differences between the stress and control subjects. One-hour treatment of pea roots with FA and *p*-CA resulted in increased activity of syr-POX in the covalently bound fraction, respectively by 92% and 47% compared with the control. A particularly high (2.5-fold) increase in activity of the enzyme was found 3 h after treatment of roots with FA, while in the roots treated with *p*-CA it was 81% compared to the control. After 5 h, there was observed a significant decrease in enzyme activity, which was higher than the control only by 24% as the average for the two stress factors.

A significant increase in radish roots of syr-POX activity in the fraction ionically bound was found after 1 h of stress (Fig. 1). It was higher than the control in the roots treated with FA by 59% and in the roots treated with *p*-CA by 113%. After 3 h, there was found a decrease in the activity of the enzyme only in the variant treated with FA. Enzyme activity was 20% higher than in the control. In other variants no statistically significant differences compared to the control were found. Analyzing syr-POX activity in radish roots in the covalently bound fraction, an increase by 28% above the control was observed at 1 h after treatment with FA and *p*-CA. Compared to untreated roots, a significant increase in activity was demonstrated even after 3 h of stress, and it was 43% and 37% greater respectively, for FA and *p*-CA. After 5 h, only the treatment with *p*-CA increased enzyme activity, by 81% compared to the control.

After 24 h, the introduction of stress in the roots of maize resulted in the induction of PAL activity (Fig. 2). It was higher than that observed in the control roots treated with FA and *p*-CA, respectively 37% and 19%. After 48 and 72 h the activity of PAL generally decreased, but under the influence of FA it still remained higher than the control by 48%, while in the roots treated with *p*-CA it reached the level of the control.

Similarly, maize pea roots after 24 h also showed an increase in PAL activity (Fig. 2). In the roots treated with FA it was 9% higher than in the control and in roots treated with *p*-CA 52% higher. After 48 and 72 h of stress, the enzyme activity decreased in all variants, but it was still higher than that observed in the control – under the influence of FA by respectively 33% and 72%, while under the influence of *p*-CA by 88% and 33%.

Also in radish roots 24 h after treatment with FA and *p*-CA, there was a strong increase in the PAL activity (Fig. 2), which was higher by 67% and 37% compared to that observed in the control roots. At other times, there were no statistically significant differences between the stress-subjected and control roots.

Four days after the introduction of stress the levels of lignin in maize roots increased by 25% under the influence of FA and 46% under the influence of *p*-CA (Fig. 3).

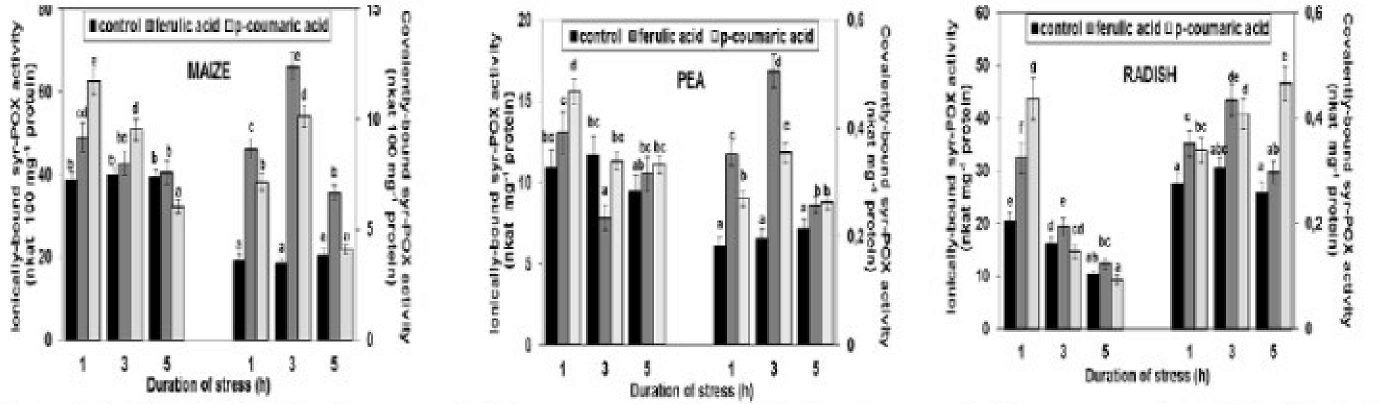

Figure 1. Activity of syr-POX in maize , pea and radish roots treated for 6 h with ferulic and *p*-coumaric acids at concentrations of 0.5 mM (maize) and 0.1 mM (pea and radish). Values marked the same letters do not differ significantly at $P \leq 0.05$.

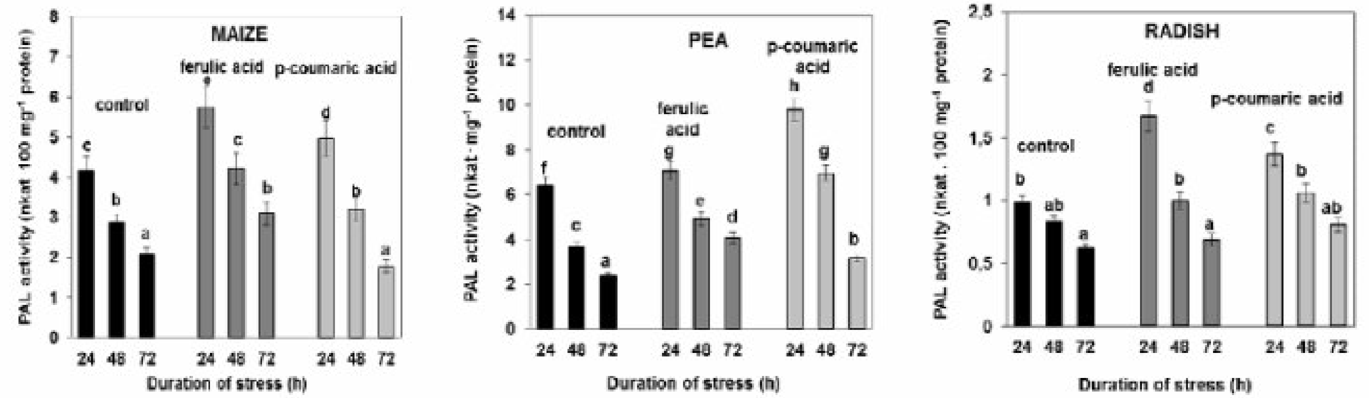

Figure 2. Activity of phenylalanine ammonia-lyase in maize, pea and radish roots treated for 6 h with ferulic and *p*-coumaric acids at concentrations of 0.5 mM (maize) and 0.1 mM (pea and radish). Values marked the same letters do not differ significantly at $P \leq 0.05$.

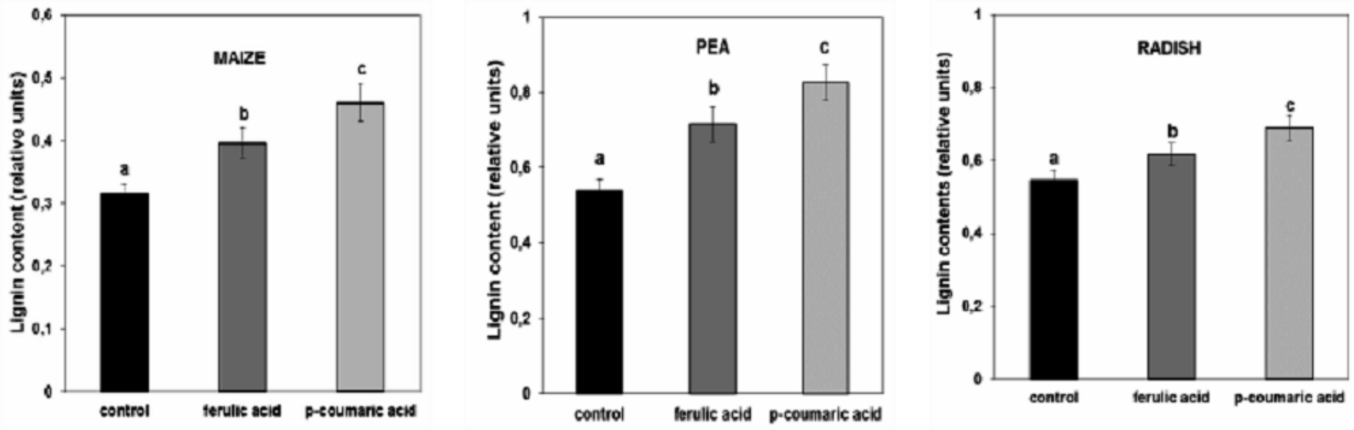

Figure 3. Lignin content in maize, pea and radish roots treated for 6 h with ferulic and *p*-coumaric acids at concentrations of 0.5 mM (maize) and 0.1 mM (pea and radish). Values marked the same letter do not differ significantly at P≤0.05.

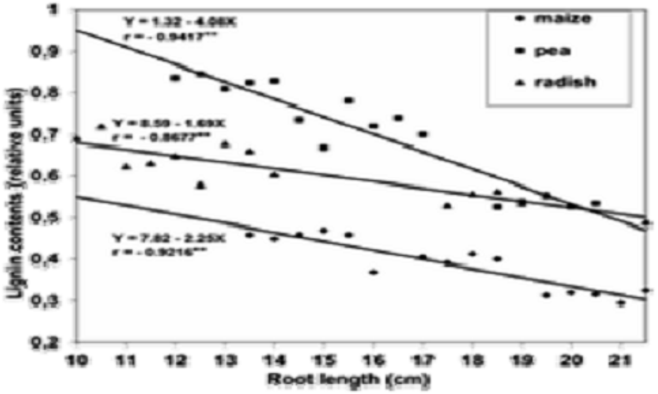

Figure 4. Correlations between the length of roots and lignin content in roots of maize, pea and radish treated ferulic and *p*-coumaric acids. ** regression coefficient at P≤0.01.

The action of FA and *p*-CA increased the level of lignin in pea roots respectively by 33% and 53% compared to the control. Treatment of radish roots with FA and *p*-CA increased the level of lignin by 12% and 25% respectively.

In the case of maize treated with FA, the root length was lower by 15%, and in the case of *p*-CA by 29% compared to the control (Table 1). Measurement of fresh weight of maize roots showed its reduction by approximately 17% and 25% with respect to the control. Also we evaluated the impact of FA and *p*-CA on the pea root growth based on measurements of their length and fresh weight in relation to the control. The inhibition of root growth under the influence of FA by 24% and under the influence of *p*-CA by 34% was demonstrated. We also observed an increase of root weight by 33% and 40%. The length of radish roots treated with FA and *p*-CA was 29% and 39% less than the roots of the control, and there was found a reduction in the fresh weight as compared to the control by the action of the two acids by 29% and 38% respectively. Finally, the analysis of regression between the length of the roots and the level of lignin showed a highly significant negative correlation (Fig. 4).

Table 1. The length and mass of seedling roots treated with ferulic and *p*-coumaric acids

Species	Treatment	Root mass/ seedling (g of FW)	Root length (cm)
Maize	Control	1.42 ±0.08 c	20.4 ±0.7 c
	0.5 mM ferulic acid	1.17 ±0.07 b	17.2 ±0.7 b
	0.5 mM *p*-coumaric acid	1.06 ±0.03 a	14.5 ±0.8 a
Pea	Control	2.84 ±0.14 c	19.1 ±0.6 c
	0.1 mM ferulic acid	1.90 ±0.16 b	14.2 ±0.6 b
	0.1 mM *p*-coumaric acid	1.61 ±0.05 a	12.5 ±0.7 a
Radish	Control	0.31 ±0.03 c	18.4 ±0.5 c
	0.1 mM ferulic acid	0.22 ±0.02 b	13.0 ±0.5 b
	0.1 mM *p*-coumaric acid	0.19 ±0.01 a	11.2 ± 0.5 a

Values in one column for one species marked the different letter differ significantly at $P \leq 0.05$.

DISCUSSION

Many authors showed that phenols are inhibitors of plant growth (1,6,9,22,26,37,42). Already in 1971 Whitmore (39) found that lignification is a process which leads to a reduction of cell wall plasticity and inhibition of cell elongation. Later, it was demonstrated that the inhibition of growth of rice coleoptiles by FA and *p*-CA was due to their inclusion in the structure of lignin and creation of bonds between lignin and saccharides of the cell wall (35,36).

Increased accumulation of lignin in the cell wall, and thus the premature termination of cell growth, may be associated with induction of the activity of syr-POX. It has been shown that syringaldazine, a non-physiological substrate, has a high affinity particularly for those POXs bound with cell walls and involved in lignification (4,12,15,18,20,24). Our previous studies showed that, in the first hour after entering the roots of cucumber, exogenous FA and *p*-CA induced activity of syr-POX covalently bound to the cell wall, and they may be substrates for the POXs (29).

Later on (after 24 h) in the roots of cucumber under FA and *p*-CA there was an increase of PAL activity and a slow increase of lignin (seen from 48-96 h), following which growth inhibition occurred (26). PAL may be induced by exogenous FA and *p*-CA; the consequence is the increased synthesis of lignin, which was found in the roots of cucumber and soybean (6,7,25,26,40). Results for pea, maize and radish obtained in these studies confirmed this relationship. The experiment demonstrated the effect of FA and *p*-CA, in the following sequence of events: increased activity of syr-POX, an increase in PAL activity, then increased intensity of lignin synthesis. Initiated effects of exogenous FA and *p*-CA – lignin deposition in the cell walls – observed in these species were negatively correlated with root growth.

PAL is the key enzyme of the phenylpropanoid pathway, which converts L-phenylalanine to trans-cinnamic acid. Derivatives of trans-cinnamic acid may be incorporated into the structure of polysaccharides and lignin (13). Our previous studies conducted on the roots of cucumber (31) and in studies of Lima *et al.* (22) conducted on the roots of soybean treated with FA and *p*-CA showed the presence of a relationship between increased PAL activity and the intensity of lignin synthesis. Introduction of the PAL inhibitor 2-amino-indan-2-phosphonic acid abrogated the effects of hydroxycinnamic acids observed in the variants without an inhibitor, i.e. increasing the activity of PAL enhanced synthesis of lignin and growth inhibition (22,31).

Analyzing the effect of derivatives of cinnamic and benzoic acids on growth of cucumber roots, we found (26) that under the influence of cinnamic acid derivatives (FA and *p*-CA) there was an increase in PAL activity and lignin content, which was accompanied by the inhibition of root growth. It was not observed under the influence of derivatives of benzoic acid. The differences between the responses of cucumber root to the derivatives of cinnamic acid and benzoic acid suggest that the inhibition of growth is the result of induction of the synthesis of lignin. Similar conclusions were reached (6) that growth reduction of soybean roots was the result of lignification caused by FA. The results obtained in the present study confirmed this relationship. Deposition of lignin initiated by FA and *p*-CA, which was found in the roots of pea~~s~~, maize and radish, was negatively correlated with the length of the roots. This means that the premature lignification of the cell wall which took place could lead to a reduction in root growth. The study showed that exogenous FA and *p*-CA can be oxidized by the syr-POX covalently bound to the cell wall. POXs' oxygen donor is hydrogen peroxide, which was generated in the first hour of treatment with FA and *p*-CA, when the induction of syr-POX occurred, as observed previously in the roots of pea, maize and radish in the same experimental system (10). The source of hydrogen peroxide (H_2O_2) could be catalyzed oxidation of polyamines by polyamine oxidase, under the influence of the induction of FA and *p*-CA as noted previously in the roots of cucumber (30). Though the changes in level of polyamines were not studied in this work, their oxidation could be a source of H_2O_2 for syr-POX. During oxidation of FA and *p*-CA, oxidative burst occurs, as evidenced by elevated levels of reactive oxygen species (ROS), i.e. $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$ found by us in the roots of cucumber (28) as well in the roots of maize, pea and radish (9). The lipid peroxidation and increasing permeability of membranes were the result of an increased level of $^{\cdot}OH$. The enzyme involved in lipid peroxidation is a lipoxygenase (LOX), which increased the activity of both the soluble and membrane-bound fractions found in roots of maize, pea and radish treated with FA and *p*-CA (11). Although the significance of reaction products catalyzed by LOX is not yet fully understood, probably they initiate a chain of reactions leading to the production of ethylene which we proved in experiments with cucumber (31). The resulting ethylene could therefore induce PAL activity. Increasing the activity of this enzyme leads to formation of lignin precursors which are transformed and incorporated into the structure of lignin. Finally the deposition of lignin in the cell wall contributes to the premature termination of cell growth.

In conclusion, premature termination of growth observed in the roots of maize, pea and radish under the influence of FA and *p*-CA was preceded by physiological changes, between which synchronization was demonstrated. Lignification induced by FA and *p*-CA action seems to be a widespread response of plants leading to growth inhibition.

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