ETHYLENE-DEPENDENT ACTIVITY OF PHENYLALANINE AMMONIA-LYASE AND LIGNIN FORMATION IN CUCUMBER ROOTS EXPOSED TO PHENOLIC ALLELOCHEMICALS

BARBARA POLITYCKA

Department of Plant Physiology, Agricultural University of Poznań
Wołyńska 35, 60-637 Poznań, Poland
E-mail: barpolit@jay.au.poznan.pl

(Received: June 30, 1998. Accepted: April 26, 1999)

ABSTRACT

Seedling roots of cucumber were subjected to allelochemical stress by treating them with 0.1 mM solutions of ferulic, p-coumaric, p-hydroxybenzoic and vanillic acids. In the experiment the influence of derivatives of cinnamic acid (ferulic and p-coumaric acids) on the activity of phenylalanine ammonia-lyase (E.C. 4.3.1.5.) (PAL), lignin content, and cucumber root growth was compared to that of benzoic acid (p-hydroxybenzoic and vanillic acids).

Roots treated with ferulic and p-coumaric acids were found to have an increased activity of PAL, a higher lignin content, and lower growth indices. No similar effect was recorded in the case of p-hydroxybenzoic and vanillic acids. The application of ethylene synthesis inhibitor, (aminooxy) acetic acid (AOA), cancelled out the effect of ferulic and p-coumaric acids on the activity of PAL. Lignin content in roots treated with ferulic acid remained on the control level when using the inhibitor, while that in roots treated with p-coumaric acid increased.

The obtained results suggest that an increase in the activity level of PAL induced by the action of ferulic and p-coumaric acids, depended on ethylene synthesis. A consequence of changes in the studied enzyme activity was an increased lignin content, and in the case of p-coumaric acid also a direct participation of that compound in their synthesis. A consequence of the above changes was inhibition of the growth of cucumber roots treated with ferulic and p-coumaric acids.

KEY WORDS: allelochemical stress, *Cucumis sativus* L., growth, lignin, phenols, phenylalanine ammonia-lyase, roots.

INTRODUCTION

Phenolic compounds, derivatives of cinnamic and benzoic acids, are commonly known allelopathic substances affecting a number of physiological processes. One of their effects is inhibition of plant growth (Einhellig 1995). Derivatives of cinnamic acid were also found to be active in this respect even at lower concentrations than derivatives of benzoic acid (Macias 1995).

In our previous paper it was shown that ferulic and p-coumaric acids caused an increase in phenylalanine ammonia-lyase (PAL) activity in cucumber roots and inhibited their growth. No similar response to the action of p-hydroxybenzoic and vanillic acids was found (Politycka 1998).

An increase in the activity of PAL may be induced by a number of factors, both external and internal. One of them is ethylene (Hyodo and Yang 1971; Hughes and Dickerson 1989). Its level increase in plants was found to be a result of various stress conditions, such as wounding, a pathogen infection, cadmium, ozone, heat shock, flooding or drought (Yang and Hoffman 1984; Morgan and Drew 1997). Hence a supposition that PAL activity observed in cucumber roots subjected to allelochemical stress may also be associated with ethylene production.

PAL is a key enzyme of the phenylpropanoid pathway leading to a synthesis of many derivatives of cinnamic acid which can be added to the structures of wall polysaccharides or lignins, thus bringing about wall stiffness and cell growth limitation (Jones 1984; Tan et al. 1992; Iiyama et al. 1994; Sasaki et al. 1996). Therefore, one of the reasons of a varying response of cucumber growth to derivatives of cinnamic and benzoic acids reported in our previous paper (Politycka, 1998) could be the differences in degree of lignification.

The purpose of the present study was to find out whether the induction of PAL activity observed in cucumber roots subjected to allelochemical stress depends on ethylene, and whether lignification is its consequence.

MATERIALS AND METHODS

Plant material

Cucumber (*Cucumis sativus* L.) seedlings of the cv. Wisconsin grew for 7 days in glass chambers on plates covered

with water-soaked filter paper, at the temperature of 27°C in luminescent light (165 W m^{-2} Philips lamp), with a 14-hour day and a 10-hour night.

Description of experiment

In the first part of the experiment, 7-day-old seedling roots were treated with 0.1 mM solutions of ferulic, p-coumaric, p-hydroxybenzoic and vanillic acids by moisturizing of filter paper with roots for 4 hours. Then the phenolic acid solutions were removed and replaced by water. Seedlings not treated with phenols were the control. Lignin content in the seedling roots was determined 24, 48, 72 and 96 hours after phenolic treatment, while the dry matter and length of the roots were determined 24, 72 and 120 hours after the treatment.

In the other part of the experiment, during a 4-hour treatment of cucumber seedling roots with the above-mentioned phenolic compounds, the inhibitor of ethylene synthesis, AOA, was introduced in the amount of 25 mg per liter. Seedlings growing without the inhibitor were the control. The influence of the inhibitor on the activity of PAL was estimated 24 hours after the introduction of the phenols, and 96 hours after lignin content was determined in the roots.

Extraction and quantification of phenylalanine ammonia-lyase

PAL was extracted at 4°C using 0.1 M Tris-HCl buffer at pH 8.9 (2.5 ml per 0.5 mg of tissue) containing 10 mmol 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). PAL activity was determined using a modified method of Cahill and McComb (1992). The incubation mixture contained 80 mmol of borate buffer, pH 8.9, 30 mmol of L-phenylalanine and 0.5 ml of enzymatic extract in a 2.5 ml volume. The reaction proceeded on for 24 hours at 30°C and was interrupted by the addition of an equal volume of 2N HCl to the incubation mixture. The product of the reaction – *trans*-cinnamic acid was determined at 290 nm (the Perkin-Elmer Lambda 11 spectrophotometer). Protein content was determined after Bradford (1976).

Determination of lignin content

Lignins were determined according to the procedure of Doster and Bostock (1988). Cucumber roots were treated for 48 hours with twice-changed methanol, using 10 ml of methanol per 1 g of tissue. Next the roots were dried in a desiccator and ground in a mortar. 20 mg of dry matter placed in teflon vials was added with 5 ml of 2N HCl and 0.5 ml of thioglycolic acid. The vials were tightly closed and heated at 95°C for 4 hours. The samples were centrifuged at 1000 g for 20 min. The precipitate was washed with de-ionised water and extracted with 5 ml of 0.5 N NaOH for 18 hours at a room temperature. The NaOH extract was separated by centrifugation. The precipitate was washed with 4 ml of de-ionised water and centrifuged, and the obtained supernatant was added to the NaOH extract. The extract was then acidified with 1 ml of concentrated HCl, placed at the temperature of 5°C for 4 hours and centrifuged. The obtained precipitate was dissolved in 10 ml of 0.5 N NaOH. Absorbance was measured at 280 nm using a Perkin-Elmer spectrophotometer (Lambda 11).

Statistical evaluation of results

Variance analysis was performed to determine the effect of AOA on the PAL activity and lignin synthesis. Regression analysis was used to determine the influence of phenolic compounds on the cucumber root growth and lignin synthesis.

RESULTS

Changes in lignin content and growth dynamics

In the first part of the experiment, changes in the lignin content of cucumber roots were analysed during four successive days after treating roots with phenolic compounds (Fig. 1). In all combinations the lignin level increased gradually. Changes occured most rapidly in the roots treated with p-coumaric acid. Already 47 hours after the introduction of phenols it differed significantly from the level observed in the other combinations. At the 96th hour a significant difference in relation to the control was found in roots treated with both p-coumaric and ferulic acids. The lignin content was 35% higher than that of the control in the case of ferulic acid and 40% higher in the case of p-coumaric acid. On the other hand, no significant differences were found between lignin content in the roots treated with p-hydroxybenzoic and vanillic acids and that of the control roots.

Treatment with phenolic compounds had a inhibitory effect on the root growth only when applying ferulic and p-coumaric acids (Fig. 2). After 120 hours, both the length and dry matter of the roots treated with these two acids were on average 20% smaller than those of the control. p-Hydroxybenzoic and vanillic acids had no influence on the growth of cucumber roots.

The effect of ethylene inhibitor on PAL activity and lignin content

In the second part of the experiment, the effect of the ethylene synthesis inhibitor – AOA on the activity of PAL 24 hours after the introduction of phenols was studied (Fig. 3). The activity of PAL in the roots treated with ferulic and p-coumaric acids without the addition of AOA increased in comparison to the control: by 31% for ferulic acid and 42% for p-coumaric acid. However p-hydroxybenzoic and vanillic acids were not found to have significant influence on the studied enzyme activity. The application of the inhibitor of ethylene synthesis to the roots treated with ferulic and p-coumaric acids caused a decrease of PAL activity to the level of the control. Other combinations were also observed to have a decrease in the studied enzyme activity.

The effect of AOA on the level of lignins measured 96 hours after the introduction of phenols (Fig. 4) was similar to that observed in the case of PAL activity. The use of the inhibitor caused a drop in the lignin content in all combinations. In the roots treated with ferulic, p-hydroxybenzoic and vanillic acids the lignin content was similar to that of the control, while in the roots treated with p-coumaric acid the lignin content exceeded that of the control by 30%.

DISCUSSION

In a number of articles it has been found that derivatives of cinnamic acid are generally stronger growth inhibitors than those of benzoic acid (Macias 1995). However, the physiological basis of this phenomenon has never been fully explained. Gerig and Blum (1991) found ferulic and p-coumaric acids to have a stronger inhibitory effect on the growth of cucumber leaves than p-hydroxybenzoic and vanillic acids. Zhu and Mallik (1994), in turn, found this observation to be valid

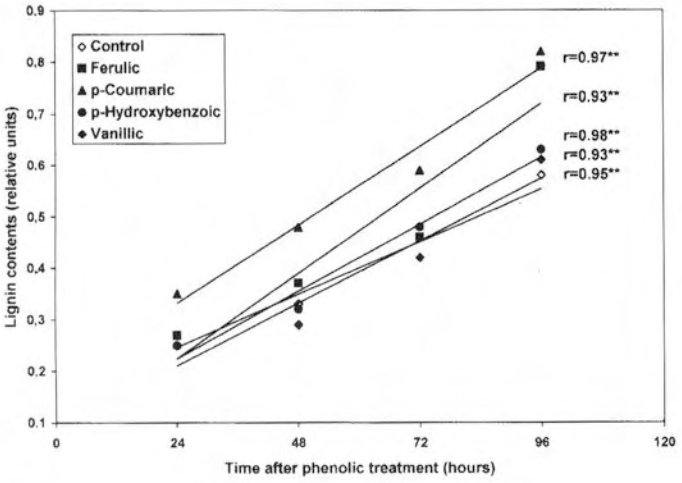

Fig. 1. Changes in the lignin content in the roots treated with ferulic, p-coumaric, p-hydroxybenzoic and vanillic acids, expressed in absorbance units obtained from 20 mg of dry matter extracted with methanol in 10 ml of 0.5 N NaOH. Regression coefficient: **statistically significant at P<0.01, *at P<0.05.

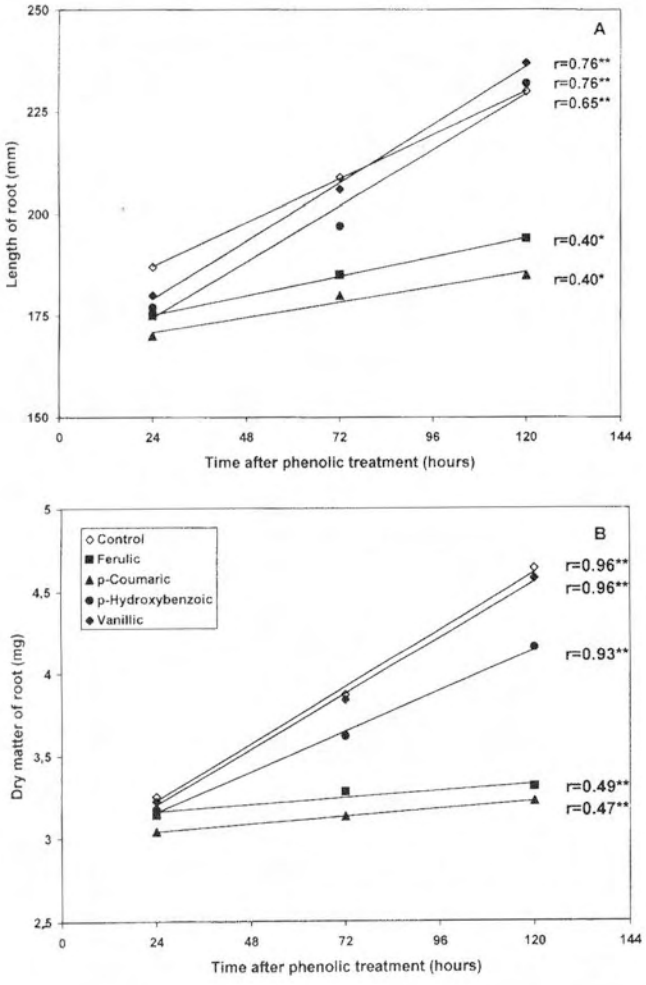

Fig. 2. Growth of cucumber roots treated with ferulic, p-coumaric acids, p-hydroxybenzoic and vanillic acids.
A. Root length.
B. Dry matter of roots. Regression coefficient: **statistically significant at P<0.01, *at P<0.05.

for the roots of pine seedlings. The results of our studies have confirmed the above findings from the literature, since cucumber roots treated with ferulic and p-coumaric acids were characterized by a limited growth, which was not observed in the case of p-hydroxybenzoic and vanillic acids (Fig. 2).

The present paper is a continuation of the earlier studies which showed that PAL activity increased in cucumber roots under the influence of ferulic and p-coumaric acids, while changes observed in the level of soluble phenols showed that they were subject to further transformations (Politycka 1997, 1998). The product of the PAL-catalysed reaction – *trans*-cinnamic acid is known to be a precursor of numerous hydroxylated and methylated derivatives (Jones 1984). These compounds can form ester bonds with polysaccharides of cell walls, can be built in lignin structures, or can create combinations of lignins with wall polysaccharides (Tan et al. 1992; Iiyama et al. 1994).

The results obtained in the present work showed a more intensified lignification in cucumber roots treated with ferulic and p-coumaric acids. A significant difference was observed in the lignin level in comparison to the control 48 hours after treating the roots with p-coumaric acid and 96 hours after treating them with ferulic acid (Fig. 1). A higher PAL activity in the roots in result of treating them with these acids was found earlier – already after 24 hours (Fig. 3, Politycka 1998).These results indicate that the intensification of lignin synthesis was largely a consequence of increased PAL activity. Similar relationships were observed by Cahill and McComb (1992) in roots of eucalyptus seedlings as a response to a stress caused by a pathogen infection. These authors found the highest PAL level 24-72 hours after the stress introduction, while the accumulation of lignins took place during the next 48 hours.

Simultaneously with changes in PAL activity and lignin content in the cucumber roots subjected to the action of ferulic and p-coumaric acids it came to growth inhibition (Fig. 2). Taking into account the fact that no similar changes were found in the studied enzyme activity and in the degree of lignification in roots treated with p-hydroxybenzoic and vanillic acids, and that growth indices of these roots did not differ from those of the control, it may be inferred that growth inhibition observed as a result of the action of ferulic and p-coumaric acids was caused by accelerated lignification. It has long been known that lignification is a limiting factor of growth, since it leads to disappearance of wall elasticity and thus inhibits cell enlargement (Whitmore 1971).

When comparing lignin content and growth indices in roots treated with ferulic and p-coumaric acids one may be surprised that a more intensified lignification induced by p-coumaric acid was not reflected in a larger difference in growth. It can be explained by the fact that the effect of phenolic compounds on growth is a more complex process because they can participate in the metabolism of auxins, change membrane permeability, affect the intensity of respiration and oxidative phosphorylation or protein synthesis (Vaughan and Ord 1991).

AOA is a well known inhibitor of ethylene synthesis in plants (Yang and Hoffman 1984). The results obtained in our experiment provided additional information on the way the derivatives of cinnamic acid affected cucumber roots. They showed that ethylene was involved in the induction of PAL activity observed as a result of treatment with ferulic and p-coumaric acids. This is proved by PAL activity decrease to the level of the control when the inhibitor was used (Fig. 3). An ethylene-induced increase in PAL activity has been re-

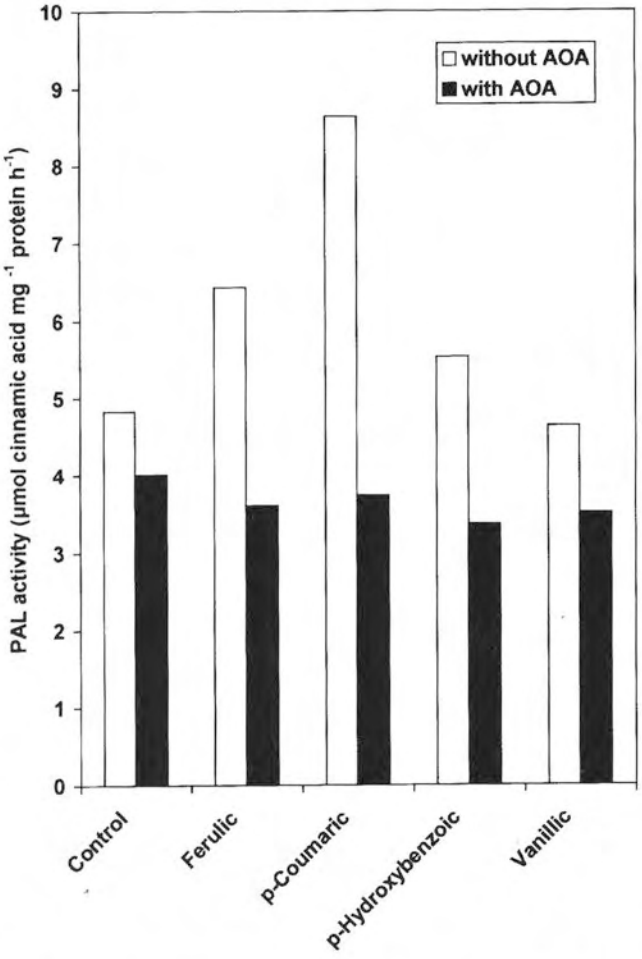

Fig. 3. The effect of AOA on the activity of PAL in cucumber roots 24 hours after treatment with ferulic, p-coumaric, p-hydroxybenzoic and vanillic acids. Values for the AOA and phenolic treatment differ significantly at $P<0.01$.

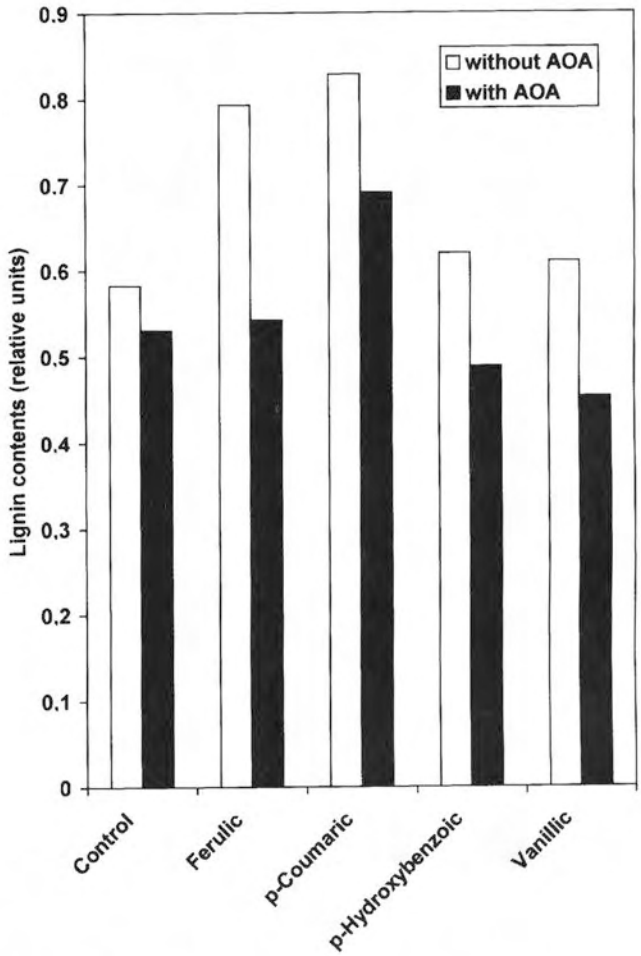

Fig. 4. The effect of AOA on the level of lignins in cucumber roots 96 hours after treatment with ferulic, p-coumaric, p-hydroxybenzoic and vanillic acids, expressed in absorbance units obtained from 20 mg of dry matter extracted with methanol in 10 ml of 0.5 N NaOH. Values for the AOA treatment differ significantly at $P<0.01$, they do not differ for the phenols.

ported by many authors (Hyodo and Yang 1971; Hughes and Dickerson 1989; Cvikrová et al. 1994). Ethylene can be synthesized in plants as a response to stress (Yang and Hoffman 1984; Morgan and Drew 1997). It seems that in cucumber roots treated with ferulic and p-coumaric acids it might be associated with free-radical reactions involving the oxidation of phenols by peroxidase and contributing to peroxidation of membrane lipids, as suggested by results of the previous paper (Politycka 1996). The ability of free-radical reactions to induce ethylene synthesis is supported by results obtained by Irigoyen and his co-workers (1992). When examining alfalfa leaves subjected to drought stress, they found that ethylene production increased with peroxidation of plasma membrane lipids and the level of hydrogen peroxides.

The application of ethylene synthesis inhibitor also affected the level of lignins (Fig. 4) and was largely a reflection of the dependence found for PAL. On the other hand, this element of the experiment has supplied a piece of new information, namely that p-coumaric acid can directly participate in lignification. This suggestion is supported by increased lignin synthesis in roots treated with p-coumaric acid (Fig. 4), despite a decline in PAL activity under the influence of the inhibitor (Fig. 3), and an early occurrence of a higher level of lignins in these roots (Fig. 1). This is also indicated by results of Tan et al. (1992), who found a significant lignin increase and incorporation of p-coumaric acid into the walls of rice seedling coleoptiles as a result of treatment with this acid.

In the present work no significant influence of p-hydroxybenzoic and vanillic on PAL activity and the lignin content, or on the growth of cucumber roots has been found. It, however, is known that these compounds may be growth inhibitors, although in higher concentrations (Macias 1995). But it is unknown, whether the inhibition of cucumber root growth, as it could be caused by these phenols, was preceded by changes similar to those found for derivatives of cinnamic acid.

ACKNOWLEDGEMENTS

The author is grateful to Mr Benedykt Matuszak for technical assistance.

LITERATURE CITED

BRADFORD M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

CAHILL D.M., MCCOMB J.A. 1992. A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophthora cinnamoni*. Physiol. Molec. Plant Pathol. 40: 315-332.

CVIKROVÁ M., HRUBCOVÁ M., VÁGNER M., MACHÁCKOVÁ I., EDER J. 1994. Phenolic acids and peroxidase activity in alfalfa (*Medicago sativa*) embryogenic cultures after ethephon treatment. Physiol. Plant. 91: 226-233.

DOSTER M.A., BOSTOCK R.M. 1988. Quantification of lignin formation in almond bark in response to wounding and infection by *Phytophthora* species. Phytopathology 78:473-477.

EINHELLIG A.F. 1995. Mechanisms of action of allelochemicals in allelopathy. In: Allelopathy: Organisms, Processes and Applications. Inderjit, K.M.M. Dakshimini and F.A. Einhellig (eds), ACS, Symposium Series 582, Washington DC. pp. 96-117.

GERIG T.M., BLUM U. 1991. Effects of mixtures of four phenolic acids on leaf area expansion of cucumber seedlings grown in Portsmouth B_1 soil material. J. Chem. Ecol. 17: 29-40.

HUGHES R.K., DICKERSON A.G. 1989. The effect of ethylene on phenylalanine ammonia-lyase (PAL) induction by fungal elicitor in *Phaseolus vulgaris*. Physiol. Molec. Plant Pathol. 34: 361-378.

HYODO H., YANG S.F. 1971. Ethylene-enhanced synthesis of phenylalanine ammonia-lyase in pea seedlings. Plant Physiol. 47: 765--770.

IIYAMA K., BACH-TUYET LAM T., STONE B.A. 1994. Covalent cross-links in the cell wall. Plant Physiol. 104: 315-320.

IRIGOYEN J., EMERICH D.W., SANCHEZ-DIAZ M. 1992. Alfalfa leaf senescence induced by drought stress: photosynthesis, hydrogen peroxide metabolism, lipid peroxidation and ethylene evolution. Physiol. Plant. 84: 67-72.

JONES D.H. 1984. Phenylalanine ammonia-lyase: Regulation of its induction, and its role in plant development. Phytochemistry 23: 1349--1359.

MACIAS F.A. 1995. Allelopathy in the search for natural herbicide models. In: Allelopathy: Organisms, Processes and Applications. Inderjit, K.M.M. Dakshini and F.A. Einhellig (eds), ACS Symposium Series 582, Washington DC. pp. 310-330.

MORGAN P.W., DREW M.C. 1997. Ethylene and plant responses to stress. Physiol. Plant. 100: 620-630.

POLITYCKA B. 1996. Peroxidase activity and lipid peroxidation in roots of cucumber seedlings influenced by derivatives of cinnamic and benzoic acids. Acta Physiol. Plant. 18: 365-370.

POLITYCKA B. 1997. Free and glucosylated phenolics, phenol b-glucosyltransferase activity and membrane permeability in cucumber roots affected by derivatives of cinnamic and benzoic acids. Acta Physiol. Plant. 19: 311-317.

POLITYCKA B. 1998. Phenolics and the activities of phenylalanine ammonia-lyase, phenol-β-glucosyltransferase and β-glucosidase in cucumber roots as affected by phenolic allelochemicals. Acta Physiol. Plant. 20: 405-410.

SASAKI M., YAMAMOTO Y, MATSUMOTO H. 1996. Lignin deposition induced by aluminium in wheat (*Triticum aestivum*) roots. Physiol. Plant. 96: 193-198.

TAN K.S., HOSON T., MASUDA Y. 1992. Effect of ferulic and p-coumaric acids on *Oryza* coleoptile growth and the mechanical properties of cell walls. J. Plant Physiol. 140: 460-465.

VAUGHAN D., ORD B.G. 1991. Extraction of potential allelochemicals and their effects on root morphology and nutrient contents. In: Plant Root Growth. An Ecological Perspective. D. Atkinson. Blackwell Scientific Publications, Oxford. pp. 399-421.

WHITMORE F. W. 1971. Lignin formation in wheat coleptile cell walls, a possible limitation of cell growth. Plant Physiol. 48: 596-602.

YANG S.F., HOFFMAN N.E. 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35: 155-189.

ZHU H., MALLIK A.U. 1994. Interactions between *Kalmia* and black spruce: isolation and identification of allelopathic compounds. J. Chem. Ecol. 20: 407-421.

ETYLENO-ZALEŻNA AKTYWNOŚĆ AMONIAKO-LIAZY FENYLOALANINOWEJ ORAZ SYNTEZA LIGNIN W KORZENIACH OGÓRKA PODDANYCH DZIAŁANIU FENOLOWYCH SUBSTANCJI ALLELOCHEMICZNYCH

STRESZCZENIE

Korzenie siewek ogórka poddano stresowi allelochemicznemu przez potraktowanie 0,1 mM roztworami kwasu ferulowego, p-kumarowego, p-hydroksybenzoesowego i wanilinowego. W doświadczeniu porównywano wpływ pochodnych kwasu cynamonowego (kwas ferulowy i p-kumarowy) i pochodnych kwasu benzoesowego (kwas p-hydroksybenzoesowy i wanilinowy) na aktywność amoniakoliazy fenylalaninowej (E.C. 4.3.1.5.) (PAL), zawartość lignin i wzrost korzeni ogórka.

W korzeniach traktowanych kwasem ferulowym i p-kumarowym obserwowano podwyższoną aktywność PAL, zwiększenie poziomu lignin oraz obniżone wartości wskaźników wzrostu. Nie stwierdzono natomiast podobnego efektu w przypadku działania kwasu p-hydroksybenzoesowego i wanilinowego. Zastosowanie inhibitora syntezy etylenu, kwasu (aminooksy)octowego (AOA), znosiło efekt wywierany przez kwas ferulowy i p-kumarowy na aktywność PAL. Zawartość lignin w korzeniach traktowanych kwasem ferulowym utrzymywała się przy zastosowaniu inhibitora na poziomie kontroli, natomiast w korzeniach traktowanych kwasem p-kumarowym pozostała na podwyższonym poziomie.

Uzyskane wyniki sugerują, że wzrost aktywności PAL wywołany działaniem kwasu ferulowego i p-kumarowego, był zależny od syntezy etylenu. Konsekwencją zmian aktywności badanego enzymu było zwiększenie poziomu lignin, a w przypadku zastosowania kwasu p-kumarowego również bezpośredni udział tego związku w ich syntezie. Następstwem wyżej opisanych zmian było hamowanie wzrostu korzeni ogórka traktowanych kwasem ferulowym i p-kumarowym.

SŁOWA KLUCZOWE: stres allelochemiczny, *Cucumis sativus* L., wzrost, ligniny, związki fenolowe, amoniako-liaza fenyloalaninowa, korzenie.