ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 57, nr 1: 177-186
1988

Phenolic compounds and phenylalanine ammonia lyase activity in two soybean (*Glycine max* L. cv. Mandarin) cell lines that differ in their ploidy levels

KHALEQUZ ZAMAN

Institute of Molecular Biology, Jagiellonian University, Mickiewicza 3, 31-120 Kraków, Poland

(Received: August 3, 1987. Revision accepted: September 22, 1987)

Abstract

Through this preliminary experiment examining different color expressions in two soybean cell lines, SB_1 and SB_3 several important features were observed. SB_3 cells, which produce much more dark brown pigments in both suspension and callus cultures, had higher total phenolics and flavonoid contents as well as higher L-phenylalanine ammonia lyase activity.

Key words: phenolic compound, phenylalanine ammonia lyase, soybean, suspension and callus cultures

INTRODUCTION

Previous studies carried out by Jain and Shargool (1987) on two soybean cell lines, SB_1 and SB_3, demonstrated that they differ from each other in several ways. SB_3 cells grow much slower than SB_1 cells in both callus and suspension cultures. Karyotypic analysis revealed that SB_3 line is hexaploid whereas the SB_1 line is tetraploid (Jain and Shargool 1987, Shargool and Jain 1984, 1986). The two cell lines differ in the fact that SB_3 cells produce a large quantity of glutamate dehydrogenase activity during growth on minus nitrate medium, which is not seen in SB_1 cells. The most visible difference between SB_1 and SB_3 cells is observed in their color expression. SB_3 cells produce much more dark brown pigments in both cultures and the production of these pigments starts much earlier in the growth cycle.

Phenolic compounds are one of the important secondary metabolites of plants which are involved in color expression (Harborne 1973a, Goodwin and Mercer 1983). Among the plant phenolic compounds, flavonoids form the largest group and main source of pigmentations (Goodwin 1965, Harborne 1973a).

L-phenylalanine ammonia lyase (PAL) is a key enzyme in the biosynthesis of phenolic compounds. It catalyzes the nonoxidative deamination of L-phenylalanine to trans-cinnamic acid which is precursor for synthesis of phenolic compounds.

It is known that PAL is the rate-limiting enzyme for synthesis of phenolic compounds in tissue culture (Goodwin and Mercer 1983). PAL activity is higher in the SB_3 cell lines.

The purpose of this experiment was to examine the levels of phenolic compounds and the PAL activity in SB_1 and SB_3 cells, which differ in their ploidy levels.

MATERIAL AND METHODS

Two lines of soybean cells referred to in this report as SB_1 and SB_3 growing in suspension and callus cultures were kindly provided by Dr. P. Shargool from Saskatechwan University, Saskatoon, Canada.

SB_1 cells attain their maximum growth in 4 days while SB_3 cells take 6 days to reach that stage (Shargool and Jain 1984, 1986). In this experiment, soybean cells were used after they reached maximum growth in both cultures.

EXTRACTION AND ESTIMATION OF TOTAL PHENOLICS IN SB_1 AND SB_3 CELLS IN SUSPENSION AND CALLUS CULTURES

Total phenolics were extracted following the method of Harborne (1973a). Soybean cells after reaching maximum growth in suspension and callus cultures were collected by filtration through miracloth. Five grams of cells were dissolved with 20 cm^3 of 2M HCl and boiled for 60 min. The extract was then filtered through cotton wool and the volume was made up to 20 cm^3 after cooling.

The total phenolic contents in the extract was estimated according to the method of Jennings (1981). One cm^3 of extract was treated with 1 cm^3 of 1N Folin-Ciocalteu reagent and then mixed with 5 cm^3 of alkaline reagent (2% sodium carbonate in 0.1N sodium hydroxide solution) and the solution was mixed well. After 30 min. the absorbance was measured at 760 nm. The same method was also used for callus cultures.

EXTRACTION AND IDENTIFICATION OF FLAVONOIDS IN SB_1 AND SB_3 CELLS WITH 95% ETHANOL

Five grams of cells were dissolved with 10 cm^3 of 95% ethanol and heated under reflux for 10 min. The extract was filtered and centrifuged at $12\,000 \times g$ for 5 min. The supernatant was subjected to spectrophotometric analysis by measuring the absorbance at wavelengths ranging from 250 nm to 610 nm. The same method also used for callus cultured.

TWO DIMENSIONAL THIN LAYER CHROMATOGRAPHY ON SILICA GEL PLATE FOR SB_1 AND SB_3 CELL LINES IN SUSPENSION AND CALLUS CULTURES

Five grams of cells were mixed with 10 cm^3 of 95% ethanol and heated under reflux for 10 min. The extract was filtered and centrifuged at 12000 × g for 10 min. The supernatant was concentrated to dryness. The concentrated supernatant was subjected to two dimensional thin layer chromatography on silica gel plates. The solvent used for developing was butanol:acetic acid:water (1:1:5) and aqueous acetic acid.

After development the spots were located under UV light with additional fuming of the chromatogram with ammonia vapors. Each group of spots was removed from the plate and the compounds were extracted by suspending the gel in 3 cm^3 of water. The suspension was spectrophometric analysis by measuring the absorbance at wavelengths ranging from 250 nm to 610 nm.

MEASUREMENT OF L-PHENYLALANINE AMMONIA LYASE ACTIVITY

Acetone powder was prepared following the method of Jain and Ullah (1979). Both SB_1 and SB_3 cells after reaching the maximum growth in suspension cultures were collected by filtration through miracloth. Forty grams of cells were homogenized in 150 cm^3 of chilled acetone for 2 min. by using a virtis homogenizer at speed 70. The homogenate was filtered through filter paper and the residue was washed three times with 30 cm^3 of cold acetone. The residue was dried between several layers of filter paper and finally in a vacuum dessicator until all the acetone was evaporated off. The dried powder was stored at 4°C in a tightly sealed container.

ENZYME ASSAY

PAL activity of the acetone powder was determined by the method of Harborne (1973b). The reaction mixture contained 0.2g of acetone powder and 4 cm^3 of 0.1% L-phenylalanine solution in 0.1M borate buffer at pH 8.8. The blank and reaction mixtures were incubated at 37°C for 2 hours, after which the reaction was arrested with 0.5 cm^3 of 2M HCl, 7 cm^3 of water was added to the blank and the reaction acidified by using HCl to pH 5 and the reaction product was extrated three times with 5 cm^3 of ether. The ether extract was evaporated to dryness using a vacuum evaporator. The dried residue was dissolved in 3 cm^3 of ethanol. The optical density was measured at 273 nm with an ethanol blank.

The concentration of cinnamic acid was calculated from its molar extinction coefficient of 20.400. One unit of specific activity of PAL is expressed as formation of 1 μmole transcinnamic acid per minute per mg protein of acetone powder at 37°C.

PROTEIN DETERMINATION

The method used for the estimation of protein in acetone powder was similar to that of Bradford (1976). Acetone powder, 0.2g was dissolved in 0.15M NaCl and the volume made up to 10 cm^3, 0.1 cm^3 of this solution was mixed with 5 cm^3 of protein reagent. After 15 min. the absorbance was measured at 595 nm and the amount of protein was determined from a standard curve by using the same method with bovine serum albumin as a standard.

RESULTS

The level of total phenolic compounds in SB_1 and SB_3 cells is represented in Table 1. A comparison of the two cell lines indicates that the level of phenolic compounds in SB_3 cells was 1.4 times higher in suspension culture and 1.8 times higher in callus cultures than in SB_1 cell lines. Flavonoid extracts of SB_1 and SB_3 cells were scanned by measuring the absorbance at wavelengths ranging from 250 nm to 610 nm. The flavonoids were indentified on the basis of comparison with standard substances, characteristic colors and R_f coefficients (Harborne 1959, 1973a).

Table 1

Level of total phenolic compounds in SB_1 and SB_3 cell lines[a]

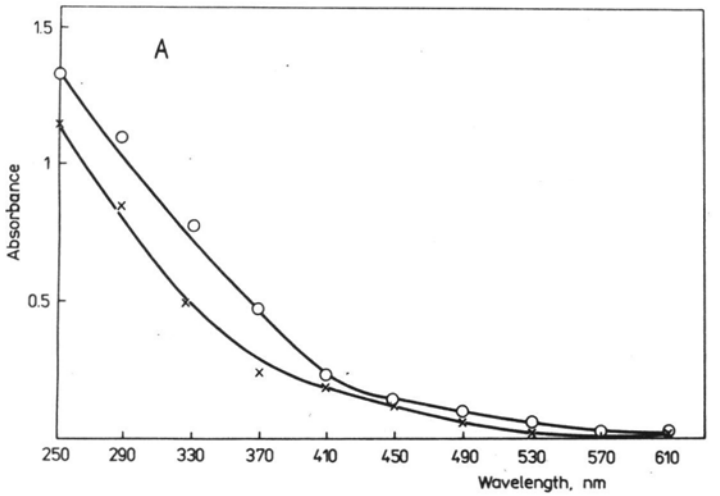
Cell lines	Optical density at 760 nm[b]	
	suspension culture	callus cultures
SB_1	0.189	0.381
SB_3	0.278	0.690

[a] SB_1 and SB_3 cells were 28-days-old in suspension culture and 65-days-old in callus culture.

[b] The levels of total phenolic compounds in SB_1 and SB_3 cells are expressed in terms of optical density at 760 nm after developing color with the Folin-Ciocalteu reagent.

Both SB_1 and SB_3 cells showed a maximum absorption at 250 nm (Harborne 1973a) and the absorbance decreased with increased wavelengths (Figs. 1A, B). This results indicates that isoflavone, flavone, biflavonyl which show absorption maximums between 250 nm and 270 nm were the most abundant classes of flavonoids in both cells (Jain and Shargool 1987). Flavanone, flavan-3-ol and flavanonol which have absorption maximums at wavelengths between 270 nm and 310 nm (Harborne 1973a) were the second in abundance.

Other classes of flavonoids such as chalcone and flavonal, absorption maximums 350 nm to 390 nm (Harborne 1973a), aurones absorption maximums of 390 nm to 430 nm (Harborne 1973a), and anthocyanine with an absorption maximums of 475 nm to 560 nm (Harborne 1973a) were



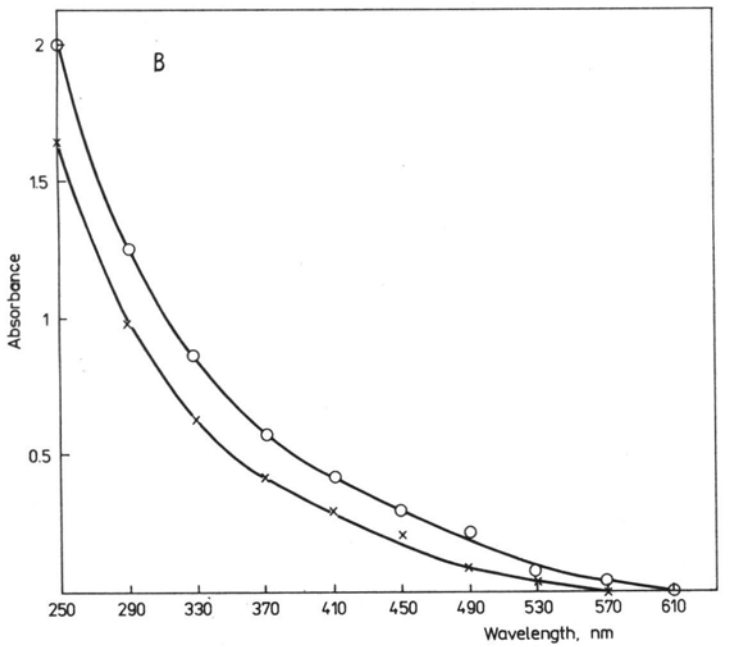


Fig. 1. Measurement of absorbance at wavelenghts ranging from 250 nm to 610 nm for 95% ethanol extract of flavonoids from SB_1 and SB_3 cell lines. A – suspension culture, B – callus culture, O – SB_3 cell line, X – SB_1 cell line

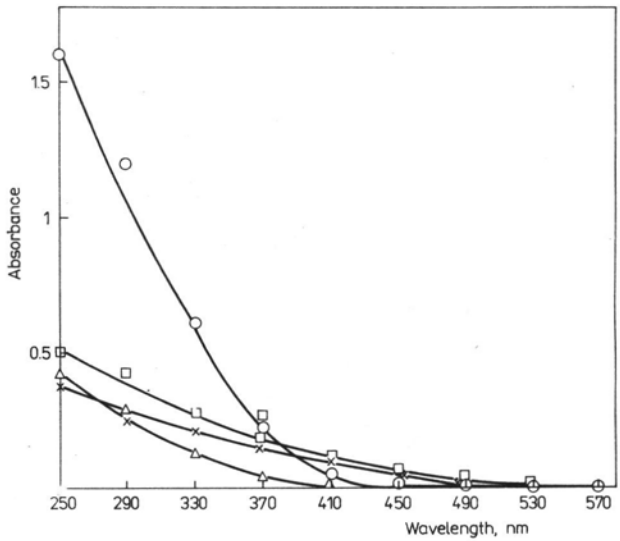


Fig. 2. Measurement of absorbance at wavelengths ranging from 250 nm to 610 nm for separated spots on a two dimensional chromatogram of 95% ethanol extract of flavonoids from SB_1 cell line in suspension culture: X———X – spot 1, Δ———Δ – spot 2, □———□ – spot 3, O———O – spot 4

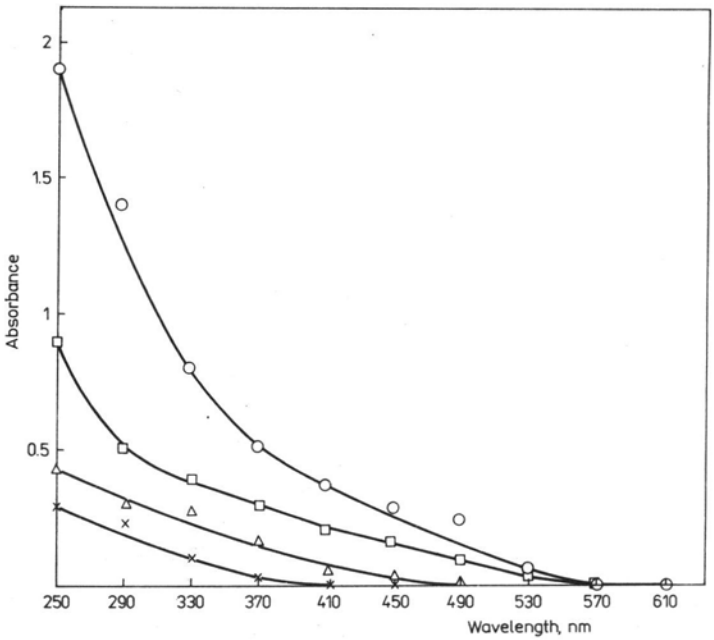


Fig. 3. Measurement of absorbance at wavelenghts ranging from 250 nm to 610 nm for separated spots on a two dimensional chromatogram of 95% ethanol extract of flavonoids from SB_3 cell line in suspension culture: X———X – spot 1, Δ———Δ – spot 2, □———□ – spot 3, O———O – spot 4

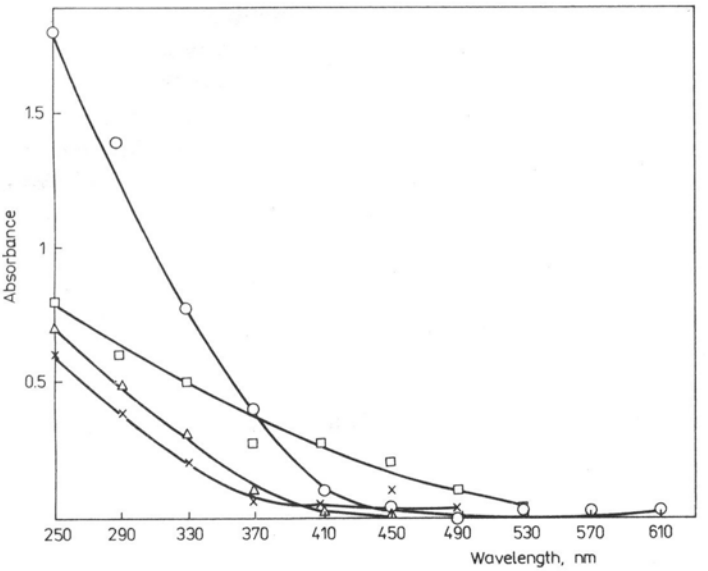


Fig. 4. Measurement of absorbance at wavelengths ranging from 250 nm to 610 nm for separated spots on a two dimensional chromatogram of 95% ethanol extract of flavonoids from SB_1 cell line in callus culture: X———X – spot 1, Δ———Δ – spot 2, □———□ – spot 3, O———O – spot 4

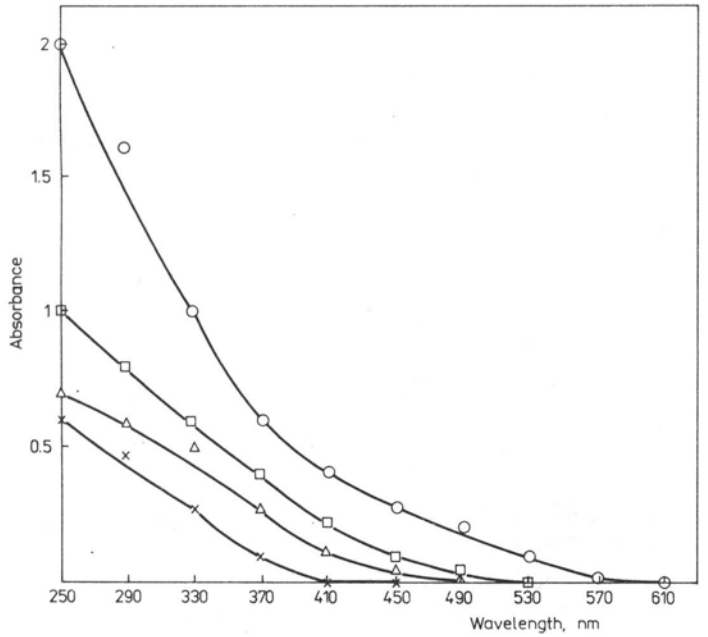


Fig. 5. Measurement of absorbance at wavelengths ranging from 250 nm to 610 nm for separated spots on a two dimensional chromatogram of 95% ethanol extract of flavonoids from SB_3 cell line in callus culture: X———X – spot 1, Δ———Δ – spot 2, □———□ – spot 3, O———O – spot 4

present in both cell lines either in little or negligible quantities. Comparison of the two cell lines showed SB_3 to be richer in flavonoids (Figs. 1A, B).

Further analysis of the concentrated flavonoids extract by two dimensional chromatography on silica gel confirmed these preliminary results. There were four groups of spots on the chromatograms of SB_1 and SB_3 cells, respectively (Figs. 2, 3, 4, 5). Each group of spots was removed separately from the plate and indentified by measuring the absorbance at wavelengths ranging from 250 nm to 610 nm. The majority of flavonoids were located at spot 4 in both cell lines. Other spots were shown to contain an insignificant amount of flavonoids. In spot 4, the main classes of flavonoids were isoflavone, flavone and biflavonyl. On comparison, SB_3 was seen to be richer in flavonoids (Figs. 4, 5).

The major classes of flavonoids that contributed to the differences between the lines were almost the same as shown in Fig. 1, except for the isoflavone and anthocyanins. This again confirms the results shown in Fig. 1, but the significance of isoflavone and anthocyanins in their difference was not shown in Fig. 1.

The most notable observation from Figs. 1, 4, 5 is that SB_3 cells are richer in flavonoids. Also, some classes of flavonoids such as flavanone and flavan-3-ols which are susceptible to oxidation and polymerizations are present in greater quantities in SB_3 cells.

DISCUSSION

The purpose of this experiment was to examine the levels of phenolic compounds and the PAL activity in SB_1 and SB_3 cells, which differ in their ploidy levels. The SB_3 cell line is a hexaploid and produces much more dark brown pigments in both suspension and callus cultures (Jain and Shargool 1987, Shargool and Jain 1984, 1986).

It was shown in this study that SB_3 cells had a higher total phenolics content (Table 1). This observation is significant in that phenolic compounds are one of the important secondary metabolites of plants involved in coloration (Goodwin and Mercer 1983). This finding leads us to the idea that some substances causing different colorations in two soybean cell lines are phenolic compounds.

Flavonoids are not directly involved in brown pigmentation in plants. Most of the brown pigments produced from flavonoids appear to be formed by the oxidation of flavonoids such as catechins, flavanones, 3-hydroxy-flavanones Siegelman 1955, Goodwin 1965). In general, flavone and 3-hydroxy-flavanones are not directly involved in coloration but they are very susceptible to oxidation and yield brown pigments in plant tissues.

Flavan-3-ols and flavan-3,4-diols are largely involved in polymerization to form oligomers and polymers and yield brown pigments. In some cases,

browning is caused when flavonoids chelate with metal ions (Goodwin 1965). Based on the observations it can be suggested that the SB_3 cell line is more likely to undergo modification of flavonoids through polymerization, oxidation and chelation. This may lead to the production of much more brown pigments in SB_3 cells.

L-phenylalanine ammonia lyase is a key enzyme in the biosynthesis of phenolic compounds. It catalyzes the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid (Loomis and Battaile 1966, Goodwin and Mercer 1983).

In dicotyledonous plants it appears to be the sole link between the shikimic acid pathway and pathways leading to cuumarins, flavonoids, condensed tannins and lignins (Maier and Hasegawa 1970). Concomitant increases in levels of PAL and phenolic compounds have been demonstrated in may plant tissues and tissue cultures (Camm and Towers 1973).

There is a direct relationship between PAL activity and the rate of naringenin glycoside (flavanone glycoside) accumulation in developing grapefruits (Maier and Hasegawa 1970). Flavonoid glycoside was accumulated with increased PAL activity in parsley culture (Hahlbrock et al. 1976), Lawton et al. (1983) observed that induction of PAL activity be elicitor in fresh been resulted in the accumulation of phytoalexin which is one of the phenolic compounds. In addition there are many examples of correlation between increased production of phenolic compounds and increases in PAL activity (Camm and Towers 1973). Because SB_3 cell lines showed higher levels of total phenolic compounds, flavonoids, tannins and PAL activity were expected to be higher in the SB_3 cell lines. The result shows that this was indeed the case. PAL activity was approximately three times higher in the SB_3 cell lines (Table 2).

Table 2

Specific activity of PAL in the acetone powders prepared from SB_1 and SB_3 cells

Cell lines	Specific activity (μmole min^{-1} mg $protein^{-1}$)
SB_1	2.89
SB_3	7.94

However, total phenolic content was also higher. This indicates that biosynthesis of different groups of phenolic compounds is not regulated by PAL activity. The result of PAL activity implicates it as a regulatory system for the synthesis of phenolic compounds but further research is warranted on its specific role.

REFERENCES

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.

Camm E. L., Towers G. H. N., 1973. Review article. L-phenylalanine ammonia lyase. Phytochemistry 12: 961-973.

Goodwin T. W., 1965. Nature and properties of flavonoids. In: Chemistry and Biochemistry of plant pigments. Academic Press, London, pp. 211-242.

Goodwin T. W., Mercer E. I., 1983. Plant phenolics. In: Introduction of plant biochemistry. (2nd ed.), Pergamon Press, Oxford.

Hahlbrock K. et al., 1976. Coordinated induction and subsequent activity changes of two groups of metabolically interrelated enzyme-light induced synthesis of flavonoid glycosides in cell suspension cultures of *Petroselium hortense*. Eur. J. Biochem. 61: 199-206.

Harborne J. B., 1959. The chromatography of the flavonoid pigments. J. Chromatogr. 2: 581-603.

Harborne J. B., 1973a. Phenolic compounds. In: Phytochemical methods. Chapman and Hall, London, pp. 33-44, 52-59.

Harborne J. B., 1973b. Isolation of plant enzyme phenylalanine ammonia lyase. In: Phytochemical methods. Chapman and Hall, London, pp. 254-255.

Jain J. C., Ullah M. R., 1979. L-phenylalanine ammonia lyase in tea leaf. Two and A bud. (India) 26: 67-69.

Jain J. C., Shargool P. D., 1987. An examination of the growth rates of two soybean cell lines that differ in their ploidy levels. J. Plant Physiol. 130: 137-146.

Jennings A. C., 1981. The determination of dihydroxy phenolic compounds in extracts of plant tissues. Anal. Biochem. 188: 396-398.

Lawton M. A., Dixon R. A., Hahlbrock K., Lamb C., 1983. Rapid induction of the synthesis of phenylalanine ammonia lyase and of chalcone synthase in elicitor-treated plant cells. Eur. J. Biochem. 129: 593-601.

Loomis W. D., Battaile J., 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry 5: 423-438.

Maier V. P., Hasegawa S., 1970. L-phenylalanine ammonia lyase activity and naringenin glycoside accumulation in developing grapefruit. Phytochemistry 9: 139-144.

Shargool P. D., Jain J. C., 1984. The effect of growth in high concentration of ammonia on enzymes involved in ammonia assimilation in soybean cells of differing ploidy. Proc. Int. Assoc. Plant Tiss. Cult. Symp. held at Saskatoon, Saskatechwan, Canada. pp. 48.

Shargool P. D., Jain J. C., 1986. Growth responses of clonal variants of soybean cells. Proc. Fed. Biol. Soc. Symp. held at Saskatoon, Saskatechawan, Canada. pp. 157.

Siegełman H. W., 1955. Detection and indentification of polyphenoloxidase substrates in apple and pear skins. Arch. Biochem. Biophys. 56: 97-102.

Badanie związków fenolowych i aktywności liazy fenyloalaninowej w dwóch liniach komórek soi (Glycine max L. cv. Mandarin) różniących się ploidalnością

Streszczenie

Podczas wstępnych doświadczeń mających na celu zbadanie różnych zabarwień w dwóch liniach komórek soi SB_1 i SB_3 zaobserwowano kilka ważnych cech. Komórki SB_3 które wytwarzają więcej ciemnobrązowych pigmentów zarówno w kulturach zawiesinowych, jak i kallusowych charakteryzowały się większą zawartością związków fenolowych i flawonoidów. Także aktywność liazy fenyloalaninowej była większa w komórkach SB_3. Aby odkryć dokładny mechanizm wytwarzania większej ilości brązowych pigmentów w komórkach SB_3 należałoby podjąć dalsze badania. Pożyteczne byłoby także zbadanie oksydazy polifenolowej.