Asian Journal of Chemistry Vol. 20, No. 3 (2008), 1848-1858

Correlation Between Cytokinins and Polyamines Contents of *Capsicum annuum* L. Callus Cultivated *in vitro*

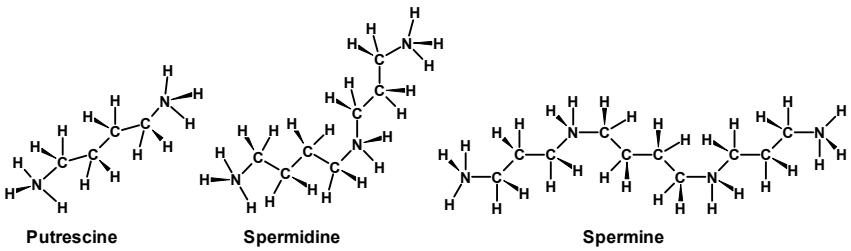
MELIHA GEMICI*, F. NIL AZERI and DILEK ÜNAL
Department of Botany, Faculty of Science, Ege University, Bornova 35100, Izmir, Turkey
E-mail: meliha.gemici@ege.edu.tr

Polyamines are naturally found both in animals and plants. They play important roles in a number of cellular processes such as replication and translation, embriyonic development, cell cycle, programmed cell death and cancer. Although the metabolic pathway of polyamines is lighten in recent years, its relationship with hormones still remains unclear. In this study, the authors investigate a correlation between polyamines and hormones. Endogen cytokinins and polyamines (spermidine and spermine) contents have determined by TLC and HPLC methods in *Capsicum annuum* L. subcallus that derived from callus formation medium consist of arginine, 2,4-Dichlorophenoxyacetic acid (2,4-D) and both of them at day 21. Phenylalanine amonium liyas (PAL) enzyme analysis have also performed. The results suggest that the use of cytokinins in cell may only related to spermine. Polyamines amount was been proportional to decrease of PAL enzyme content. When cytokinin and PAL enzyme content was compared, the consumption of cytokinin in callus which placed applicaton medium, was likely to decrease PAL activity.The results suggest that the use of cytokinins in cell may only related to spermine.

Key Words: Polyamine, *Capsicum annuum* L., Cytokinin.

INTRODUCTION

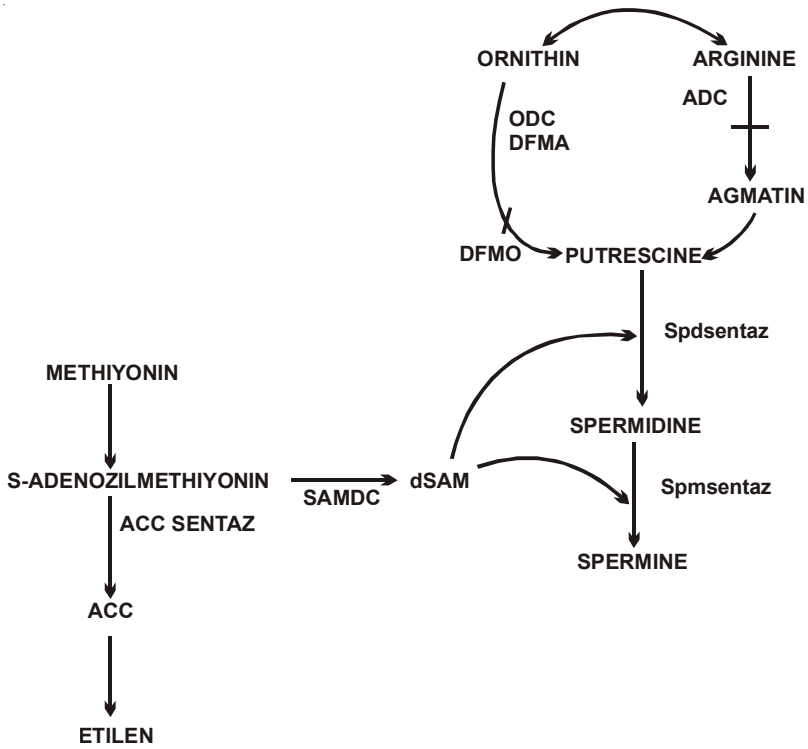
In recent years, a new group of substances called polyamines have been added to the list of growth regulating substances. Essential for life and naturally found in all living organisms, polyamines are multifunctional compounds containing one or more amino groups (**Scheme-I**).



Scheme-I

Four types of polyamines, namely putrescine (put), spermidine (spd), cadaverine (cad) and spermine (spm), are found in all eukaryotes in millimolar concentrations. The pathway of putrescine and spermine was first determined in fungus[1].

Putrescine is derived from arginine in two way; first is the change of arginine losing urea to ornithine and so of ornithine to putrescine by ornithine decarboxylase (ODC) *via* release of CO_2. In second way, arginine is decarboxylated by arginine decarboxylase (ADC) to agmatine and after this, putrescine is formed from agmatine (**Scheme-II**). Further, it is more valid for plants. Thereafter spermine and spermidine are derived from putrescine. Due to the fact that polyamines demonstrate polycationic characteristics in their cellular pH values, they can be easily bound to cellular polyanions, DNA, RNA, phospholipids, asitic proteins and cell wall compounds[2,3]. Polyamines can effect the partial phases (stages) of mitosis and mayosis, cell membrane permeability and the activity and synthesis of macromolecules. Moreover, polyamines are known to play important roles in vascular differentiation, root initiation, stem formation, flower initiation and development, fruit growth, senescence and embryo formation in tissue cultures in higher plants[3].



Scheme-II

Because of its property indicated above, polyamines are also required for optimum growing and development of a living organism. For example, in the light of investigations which perform in the no synthesized polyamines mutant species of *Escherichia coli* and *Sacchoramyes cerevisia*, it is indicated that they are not show normal growing and development[4-6]. Optimum growing occurs in the high levels of polyamine and its level keep parallel way when mitotic activity starts. This relationship between polyamine concentration and cell grow have been first investigated in chicken embryo. In the result of this studies, an increase in the activity of enzymes related to polyamine biosynthesis are determined in early development state of chicken embryo[3]. In addition to this, it is determined by using partial liver excise method that polyamine and enzymes are present more in new divided cells[7,8]. Similarly, in plant, particularly in tissue just starting divide, meristem and also embryo, the amount of polyamine is high[8,9]. Besides, acceleration of mitotic activity *via* spermine was indicated in the light of the investigation made in ovul of chickpea seed[9]. This increase occur in polyamine (spermine and spermidine) biosynthesis and mitotic activity is seen especially in passing of cell cycle from G_1 to S phase[2,10,11]. Moreover, the obstructed of spermine and spermidine biosynthesis in G_1 phase of cell cycle show that these polyamines are necessary for nucleic acid synthesis and completion of cell cycle[11].

The relationships between cytokinins and these compounds, which have assumed this much important roles for life, both in animals and plants haven't been fully brought to a definiteness yet[9,12,13].

As is known, cytokinins have important roles in promoting plant growth and development. As well as they function as hormone triggering *in vitro* cell division[14], cytokinins also play an important role in regulation of mytose cell division and cytokinesis[15]. As the result of studies carried out, it was determined that cytokinins augmented polyamine synthesis[16]. In addition, at the beginning 1980's, it have been suggested by Galaston that polyamines may function as secondary messengers for some phytohormones, especially cytokinins[17]. In the light of research carry out in animals, it is proposed that polyamines incite hormone activity as well as hormone affect in polyamines synthesis[9]. In plant, although the existing of cytokinin and polyamines (spermidine) opposite effects in molecular level have been determined especially in control of membrane permeability, its intracellular interaction ways haven't been explained yet[17,18]. But polyamines perform this roles, have been undertaken, only when they are found in free form in cell. If they join with phenolic acids, aren't been active in cell[19,20]. However, the concentration of free forms of polyamines in cell importance from the point of view of cell division control[19]. Besides, the probable effects of polyamines can be obstructed by crossing polyamines at phenolics-bound situation in the result of operating of phenylpropanoid way[19]. In addition

this, it was reported that in the following external auxin application on Helianthus tubers, polyamines metabolic enzymes and polyamine itself amount in divided cell increase before S phase and throughout the S phase[21]. In the light of all this results, obtained from former studies, we suggest that cytokinin may work in the existing of polyamines in free form in divided cell and so may be active in cell cycle arrangement by binding both DNA and cyclic proteins and we also thought that the balance between phenolic compounds and cytokinin contents within cell affect on free polyamine content. In this respect, in present study, along with giving external arginine, polyamines precursor substance, it's effect on inner cytokinin, polyamine and phenylalanine amonium liyaz enzyme changes and on callus development have been brought up. The correlation between polyamines and cytokinin, a growth hormone and one of factors that control cell cycle, was investigated.

EXPERIMENTAL

In this study, the seeds of an ACI-ILICA 256 cultivar of *Capsicum annuum* L. species belonging in family *Solanaceae* were used as study material. The seedlings were grown *in vitro* inside M.S.[11] culture medium in the photoperiod chamber under 16 h light, 8 h dark long day conditions. The seeds of an ACI-ILICA 256 cultivar of *Capsicum annuum* L. species was sterilized superficially, washed with sterilized distilled water and dried well on filter paper. Dried seeds placed into erlens in shape that each consist of four seed. Modified M.S.[22] medium was used[23]. Erlens transferred into incubation chamber under 16 h light, 8 h dark long day conditions.

Constitution of callus: Explants by excising from obtained seedlings was transferred into callus medium (M.S. + 1 mg IAA/lt; standard callus development medium). Callus, acquired from this medium, were multiplied *via* sub-culture. Constituted third sub-culture transferred into application (test) medium and harvested at day 21. Treatment environments were arranged as follows: 1st series: M.S. and 1 mg IAA (standard callus development medium), 2nd series: 2 mg arginine, 3rd series: 2 mg 2,4-D, 4th series: 2 mg 2,4-D and 2 mg arginine.

Fresh weight of callus: The weight of callus harvested at day 21 from series are measured and results were utilized for drawing a graph.

Cytokinins hormone analysis: Scott and Jacob[24] method was used with some modifications in the internal hormone extraction in 21-day-old seedlings and Nitsch and Nitsch[25] method was utilized in the thin-layer chromatography performed for the isolation of the cytokinins in the material. Wheat leaf biological aging test, which is based on the chlorophyll-breaking and age-retarding effect of the cytokinin, was used for the determination of the cytokinins[26,27].

Polyamine analysis: Analysis of polyamines in callus was performed *via* high performance liquid chromatography analysis method and thin layer chromatography analysis method as described by Flores and Galston[28].

TLC Analysis: The TLC analysis was performed according to Flores and Galston[28]. The callus tissues were extracted in 5 % cold $HClO_4$ (100 mg/mL). After extraction for 1 h in an ice bath, samples were centrifuged at 25,000 g × 20 min. The supernatant phase was stored frozen at -20 ºC in plastic vials (the supernatant phase can be stored frozen at -20 ºC for more than 6 months). 200 µL of $HClO_4$ extract were mixed with 400 µL dansyl chloride (5 mg/mL in acetone, prepared fresh) and 200 µL of saturated sodium carbonate were added. The mixture was incubated in darkness at room temperature overnight. And so 100 µL of proline (100 mg/mL) was added and incubation for 0.5 h. Dansylpolyamines were extracted in 0.5 mL benzene and vortexed for 30 s. The organic phase was collected. TLC was performed on high resolution LK6D silica gel plates. Danylated extract were loaded on plates and the chromatogram was developed for about 1 h with solvent system:chloroform:triethylamine (25:2, v/v). The dansylpolyamine bands were scraped and eluted in 2 mL ethyl acetate and quantified with spectrophotofluorometer at 350 nm and 495 nm. The results were interpreted.

HPLC Analysis: With some modification, the method of Flores and Galston[28] was used for HPLC analysis. Extraction process was performed as in TLC. Extracts were centrifuged at 21,000 g × 5 min. 250 mL $HClO_4$ extract and 1 mL 2 N NaOH were mixed. After addition of 10 µL benzoyl chloride, vortexing for 10 s and incubation for 20 min at room temperature, 2 mL saturated NaCl was added in mixture. Then benzoyl-polyamines were extracted in 2 mL diethyl ether. Mixture was centrifugated at 1500 g × 5 min, 1 mL of ether phase was collected, evaporated and redissolved in 100 µL methanol. HPLC analysis was maintained as described by Flores and Galston[28].

Phenylalanine ammonium liyaz enzyme analysis: 150 mg leaf tissue was extracted in *tris*-HCl buffer (50 mM, pH 8.5), 14.4 mM 2-mercapto-ethanol, 5 % (w/v) Dowex 1 × 200 and 5 % (w/v) polyvinylpyrrolidone mixture. The mixture was centrifugated at 12,000g × 15 min. Supernatant after incubation at 40 ºC for 2 h, 2 mL 6 N HCl was added and the reaction was stopped. The reaction mixture was diluted with 0.88 mL distilled water and so the measuring was performed in 290 nm. The results were interpreted.

RESULTS AND DISCUSSION

Fresh weight results of callus from (in) different application medium: The weight of callus obtained from constituted third sub-culture of *Capsicum annuum* L. callus were measured during harvest at 21 d (Fig. 1). A clear

increase between control and other all application groups was noticed when the results were compared. According to result of control group, there is an increase of 33 % in arginine group, of 77 % in 2,4-D + arginine group and most increase of 116 % in 2,4-D group.

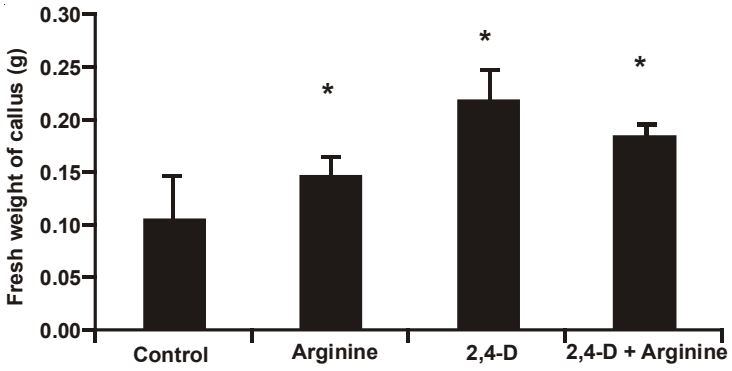


Fig. 1. Fresh weight of *Capsicum annuum* L. callus in different application medium. (*Statistically significant (p 0.05) difference in comparison to control medium)

Endogen cytokinin content of callus from different application medium: Fig. 2. illustrates the inner cytokinin content of callus in control, arginine, 2,4-D and 2,4-D + arginine group application medium. When control and other application groups are compared, a clear decrease is attractive in callus harvested in the end of 21 d as opposite to increase that is observed in fresh weight results of callus. As to percentage, according to control, this decrease was determined as 77 % in arginine group, 85 % in 2,4-D group and 85 % in 2,4-D + arginine group.

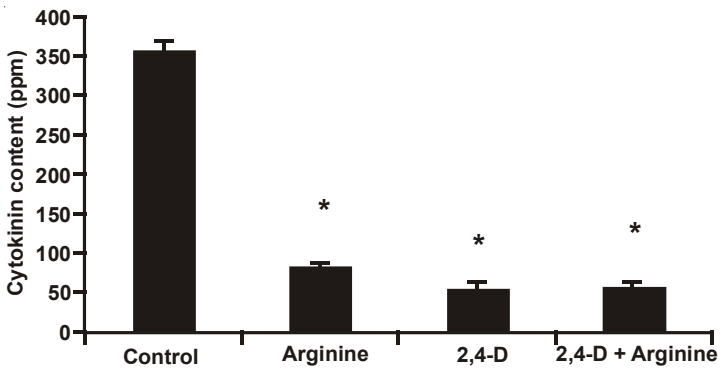


Fig. 2. Endogen cytokinin content of callus in different application medium. (*Statistically significant (p 0.05) difference in comparison to control medium)

Polyamine content in *Capsicum annuum* L. callus from different application medium

Polyamine content determined by using of TLC analysis method: As mentioned in materials and methods, polyamine contents were determined in the end of 21 d by using TLC analysis method. The results are given in Fig. 3.

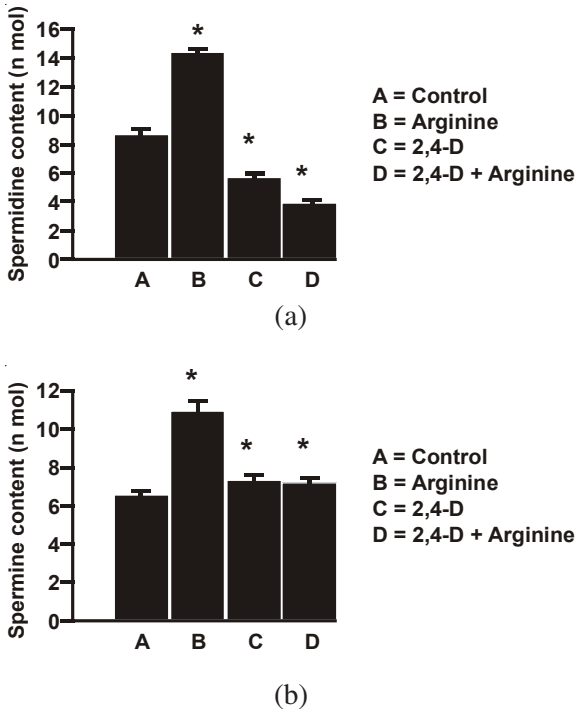


Fig. 3. Endogen spermidine (a) and spermine (b) content of callus in different application medium by using TLC. (*Statistically significant (p 0.05) difference in comparison to control medium)

When present results are evaluated, it is seen that there is an increase of 63.96 % in arginine group and a decrease of 33.38 % in 2,4-D, of 51.5% in 2,4-D + arginine group. However, as for spermine, an increase of 65.22 % in arginine group, of 10.12 % in 2,4-D and of 9.36 % in 2,4-D + arginine group is observed.

Polyamine content determined by using of HPLC analysis method: HPLC procedure was performed in sub-culture of *Capsicum annuum* L. plant. Obtained HPLC analysis results was found similar for increasing spermine values in arginine group but this value decreased in 2,4-D and 2,4-D + arginine groups according to control when compared with TLC analysis results (Fig. 4).

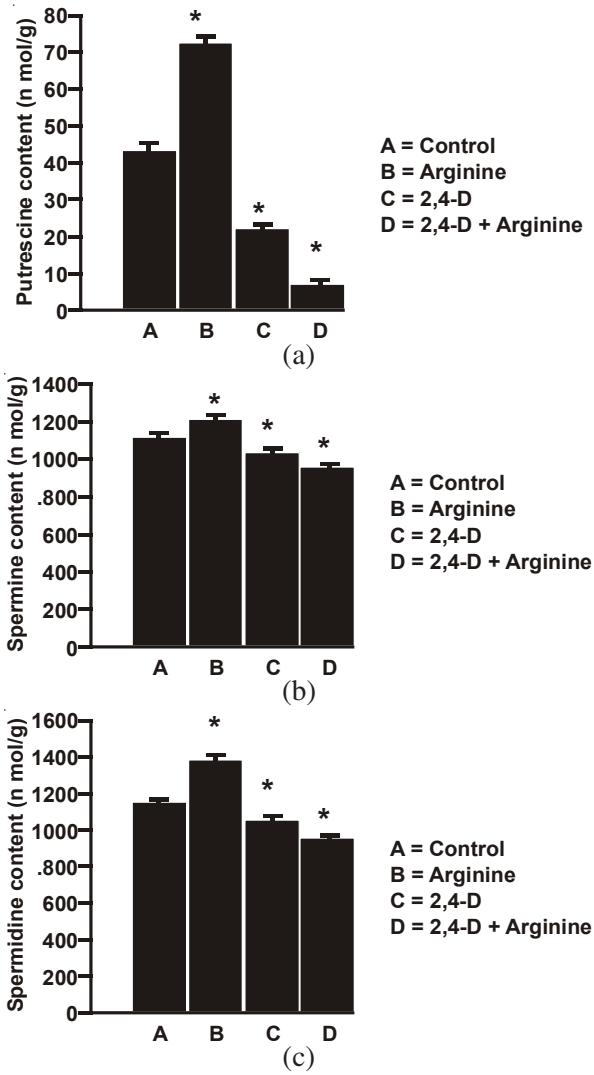


Fig. 4. Endogen putrescine (a), spermine (b), spermidine (c) content of callus in different application medium by using HPLC. (*Statistically significant (p 0.05) difference in comparison to control medium)

Phenylalanine ammonium liyas enzyme content of callus from different application medium: PAL content was showed a decrease at all application groups according to control (Fig. 5). As to percentage, the rate of this decrease was determined as 2.58 % in arginine group, 20 % in 2,4-D group, 29 % in 2,4-D + arginine group. It was obtained that callus development is well in application group when compared with control. Similarly, cell division activity is higher in callus of application groups. So PAL content, decrease in callus according to control, is directly proportional with this results.

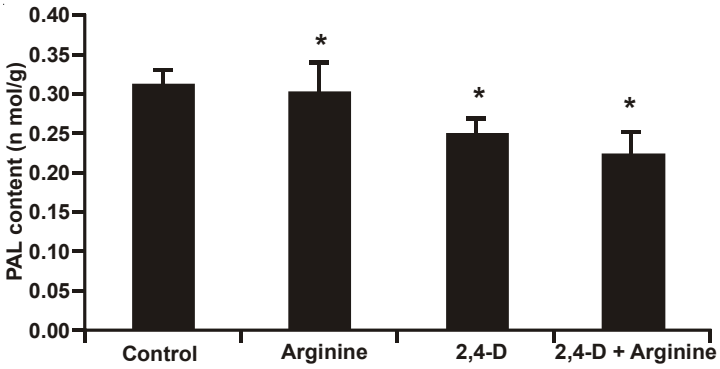


Fig. 5. Phenylalanine amonium liyas (PAL) enzyme content of callus in different application medium. (*Statistically significant (p 0.05) difference in comparison to control medium)

Percentage increase-decrease values: With all data are considered, the results (Table-1) have been able to investigate by using percentage increase-decrease values. Spermine and spermidine contents increased as parallel to callus weight increase. However, PAL enzyme content decrease. The decrease of inner cytokinin is indicate that cytokinin is utilized inside cell. As coherent to this, a decrease was also determined in PAL content. Both in 2,4-D and 2,4-D + arginine group, spermidine amount decrease. As for spermine amount, it increase in 2,4-D + arginine and 2,4-D group according to control. When compared to cytokinin results, it is observed that the consumption of cytokinin inside cell may only be related to spermine.

TABLE-1
PERCENTAGE INCREASE-DECREASE VALUES OF CALLUS IN DIFFERENT APPLICATION MEDIUM

	Callus weight	Cytokinine	PAL	Spermidine	Spermine
Arginine	33	77*	2.58*	63.96*	65.22
2,4-D	116	88*	20.00*	33.38*	10.12
2,4-D + Arginine	77	88*	29.00*	51.50*	9.36

*Percentage of decrease values according to control.

Studies conducted on the relationship between polyamines and plant growth has shown the presence of a correlation between the increase in polyamines and the increase in internal hormones. Similarly, researchers determined that internal indole acetic acid (IAA) and polyamine synthesis increased with the application of naphthalene acetic acid (NAA) and kinetine to glycine max calluse[7]. In addition, it was reported that polyamine metabolic

enzymes (*i.e.*, ODC, ADC) and polyamine quantity increased in the cell divided following insertion of auxins into Helianthus tubers before and during the S phase. It is suggested that this increase induced by external hormone application is due to the fact that kinetine and regulate the plant regulators regulating the genes responsible for the activation of metabolic enzymes or the activities of these enzymes[7]. However, studies conducted on animals have shown that the increase that occurs in the polyamine biosynthesis is not only a response to hormones, but also a factor necessary for hormone activity[29]. In plants, on the other hand, most data indicate that the change in the polyamine quantity is a response to internal hormones (auxins, cytokinins and gibberelins). In recent years, however, some researchers have suggested that polyamines mediate for plant hormone activation or their signal molecules[9]. Similarly, in a reseach conducted on the genes that are responsible for synthesis of cytokinin precursor, an antogonism was determined between spermine and cytokinins[17]. Also, an effect of spermine that act as opposite to kinetine was determined in a research conducted on membrane permeability in beet root cells[18]. In present examination, it was determined that cytokinin cause 10.12 % increase in spermine amount according to control in callus and so it was harmonious with increase of mitotic activity, but that there was no any correlation related to spermidine. Because arginine is a precursor substance of polyamine biosynthesis, both spermine and spermidine amount increased in arginine group. In addition to cytokinin and polyamine content, the analysis PAL enzyme which play role in was performed in our study. PAL is first enzyme that regulates synthesis of phenolic compounds phenyl propanoide biosynthesis pathway. As a result of bounding of polyamines and phenolic compounds each other bounded-polyamine's are formed[19]. It was reported that free spermidine amount increased by application of 2-aminoidan-2-phosphonic acid, a inhibitor of phenyl propanoide biosynthesis pathway, on oak somatic embryo[20]. Also, in present investigation, polyamine have been proportional to decrease of PAL enzyme content. When cytokinin and PAL enzyme content was compared, even though the absence of a direct correlation between phenyl propanoide pathway and cytokinins was reported in a investigation conducted on alfalfa cell[30], the consumption of cytokinin in callus which placed application medium, was likely to decrease of PAL activity.

REFERENCES

1. Y. Akman, C. Darici, Bitki Fizyolojisi (Beslenme ve Gelisme Fizyolojisi), Kariyer Matbaasi, Ankara (1998).
2. T. Thomas and T.J. Thomas, *Cell. Mol. Life Sci.*, **58**, 244 (2001).
3. N. Ünsal-Palavan, H. Büyüme and K. Polaminler, *Turk. J. Med. Sci.*, **20**, 141 (1990).
4. M.V. Rajam and A.W. Galston, *Plant Cell Physiol.*, **26**, 683 (1985).

5. H. Birecka, M.O. Garraway, R.J. Baumann and P.P. McCann, *Plant Physiol.*, **80**, 798 (1986).
6. S.B. Gioconda, S.-B. Felipe, S. Françoise, M. Belisario and R.-H. José, *Arch. Microbiol.*, **166**, 441 (1997).
7. L. Zin-Huang, W. Wei-Chang and Y. Sho-Yun, *Bot. Bull. Acad. Sin.*, **38**, 171 (1997).
8. F.L. Meyskens, Jr. Gerner and W. Eugene, *Clin. Cancer Res.*, **5**, 945 (1999).
9. A. Bouchereau, A. Aziz, F. Larher, J. Martin-Tanguy, *Plant Sci.*, **140**, 103 (1999).
10. J. Laitinen, K. Stenius, T.O. Elorantha and E. Hölttä, *J. Cell. Biochem.*, **68**, 200 (1998).
11. S. Iwata, Y. Sato, M. Asada, M. Takagi, A. Tsujimoto, T. Inaba, T. Yamada, S. Sakamoto, J.I. Yata, T. Shimogori, K. Igarashi and S. Mizutani, *Oncogene*, **18**, 165 (1999).
12. J. Legocka and A. Zarnowaka, *Acta Physiol. Plant.*, **22**, 395 (2000).
13. J. Legocka and A. Zarnowska, *Acta Physiol. Plant.*, **21**, 349 (1999).
14. S.A. Carle, G.W. Bates and T.A. Shannon, *J. Plant Growth Regul.*, **17**, 221 (1998).
15. R.P. Huntley and J.A.H. Murray, *Curr. Opin. Plant Biol.*, **24**, 440 (1999).
16. N. Ünsal-Palavan, Bitki Büyüme Maddeleri, Üniversite Yayin No: 3677 Istanbul, p. 302 (1993) (In Turkish).
17. N. Yu. Rakova and G.A. Romanov, *Russ. J. Plant Physiol.*, **52**, 50 (2005).
18. G.A. Romanov and N. Yu, *Doklady Biochem. Biophys.*, **398**, 288 (2004).
19. J.C. Mader and D.E. Hanke, *J. Plant Growth Regul.*, **16**, 89 (1997).
20. M. Cvikrová, J. Malá, M. Hrubcová, J. Eder, J. Zo'n and I. Macháckova, *Plant Physiol. Biochem.*, **41**, 251 (2003).
21. P. Torrigian, D. Serafini-Fracassini and N. Bagni, *Plant Physiol.*, **84**, 148 (1987).
22. T. Murashige and F. Skoog, *Physiol. Plant*, **154**, 73 (1962).
23. C. Aki, *Capsicum annuum* L.' un Bazi Varyeteleri Üzerinde Doku Kültürü Çalismalari. Ege Ünv. Doktora Tezi (1997) (In Turkish).
24. T.K. Scott and W.P. Jacops, Critical Assesment of Techniques for Identifying the Physiologically Significant auxin in plants, In: Régulateurs Naturels de la croissance Végétale, eds C.N.R.S., Paris, pp. 457-474 (1964).
25. J.P. Nitsch and C. Nitsch, *Beitr. Biol. Pflanzen*, **31**, 387 (1955).
26. M. Gemici, *Phaseolus vulgaris* L. Fidelerinde Beslenmeye Bagimli Olarak Korelatif Iliskilerdeki Hormonal Etmenlerin Rolü. Ege Ünv.Doktora Tezi (1987) (In Turkish).
27. P. Abramoff and R.G. Thompson, Elementray Statistical Analysis An Experimental Approach to Biology, pp. 251-253 (1966).
28. H.E. Flores and A.W. Galston, *Plant Physiol.*, **69**, 701 (1982).
29. O. Heby, *Differentiation*, **19**, 1 (1981).
30. M. Cvikrová, P. Binarová, J. Eder, M. Vágner, M. Hrubcová, J. Zo'n and I. Macháckova, *Physiol. Plant.*, **107**, 329 (1999).

(*Received*: 20 February 2007; *Accepted*: 2 November 2007) AJC-6052