Proc. Estonian Acad. Sci. Biol., 1995, 44, 3/4, 109—118
https://doi.org/10.3176/biol.1995.3/4.06

HETEROGENEITY IN BUCKWHEAT SEEDLING POPULATION IN RELATION TO LIGHT- AND KINETIN-INDUCED ANTHOCYANIN ACCUMULATION

Ants TOHVER, Lembe LAANEST, and Tiiu VAINJÄRV

Eesti Teaduste Akadeemia Eksperimentaalbioloogia Instituut (Institute of Experimental Biology, Estonian Academy of Sciences), Instituudi tee 11, EE-3051 Harku, Harjumaa, Eesti (Estonia)

Presented by U. Margna

Received 22 February 1995, revised version received 10 April 1995, accepted 11 April 1995

Abstract. The production of anthocyanin in lower epidermis of the cotyledons in response to illumination varied widely between individual dark-grown seedlings of a buckwheat (*Fagopyrum esculentum* Moench) population. No significant anthocyanin colouration developed in 20—30% of the cotyledons. Nevertheless, the seedlings did not differ in the content of glycosylflavones and rutin. A combined treatment with kinetin and light induced anthocyanin accumulation in all cotyledons of the population. Anthocyanin accumulated also in the upper epidermis in an amount comparable with the lower epidermis. The conclusion has been drawn that kinetin causes the appearance of competence for anthocyanin synthesis in response to illumination in cotyledons and tissues lacking it for some genetical and/or developmental reasons.

Key words: anthocyanin, buckwheat, light, kinetin, competence, population.

INTRODUCTION

The synthesis and accumulation of anthocyanin pigments in plants are regulated by both environmental and developmental signals (Mohr, 1972; Murray et al., 1994). Light is the most important factor in the control of anthocyanin synthesis, and evidence of the involvement of at least three photomorphogenic photoreceptors has been obtained (Mancinelli, 1985; Oelmüller & Mohr, 1985). Phytohormones, such as gibberellic acid (Weiss et al., 1990; Ozeki & Komamine, 1986), and kinetin and other cytokinins (Klein & Hagen, 1961; Pecket & Bassim, 1974; Nakamura et al., 1980; Margna & Vainjärv, 1983) can also substantially promote the accumulation of anthocyanins in various plant organs. Among kinetin effects on the phenolic metabolism, perhaps the most striking one is a 9-fold increase in anthocyanin accumulation observed in excised cotyledons of dark-grown buckwheat seedlings (Margna & Vainjärv, 1983).

In previous studies of light- and kinetin-dependent anthocyanin accumulation, 20 to 50 seedlings, constituting a sample, were analysed together. However, visual observations of the buckwheat seedling population used in our studies have shown that they display a remarkable heterogeneity in anthocyanin accumulation under different treatments. Therefore it cannot be excluded that average data obtained with heterogenous material disguise some essential features of the studied effects. In this work, we have checked whether the individual seedlings differ in their response to illumination and kinetin.

MATERIAL AND METHODS

Buckwheat (*Fagopyrum esculentum* Moench cv. Victoria) seeds were germinated in the dark on moistened filter paper at 25 °C. From 96-h-old dark-grown seedlings cotyledons were excised and incubated in water or 0.3 mM kinetin solution in the dark or in a phytotron (light of white fluorescent tubes, 30 Wm^{-2}) at 25 °C. With seedlings incubated in darkness, the excision was performed in dim green safelight. After incubation (20 or 44 h) the cotyledons of each individual seedling were separately fixed in 3.5 ml of 1% HCl in 50% ethanol on a boiling water bath for 1.5 min and thereafter extracted overnight at 10 °C in the same mixture. After centrifugation the absorbancy of the extracts at 546 nm was determined and taken as an estimate of anthocyanin content in a pair of cotyledons. The frequency distribution curves of anthocyanin content include the data of 200—450 seedlings.

The anthocyanin content in the upper and lower epidermis was compared as follows. Cotyledons were floated for 1—2 min in 5% HCl in 50% ethanol, covered with glass dust, laid between sheets of filter paper, and pressed to obtain imprints. The imprints of the upper and lower side of cotyledons and the tissue residue were extracted with 1% HCl in 50% ethanol, and the absorbancy of the centrifuged extracts at 546 nm was determined.

Flavonoids were determined spectrophotometrically after their paper chromatographic separation (Margna & Margna, 1969; Margna & Vainjärv, 1983). Statistical analysis was performed using Statgraphics 4.2.

RESULTS

Visual observations revealed that individual buckwheat seedlings differ significantly in their ability to produce anthocyanin in response to illumination. While hypocotyls and petioles became always pigmented when illuminated, almost a third of the seedlings showed no visible red pigmentation in leaf blades of their cotyledons. Among the red-pigmented cotyledons there were those whose lower epidermis was rather weakly and unevenly red. The observed variation in the pigmentation of cotyledons is documented using the relative frequency distribution curve (polygon) of the anthocyanin content of cotyledons. Each point of the curves represents the relative frequency (percentage) of cotyledons whose anthocyanin content falls into a given category (length 0.03 or 0.04 OD units).

It must be noted here that the measured absorbancy of cotyledon extracts includes light scattering and the contribution of anthocyanin originated not only from laminae but also from petioles which became pigmented in all seedlings during the illumination. The average absorbancy of anthocyanins from petioles equalled 0.012 OD units per seedling. The share of light scattering at 546 nm estimated by the base line of the absorbancy spectrum of extracts and by absorbancy measurements of H_2O_2-bleached extracts was found to be 0.03 on average. This means that the cotyledons with the absorbancy value of the extract below 0.04 may be considered unpigmented. This conclusion is in agreement with visual observations.

The relative frequency distribution of the anthocyanin content in cotyledons demonstrates the extent of differences in the ability of individual seedlings to produce anthocyanin in response to a certain light and/or kinetin treatment. After a 20-h light incubation of cotyledons in water or after dark incubation with kinetin the distribution polygon of the corresponding data points is markedly asymmetrical (Fig. 1), its maximum

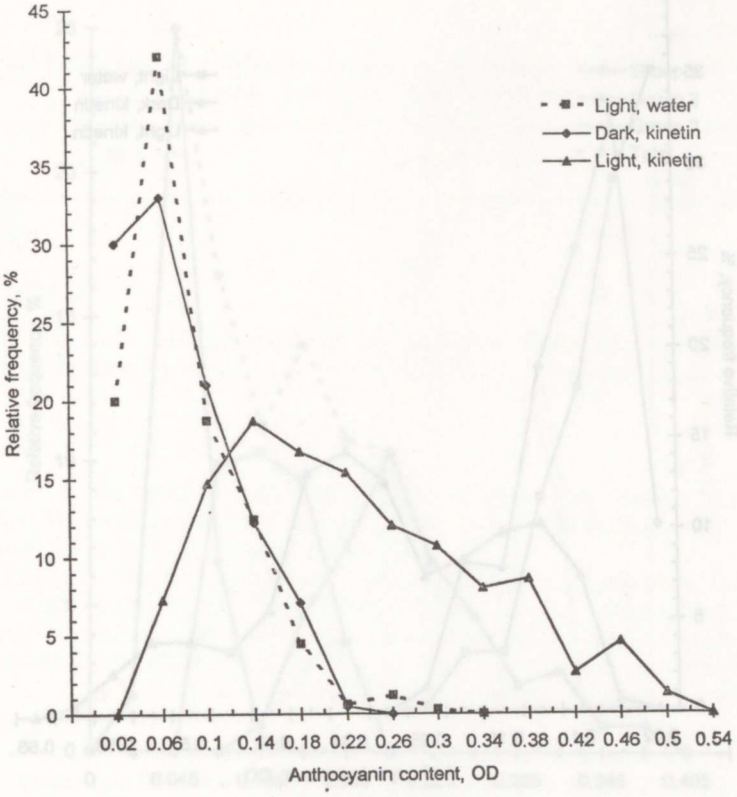

Fig. 1. The frequency distribution of the anthocyanin content in buckwheat cotyledons after a 20-h incubation. Each point on the curves represents the percentage of cotyledons whose anthocyanin content falls into a given category (length 0.04 OD units).

being located at the beginning of the distribution range: 20—30% cotyledons fall into the category of unpigmented ones (OD<0.04). Prolongation of illumination of cotyledons from 20 to 44 h did not alter the general character of the distribution curve (Fig. 2). This means that the anthocyanin accumulation in cotyledons was completed within the first 20 h of illumination in water. However, when incubated with kinetin in the dark, the anthocyanin accumulation in cotyledons proceeded longer than 20 h. As a result, the obtained frequency distribution polygon is shifted towards higher OD values and the mean anthocyanin content rises markedly (Table 1).

The relative frequency polygon of the anthocyanin content of cotyledons after a 20-h incubation with kinetin solution in the light (Fig. 1) differs substantially from that obtained under the impact of light or kinetin alone, being much more uniform and stretching over a broad interval of OD values over 0.5 OD units. It is remarkable that no unpigmented cotyledons (OD<0.04) were discovered. The prolongation of the treatment from 20 to 44 h (Fig. 2) changed markedly the shape of the frequency polygon and shifted it towards greater OD values. It is important to stress that after 44-h treatment the frequency polygon obtained three peaks.

To find out whether there is a correlation between the light- and kinetin-induced anthocyanin accumulation in an individual cotyledon, the treatment of cotyledons (with light and kinetin) was carried out according

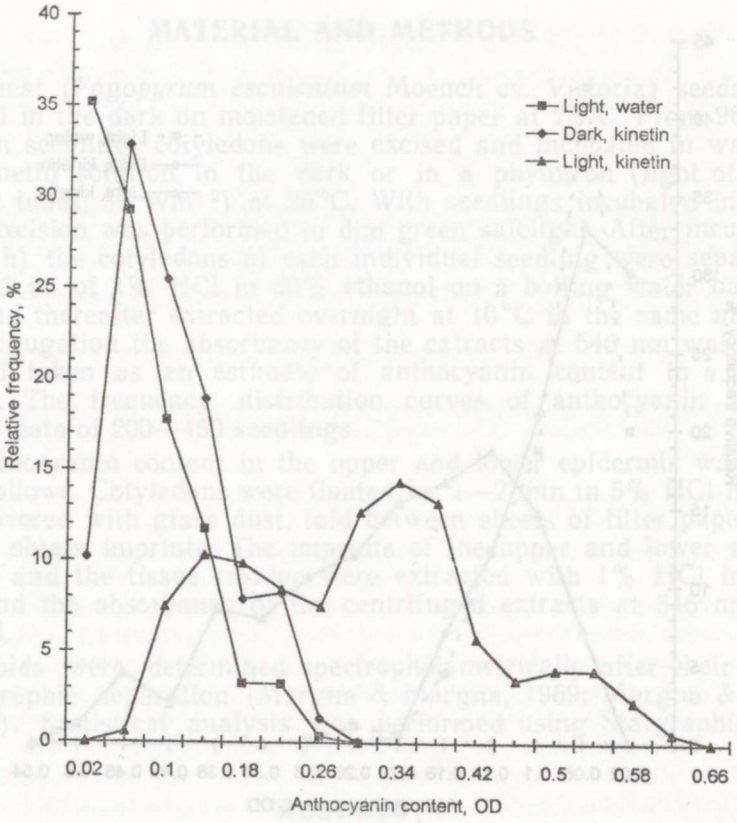

Fig. 2. The frequency distribution of the anthocyanin content in buckwheat cotyledons after a 44-h incubation. Category length 0.04 OD units.

Table 1

Comparison of the effect of kinetin and light on anthocyanin production in buckwheat cotyledons (OD units) by the statistical parameters of the population

Treatment	Mean	Standard deviation
20 h light	0.079	0.049
44 h light	0.073	0.051
20 h kinetin & light	0.216	0.110
44 h kinetin & light	0.311	0.149
20 h kinetin & dark	0.072	0.047
44 h kinetin & dark	0.118	0.072

to the following scheme. The cotyledons kept 20 h in the light in water were divided, after the visual estimation of anthocyanin colouration, into three groups (group 1 — unpigmented, group 2 — unevenly pigmented, group 3 — fully pigmented), transferred onto kinetin solution, and illuminated further for 24 h. The frequency distribution of anthocyanin in the cotyledons of these three groups after 20 h of illumination and after the subsequent combined light and kinetin treatment are given respectively in Fig. 3 and Fig. 4. This graphical presentation and also

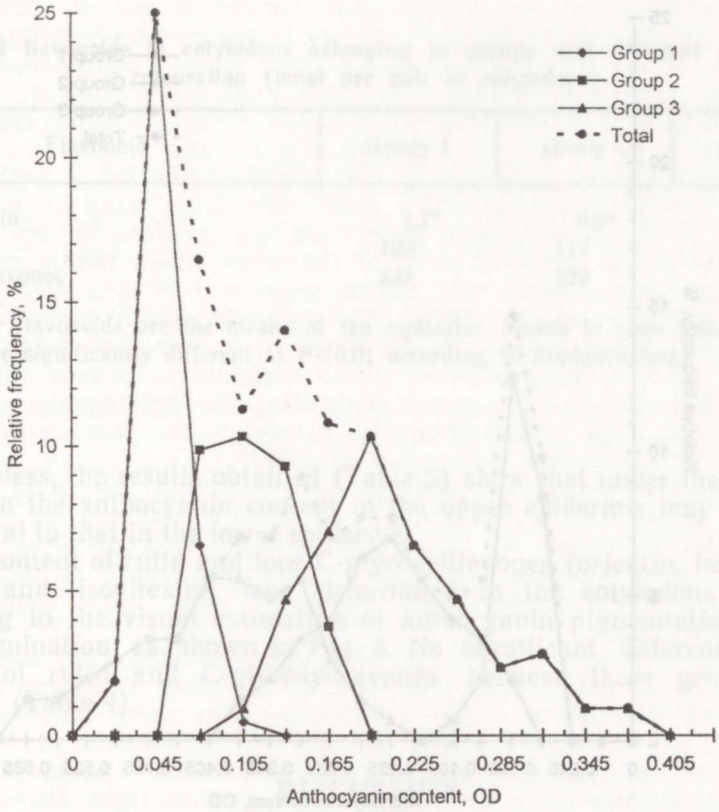

Fig. 3. The frequency distribution of the anthocyanin content in groups of buckwheat cotyledons formed according to visual estimation of pigmentation after a 20-h incubation in water in the light. Group 1, unpigmented; group 2, unevenly pigmented; group 3, fully pigmented cotyledons. Category length 0.03 OD units.

Table 2

The average kinetin and light effect on anthocyanin production (OD units) in cotyledons belonging to groups with different responsiveness

Treatment	Group 1	Group 2	Group 3
Grouping after a 20-h illumination without kinetin			
20 h light	0.053	0.114	0.214
20 h light + 24 h light & kinetin	0.076	0.223	0.419
Grouping after a 20-h kinetin treatment in the dark			
20 h kinetin & darkness	0.022	0.071	0.117
20 h kinetin & darkness + 24 h kinetin & light	0.135	0.342	0.523

statistical parameters given in Table 2 show that the responses of a given group to illumination and kinetin are correlated. An experiment with the reversed order of treatment, with kinetin and light, when the cotyledons were grouped on the basis of the colouration after a 20-h dark incubation with kinetin and thereafter illuminated, gave similar results (Table 2).

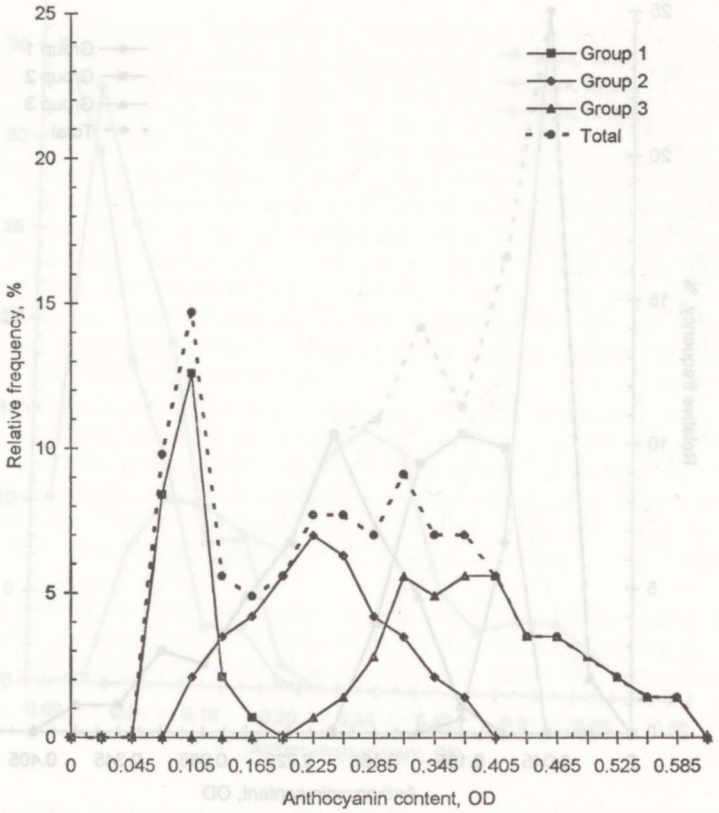

Fig. 4. The frequency distribution of the anthocyanin content in groups of buckwheat cotyledons formed after a 20-h illumination (grouping as in Fig. 3) and incubated thereafter 24 h on kinetin solution in the light. Category length 0.03 OD units.

Table 3

Content of anthocyanin in the tissue prints of cotyledons (percentage of the total content of a cotyledon)

Treatment	Upper side	Lower side	Residue
44 h light & water	5	39	56
44 h light & kinetin	19	22	59
44 h dark & kinetin*	9	31	60

* Excised in dim green safelight.

Illumination induces anthocyanin accumulation in the lower epidermis. Kinetin caused the accumulation of anthocyanin also in the upper epidermis. For demonstration and documentation of this phenomenon, the cotyledons most intensively coloured after 44 h of illumination with and without kinetin were analysed by the tissue printing method described above. This method is not strictly quantitative because of a considerable amount of anthocyanin remaining in the tissue residue, and the cell sap from upper and lower epidermises getting mixed in the marginal region.

Table 4

Content of flavonoids in cotyledons belonging to groups with different anthocyanin colouration (nmol per pair of cotyledons)

Flavonoid	Group 1	Group 2	Group 3
Anthocyanin	1.7*	9.6*	22.5*
Rutin	102	117	109
Glycosylflavones	232	229	238

Values for flavonoids are the means of ten replicates. Means in rows followed by an asterisk are significantly different at $P<0.01$ according to Student's test.

Nevertheless, the results obtained (Table 3) show that under the influence of kinetin the anthocyanin content in the upper epidermis may reach the level equal to that in the lower epidermis.

The content of rutin and four C-glycosylflavones (orientin, iso-orientin, vitexin, and isovitexin) was determined in the cotyledons grouped according to the visual estimation of anthocyanin pigmentation after a 20-h illumination as shown in Fig. 3. No significant differences in the content of rutin and C-glycosylflavones between these groups were observed (Table 4).

DISCUSSION

Light as a photomorphogenic signal plays a central role in the control of developmental events including metabolic differentiation of cells and tissues. The metabolic differentiation of cells includes spatial and temporal regulation of the genes that encode enzymes of the biosynthetic pathway of anthocyanin biosynthesis and the sensitivity of these processes to light and various other external and internal factors (Murray et al., 1994; Taylor & Briggs, 1990). By the time the dark-grown buckwheat seedlings in our experiments were exposed to light they had, according to the endogenous differentiation programme of morphogenesis, acquired the ability to synthesize anthocyanin in response to illumination in the epidermal cells of the hypocotyls and the lower side of the cotyledons but not in the upper epidermis.

The results presented in this article indicate that in addition to the above-mentioned differentiation pattern of tissues there exists also a remarkable diversity between individual cotyledons of dark-grown seedlings in terms of their competence to produce anthocyanin in response to light. The frequency distribution of anthocyanin content of cotyledons illuminated without kinetin for 20 or 44 h (Figs. 1 and 2) demonstrates the extent of this metabolic difference in the buckwheat population. A remarkable proportion (20—30% cotyledons) is not competent to accumulate anthocyanins in response to light. Though these light-insensitive cotyledons acquire the ability to produce anthocyanin when kinetin is applied together with light, the extent of variation in anthocyanin accumulation does not decrease, but, on the contrary, increases greatly. It seems to be informative that the frequency distribution curves of the anthocyanin content in cotyledons never displayed a normal distribution. Moreover, after 44 h of combined treatment with light and kinetin the frequency distribution curve had three separate peaks. These facts give evidence

that the variation of the anthocyanin content is not of a completely stochastic nature, but seems to be related to the genetic heterogeneity of the population. The included subpopulations were distinguished by the anthocyanin accumulation rate during the combined treatment of cotyledons. These subpopulations may differ in the regulatory genes controlling the expression of anthocyanin synthesis. For instance in maize, an extensively studied species in respect to the genetic regulation of anthocyanin synthesis, two families of activator genes have been found to control the expression of all the structural genes that are required for anthocyanin biosynthesis in the different parts of the plant. Each family comprises multiple homologous genes that control pigmentation in a specific tissue or organ (Dooner et al., 1991). The light induction of anthocyanin synthesis and its dependence on the fluency rate were found to be determined by allelic combination of these regulatory locuses (Taylor & Briggs, 1990). Besides, the genes controlling the anthocyanin synthesis show a broad range of variation with respect to the developmental and tissue-specific expression (Murray et al., 1994).

The presented results demonstrate that cotyledons with greatly different ability to produce anthocyanin in response to illumination are indistinguishable in respect to the accumulation of C-glycosylflavones and rutin. This fact indicates that the observed difference in anthocyanin accumulation is referrable to the control of some later steps of the synthetic pathway.

The difference in the effects of light and combined light and kinetin treatment on the anthocyanin synthesis in the upper epidermis suggests participation of different action mechanisms. The same conclusion may be drawn from the data in Table 2 showing that the effect of the combined light and kinetin treatment following a pretreatment with kinetin alone is markedly greater than after a pretreatment with light. However, the obtained results do not allow of the specification of the site(s) of action and mechanism(s) through which kinetin affects the light control of anthocyanin synthesis. Margna and Vainjärv (1983) found that kinetin promotes also the formation of C-glycosylflavones and rutin in buckwheat cotyledons in the dark and, to a lesser extent, in the light. This suggests that the expression of some earlier steps of the synthetic pathway is affected by kinetin.

The statistical approach applied in the present work enabled us to demonstrate, therefore, heterogeneity of the buckwheat population studied. It includes several (three) subpopulations differing in the level of the light-induced anthocyanin accumulation. This difference in light sensitivity is obviously associated with the control of final steps in anthocyanin biosynthesis. Kinetin enhances anthocyanin accumulation and increases the effectiveness of light action resulting in the enhancement of differences in the synthetic capacity of these subpopulations.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. U. Margna and J. Viil for critical reading of the manuscript, and Mrs. E. Margna and Mr. E. Palm for technical assistance. This work was partly supported by grant No. 115 of the Estonian Science Foundation.

REFERENCES

Dooner, H. K., Robbins, T., Jorgensen, R. 1991. Genetic and developmental control of anthocyanin biosynthesis. — Annu. Rev. Genet., **25**, 173—199.

Klein, A. O., Hagen, C. W. 1961. Anthocyanin production in detached petals of *Impatiens balsamina* L. — Plant Physiol., **36**, 1, 1—9.

Mancinelli, A. L. 1985. Light-dependent anthocyanin synthesis: A model system for the study of plant photomorphogenesis. — Bot. Rev., **51**, 1, 107—157.

Margna, U., Margna, E. 1969. A suitable chromatographic method for quantitative assay of rutin and flavone C-glycosides in buckwheat seedlings. — ENSV TA Toimet. Biol., **18**, 1, 40—50.

Margna, U., Vainjärv, T. 1983. Kinetin-mediated stimulation of accumulation of buckwheat flavonoids in the dark. — Z. Naturforsch., **38c**, 9/10, 711—718.

Mohr, H. 1972. Lectures on Photomorphogenesis. Springer Verlag, Heidelberg.

Murray, J. R., Smith, A. G., Hackett, W. P. 1994. Differential dihydroflavonol reductase transcription and anthocyanin pigmentation in the juvenile and mature phases of ivy (*Hedera helix* L.). — Planta, **194**, 1, 102—109.

Nakamura, N., Nakamae, H., Maekawa, S. 1980. Effect of light and kinetin on anthocyanin accumulation in the petals of *Rosa hybrida* Hort cv. Ehigasa. — Z. Pflanzenphysiol., **98**, 3, 263—270.

Oelmüller, R., Mohr, H. 1985. Mode of co-action between blue/UV light and light absorbed by phytochrome in light-mediated anthocyanin formation in the milo (*Sorghum vulgare* Pers.) seedling. — Proc. Natl. Acad. Sci. USA, **82**, 18, 6124—6128.

Ozeki, Y., Komamine, A. 1986. Effects of growth regulators on the induction of anthocyanin synthesis in carrot suspension cultures. — Plant Cell Physiol., **27**, 7, 1361—1368.

Pecket, R. C., Bassim, T. A. H. 1974. The effect of kinetin in relation to photocontrol of anthocyanin biosynthesis in *Brassica oleracea*. — Phytochemistry, **13**, 8, 1395—1388.

Taylor, L. P., Briggs, W. R. 1990. Genetic regulation and photocontrol of anthocyanin accumulation in maize seedlings. — Plant Cell, **2**, 1, 115—127.

Weiss, D., Van Tunen, A. J., Halevy, A. H., Mol, J. N. M., Gerats, A. G. M. 1990. Stamens and gibberellic acid in the regulation of flavonoid gene expression in the corolla of *Petunia hybrida*. — Plant Physiol., **94**, 2, 511—515.

TATRAIDANDITE POPULATSIOONI HETEROGEENSUS VALGUSE JA KINETIINI POOLT INDUTSEERITAVA ANTOTSÜANIINIDE AKUMULATSIOONI SUHTES

Ants TOHVER, Lembe LAANEST, Tiiu VAINJÄRV

Etioleerunud tatraidandite idulehtedes valguse mõjul akumuleerunud antotsüaniini hulk varieerus populatsiooni piires suures ulatuses. 20—30 protsendil idulehtedest jäid lehelabad praktiliselt antotsüaanpigmendita. Erinevalt värvunud idulehtede glükosüülflavoonide sisalduses olulist erinevust ei täheldatud. Valguse ja kinetiini koostoimel ilmus antotsüaanpigmentatsioon kõigis idulehtedes üle populatsiooni. Nende kahe teguri koosmõju põhjustas antotsüaniini akumulatsiooni induktsiooni ka ülemises epidermises alumise epidermisega lähedasel määral. Järeldatakse, et kinetiini toimel laieneb kompetentsus antotsüaniini sünteesiks valguse mõjul ka ülemise epidermise rakkudele ja neile idulehtedele, milles geneetilistel põhjustel antotsüaniini valgustamisel ei teki.

ГЕТЕРОГЕННОСТЬ ПОПУЛЯЦИИ ПРОРОСТКОВ ГРЕЧИХИ ОТНОСИТЕЛЬНО НАКОПЛЕНИЯ АНТОЦИАНИНА ПОД ДЕЙСТВИЕМ СВЕТА И КИНЕТИНА

Антс ТОХВЕР, Лембе ЛААНЕСТ, Тийу ВАЙНЯРВ

Количество антоцианина, накопленного в ответ на действие света в семядолях (в нижнем эпидермисе) отдельных проростков гречихи, сильно варьировалось в пределах популяции. У 20—30% семядольных листьев образования антоцианового пигмента практически не наблюдалось. В семядолях с различной антоциановой пигментацией не обнаружили существенной разницы в содержании гликозилфлавонов и рутина. При совместном действии света и кинетина накопление антоцианина произошло во всех без исключения семядольных листьях популяции, причем накопление антоцианина наблюдалось также в верхнем эпидермисе приблизительно в равном количестве с таковым в нижнем эпидермисе. Делается вывод, что кинетин вызывает компетенцию к образованию антоцианина на свету и в верхнем эпидермисе, а также в тех семядольных листьях, которые по генетическим причинам лишены этой способности.