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COMPARISON OF LIGHT AND KINETIN EFFECTS ON ANTHOCYANIN BIOSYNTHESIS IN BUCKWHEAT SEEDLINGS

Ants TOHVER, Lembe LAANEST, and Elmo PALM

Institute of Experimental Biology, Instituudi tee 11, EE-3051 Harku, Harjumaa, Estonia; e-mail: ebi@ebi.estnet.ee

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Abstract. The time courses of light- and kinetin-induced anthocyanin accumulation in buckwheat cotyledons were compared by two characteristic parameters: duration of the lag phase and the steady-state accumulation rate. Light and kinetin were applied either separately, simultaneously, or in succession with the second factor applied after the full development of the response to the first one. The lag phase of the response after onset of light was 4 h, and after kinetin application 7 h. Simultaneous treatment of cotyledons with light and kinetin did not alter the rapidity of their responses. Prolonged pretreatment of cotyledons with light or kinetin prior to the application of the other factor did not eliminate the lag phase in the appearance of the response to the factor applied later. The accumulation rate of anthocyanin in response to combined treatment after complete development of the response was in all treatment regimes greater than the sum of separate light and kinetin effects. Our results indicate that the action of light on anthocyanin accumulation is not related to kinetin, but kinetin action is favoured by light. It is suggested that the enhancement of light- and kinetin-induced anthocyanin accumulation in cotyledons pretreated with the other factor reflects an increase in the number of anthocyanin-producing cells.

Key words: anthocyanin, buckwheat, light, kinetin, co-action.

INTRODUCTION

Plant morphogenesis, which includes both anatomical and biochemical differentiation, proceeds according to the encoded genetic programme whose expression is controlled by environmental factors, with light being the most crucial one. A well-known example of light-regulated biochemical differentiation

of seedlings is anthocyanin accumulation in their epidermal cell layers. Light-regulated tissue-specific accumulation of other flavonoids has also been described in several studies (for review see Wiermann, 1981; Beggs & Wellmann, 1994).

The increase in anthocyanin accumulation is also one of the responses to cytokinin treatment in many plants. Kinetin was shown to promote anthocyanin synthesis in petals of *Impatiens balsamina* (Klein & Hagen, 1961) and *Rosa hybrida* (Nakamura et al., 1980), and in seedlings of *Brassica oleracea* (Pecket & Bassim, 1974), *Fagopyrum esculentum* (Margna & Vainjärv, 1983), and *Arabidopsis thaliana* (Deikman & Hammer, 1995). An increase in the accumulation of other flavonoid compounds such as rutin and glycosylflavones in *Fagopyrum esculentum* cotyledons (Margna & Vainjärv, 1983, 1989) and glycosylflavones in *Hordeum vulgare* leaves (Laanest & Margna, 1985) as a response to kinetin treatment has also been observed. However, there are examples where cytokinin inhibited anthocyanin formation, for example, in the cotyledons of *Sinapis alba* (Tong et al., 1983).

The molecular basis of light-regulated biosynthesis of flavonoids has been shown to involve modulation of gene expression. A number of reports demonstrate that the light-caused increase in flavonoid biosynthesis is preceded by the induction of groups of co-ordinatedly regulated enzymes based on changes in the amounts and rate of synthesis of the corresponding mRNAs (Chappell & Hahlbrock, 1984; Takeda, 1990; Boss et al., 1996). Similarly, cytokinin regulation of flavonoid biosynthesis on the transcriptional level in *Arabidopsis thaliana* plants was recently demonstrated (Deikman & Hammer, 1995).

Genetic studies and single-cell microinjection assays of photoreceptor mutants with putative signalling intermediates or their analogues have made possible the identification of several elements of light signal transduction chains that control the switch between light-independent and light-dependent seedling development including the regulation of the expression of structural genes of flavonoid biosynthesis (for reviews see Chory et al., 1995; Holton & Cornish, 1995; McNellis & Deng, 1995). It was found that in mustard seedlings phytochrome action cannot be affected by the application of cytokinin (Kasemir & Mohr, 1982; Tong et al., 1983). However, cytokinins were shown to be involved in de-etiolation of *Arabidopsis* seedlings (Chory et al., 1994), and it was suggested that light might act via changes in cytokinin metabolism (Chory et al., 1995). Although the mode of light interaction with the hormone signal-transduction pathway is not yet understood, some tentative models for the functional relationships among genes conferring kinetin-altered light responses were proposed (Chory et al., 1994; Chin-Atkins et al., 1996).

If the accumulation of anthocyanin or some other end product of flavonoid pathway occurs in response to light or cytokinin action, posttranslational control mechanisms may also be involved. Earlier physiological and biochemical studies have pointed to phenylalanine supply and intracellular transport as a potential

regulatory factor for flavonoid synthesis (Margna, 1977). This, in its turn, may be regulated by light and kinetin (Margna & Vainjärv, 1983, 1989; Tohver, 1990; Tohver et al., 1996).

Whatever the actual mechanisms of action may be, the overlapping roles of light and kinetin in phenolic biosynthesis raise the question of whether light and hormone act independently or whether kinetin is involved in the sequence of events initiated by physiologically active photoreceptors.

To elucidate the problem, we adopted a physiological approach: comparison of the time courses of kinetin- and light-stimulated anthocyanin biosynthesis in buckwheat cotyledons. Anthocyanin accumulation in buckwheat seedlings is a sufficiently fast and intense response to both kinetin and light action for its quantitative characterization by simple methods.

MATERIAL AND METHODS

Buckwheat (*Fagopyrum esculentum* Moench cv. Victoria) seedlings were grown in darkness at 25 °C on filter paper moistened with tap water. 96 h after sowing cotyledons were excised in darkness, placed on filter paper moistened with distilled water or 0.3 mM kinetin solution, and incubated in the dark or in the light at 25 °C. The irradiation was carried out under white light fluorescent tubes at fluence rate 30 W m^{-2}. Preliminary experiments demonstrated that the stimulatory effect of kinetin on anthocyanin accumulation in buckwheat cotyledons can be observed already at 10^{7} M concentration of kinetin in the incubation medium. The dependence of anthocyanin accumulation on kinetin concentration was similar both in the dark and in the light with an optimum at 3×10^{-4} M. This concentration was chosen for the kinetic experiments.

Incubated cotyledons were fixed in boiling 1% HCl solution in 50% ethanol, homogenized, and extracted twice with 1% HCl in 50% ethanol. 95% ethanol was added to the combined extract. The extracts were stored overnight at –10 °C and centrifuged. The anthocyanin content of the supernatant was determined by the absorbancy at 546 nm.

Each sample consisted of 25 pairs of cotyledons. The presented data are mean values from at least 5 experiments.

The lag phase, the time gap between the onset of light or kinetin treatment and the start of the anthocyanin accumulation, and the anthocyanin accumulation rate were determined using linear regression lines to which the accumulation curves were approximated. The lag phase was defined as the *x*-coordinate of the intersection point of two successive regression lines, and the rate of steady-state anthocyanin accumulation was measured as the slope of the corresponding regression line.

RESULTS

The light or kinetin effect on anthocyanin accumulation in etiolated seedlings is quantitatively characterized by two parameters of the accumulation curve: duration of the lag phase before detectable increase in pigment formation and the rate of anthocyanin formation during the linear accumulation phase.

In our experiments the lag phase of light effect on anthocyanin accumulation in cotyledons of dark-grown seedlings was about 4 h (Fig. 1, curve *1*). The lag phase of kinetin response was found to be nearly three hours longer, about 7 h (Fig. 1, curve *2*). The time course of anthocyanin accumulation in the case of simultaneous application of light and kinetin demonstrates successive appearance of the response to light and kinetin action, respectively, and can readily be approximated to a broken line consisting of segments of three regression lines (Fig. 2). From this broken line it is possible to estimate two lag periods. The first one, which corresponds to the manifestation of light action, is equal to 4 h, and the second one, corresponding to the appearance of kinetin action (the time before reaching the final rate of accumulation is reached), is 7 h. Thus, simultaneous application of kinetin and light did not alter the lag time of their respective anthocyanin response.

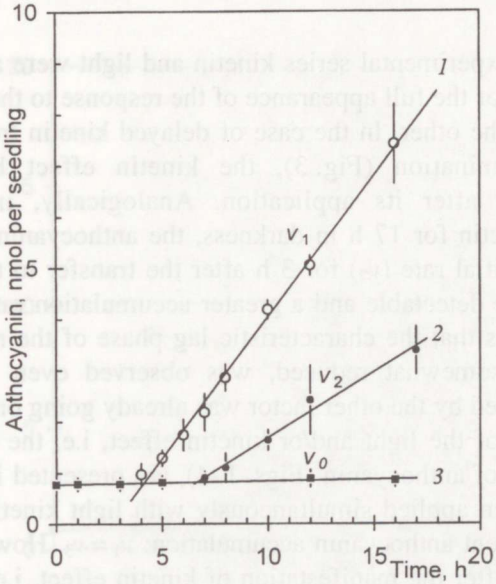

Fig. 1. The course of anthocyanin accumulation in buckwheat cotyledons. *1*, water, light; *2*, kinetin, dark; *3*, water, dark. $v_0 - v_2$, steady-state accumulation rates.

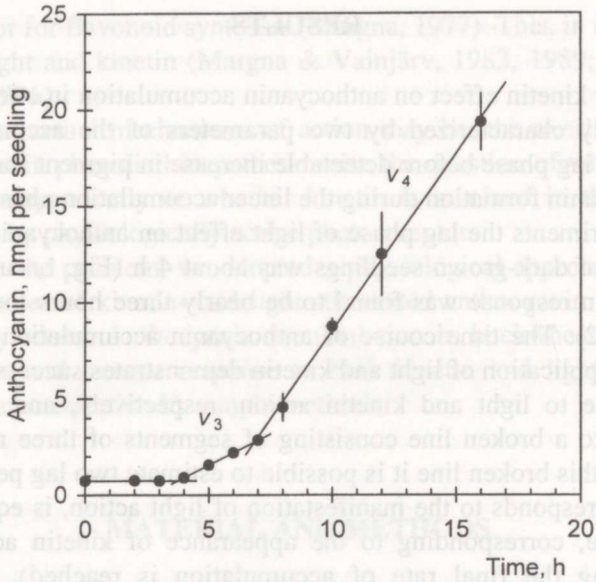

Fig. 2. The course of anthocyanin accumulation in buckwheat cotyledons incubated in kinetin solution in the light. v_3, accumulation rate before the appearance of kinetin response; v_4, the final accumulation rate.

In two further experimental series kinetin and light were applied with a time interval sufficient for the full appearance of the response to the first factor before the application of the other. In the case of delayed kinetin treatment, 12 h after the onset of illumination (Fig. 3), the kinetin effect became detectable approximately 5 h after its application. Analogically, in cotyledons first incubated with kinetin for 17 h in darkness, the anthocyanin accumulation still continued at the initial rate (v_7) for 3 h after the transfer to the light before the light action became detectable and a greater accumulation rate (v_8) was reached (Fig. 4). This means that the characteristic lag phase of the response to light or kinetin, although somewhat reduced, was observed even when anthocyanin accumulation induced by the other factor was already going on at full speed.

The magnitude of the light and/or kinetin effect, i.e. the rates of the steady state accumulation of anthocyanin (Figs. 1–4), are presented in the Table. These data show that when applied simultaneously with light kinetin did not alter the rate of light-dependent anthocyanin accumulation: $v_1 = v_3$. However, the increased accumulation rate after the manifestation of kinetin effect, i.e. the magnitude of kinetin effect in illuminated cotyledons ($v_4 - v_3 = 1.189$) exceeds 4.6-fold the kinetin effect in darkness ($v_2 = 0.259$). As a result, the accumulation rate of anthocyanins in response to combined light and kinetin treatment after full appearance of the response ($v_4 = 1.826$) was twice the sum of separate kinetin and light effects ($v_1 + v_2 = 0.894$).

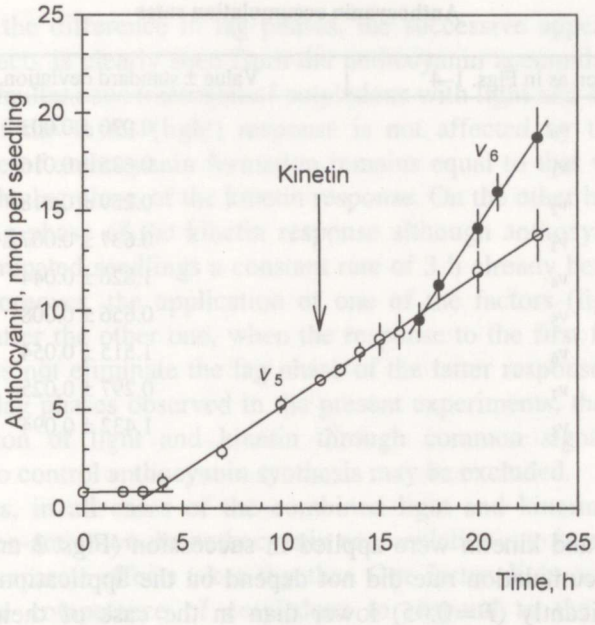

Fig. 3. The course of anthocyanin accumulation in illuminated buckwheat cotyledons. Kinetin treatment at 12 h. v_5, accumulation rate before kinetin treatment; v_6, the final accumulation rate.

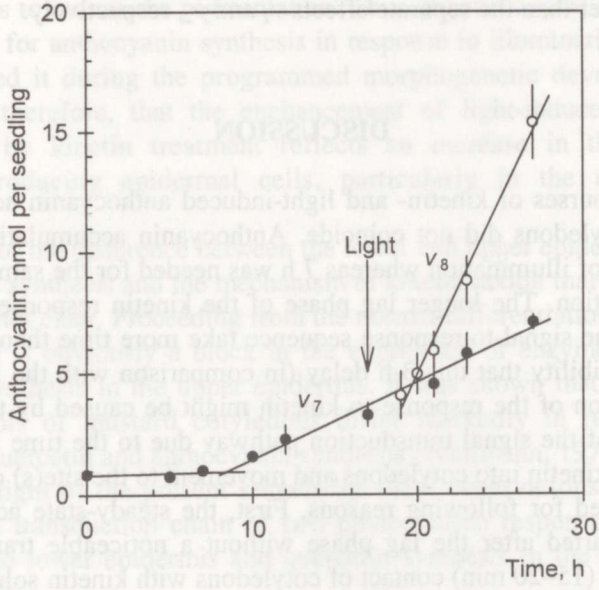

Fig. 4. The course of anthocyanin accumulation in kinetin-treated buckwheat cotyledons. Onset of illumination at 17 h. v_7, accumulation rate in the dark; v_8, the final accumulation rate.

Anthocyanin accumulation rates

Designation as in Figs. 1–4	Value ± standard deviation, nmol h^{-1}
v_0	0.030 ± 0.003
v_1	0.635 ± 0.016
v_2	0.259 ± 0.018
v_3	0.637 ± 0.007
v_4	1.826 ± 0.044
v_5	0.656 ± 0.008
v_6	1.515 ± 0.054
v_7	0.297 ± 0.025
v_8	1.432 ± 0.094

When light and kinetin were applied in succession (Figs. 3 and 4), the final anthocyanin accumulation rate did not depend on the application order ($v_6 = v_8$) but was significantly ($P = 0.05$) lower than in the case of their simultaneous application (v_4) although still much greater than the sum of the separate effects ($v_1 + v_2$).

Likewise, the light effect in kinetin-treated cotyledons ($v_8 - v_7 = 1.135$) and kinetin effect in illuminated cotyledons ($v_6 - v_5 = 0.859$) were significantly ($P = 0.05$) higher than the separate effects v_1 and v_2, respectively.

DISCUSSION

The time courses of kinetin- and light-induced anthocyanin accumulation in buckwheat cotyledons did not coincide. Anthocyanin accumulation started 4 h after the onset of illumination whereas 7 h was needed for the same process after kinetin application. The longer lag phase of the kinetin response indicates that the events in the signal-to-response sequence take more time than in the case of light. The possibility that this 3-h delay (in comparison with the light effect) of the manifestation of the response to kinetin might be caused by the shift in the starting point of the signal transduction pathway due to the time needed for the penetration of kinetin into cotyledons and movement to the site(s) of its reception may be excluded for following reasons. First, the steady-state accumulation of anthocyanin started after the lag phase without a noticeable transition period. Second, a brief (15–20 min) contact of cotyledons with kinetin solution results in an intensive anthocyanin accumulation (Margna & Vainjärv, 1983) which is close to the result of a prolonged incubation (our unpublished results). It is evident, therefore, that kinetin reaches its receptors without marked difficulties.

Because of the difference in lag phases, the successive appearance of light and kinetin effects is clearly seen from the anthocyanin accumulation curves in the case of a simultaneous treatment of cotyledons with light and kinetin (Fig. 2). The kinetics of the faster (light) response is not affected by the addition of kinetin: the rate of anthocyanin formation remains equal to that without kinetin till the end of the lag phase of the kinetin response. On the other hand, light does not alter the lag phase of the kinetin response although anthocyanin formation reaches in illuminated seedlings a constant rate of 3 h already before the end of kinetin lag. Moreover, the application of one of the factors (light or kinetin) several hours after the other one, when the response to the first factor has fully developed, does not eliminate the lag phase of the latter response. Thus, on the ground of the lag phases observed in the present experiments, the possibility of sequential action of light and kinetin through common signal transduction intermediates to control anthocyanin synthesis may be excluded.

Nevertheless, in all cases of the combined light and kinetin treatment the effect of their co-action on the anthocyanin accumulation rate is more than twice as high as the separate effects taken together. One factor, light or kinetin, seems to enhance the competence of cotyledons to respond to the other one by anthocyanin accumulation.

This result may be explained with the help of our previous findings (Tohver et al., 1995). It was demonstrated that under combined light and kinetin treatment anthocyanin accumulates substantially also in the upper side of cotyledons where light alone fails to induce the pigmentation. Thus, kinetin causes the appearance of competence for anthocyanin synthesis in response to illumination of cells not having acquired it during the programmed morphogenetic development. One may suggest, therefore, that the enchancement of light-induced anthocyanin accumulation by kinetin treatment reflects an increase in the number of anthocyanin-producing epidermal cells, particularly in the upper side of cotyledons.

The nature of the difference between the lower and upper epidermis in respect to anthocyanin synthesis and the mechanism of kinetin action that eliminates this difference are not clear. Proceeding from the normal differentiation pattern of the seedling, there is obviously a block in the expression of enzyme(s) specific to anthocyanin synthesis in the upper epidermis. It was shown that the upper and lower epidermis of mustard cotyledons differ markedly in respect to light regulation of quercetin and anthocyanin synthesis (Wellmann, 1974; Beggs et al., 1987). In the light of the present knowledge these observations give evidence that the signal transduction chain of two phytochrome responses, anthocyanin synthesis in the lower epidermis and quercetin synthesis in the upper one, are different.

Little is as yet known about the molecular mode of cytokinin action (Hobbie et al., 1994). Evidence has been recently obtained that cytokinin treatment of *Arabidopsis* (Chory et al., 1994) or a mutation that results in an increase in

cytokinin level of this plant (Chin-Atkins et al., 1996) is sufficient to override the light recquirement for leaf and chloroplast development and anthocyanin synthesis. Several models have been proposed (Chory et al., 1994) in which light and cytokinin act independently or sequentially through common signal transduction intermediates to control light-regulated responses. As shown above, our results indicate that cytokinin and light signal transduction pathways are different. The observed interdependence of light and kinetin action in the determination of anthocyanin accumulation suggests that light regulates the cytokinin signal transduction pathway, possibly according to the model proposed by Chin-Atkins et al. (1996) where an increase in endogenous cytokinin levels in turn results in altered light-regulatory response pathways. It is possible that kinetin treatment sets up a sufficiently high hormone level in cells to initiate the inductive signal. Exogenously applied kinetin can induce anthocyanin synthesis in buckwheat cotyledons; however, the response in the absence of light is weak. Light amplifies significantly the kinetin effect. This light amplification of kinetin action could be considered an alternative mechanism for light regulation of anthocyanin synthesis.

A similar phenomenon might be observed if light and kinetin signals regulated the activity of different enzymes in the pathway of anthocyanin synthesis. The study of light and kinetin effects on the synthesis of other flavonoid substances in buckwheat seedlings is now in progress. Its aim is to locate the possible light- and/or kinetin-sensitive regulatory step(s) in the branched flavonoid pathway.

REFERENCES

Beggs, C. J. & Wellmann, E. 1994. Photocontrol of flavonoid biosynthesis. In *Photomorphogenesis in Plants* (Kendrick, R. E. & Kronenberg, G. H. M., eds.). Kluwer Acad. Publishers, Dordrecht, 733–751.

Beggs, C. J., Kuhn, K., Böcker, R. & Wellmann, E. 1987. Phytochrome-induced flavonoid biosynthesis in mustard (*Sinapis alba* L.) cotyledons. Enzymic control and differential regulation of anthocyanin and quercetin formation. *Planta*, **172**, 1, 121–126.

Boss, P. K., Davies, C. & Robinson, S. P. 1996. Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv Shiraz grape berries and the implications for pathway regulation. *Plant Physiol.*, **111**, 4, 1059–1066.

Chappell, J. & Hahlbrock, K. 1984. Transcription of plant defence genes in response to UV light or fungal elicitor. *Nature*, **311**, 5981, 76–78.

Chin-Atkins, A. N., Craig, S., Hocart, C. H., Dennis, E. S. & Chaudhury, A. M. 1996. Increased endogenous cytokinin in the *Arabidopsis amp 1* mutant corresponds with de-etiolation responses. *Planta*, **198**, 4, 549–556.

Chory, J., Cook, R. K., Dixon, R., Elich, T., Li, H. M., Lopez, E., Mochizuki, N., Nagpal, P., Pepper, A., Poole, D. & Reed, J. 1995. Signal-transduction pathways controlling light-regulated development in *Arabidopsis*. *Phil. Trans. R. Soc. Lond.* B, **350**, 59–65.

Chory, J., Reinecke, D., Sim, S., Washburn, T. & Brenner, M. 1994. A role for cytokinins in de-etiolation in *Arabidopsis: det* mutants have an altered response to cytokinins. *Plant Physiol.*, **104**, 2, 339–347.

Deikman, J. & Hammer, P. E. 1995. Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana. Plant Physiol.,* **108**, 1, 47–57.

Hobbie, L., Timpte, C. & Estelle, M. 1994. Molecular genetics of auxin and cytokinin. *Plant Mol. Biol.,* **26**, 5, 1499–1519.

Holton, T. A. & Cornish, E. C. 1995. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell*, **7**, 7, 1071–1083.

Kasemir, H. & Mohr, H. 1982. Coaction of three factors controlling chlorophyll and anthocyanin synthesis. *Planta,* **156**, 3, 282–288.

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

Laanest, L. & Margna, U. 1985. Kinetin as an efficient stimulator of the formation of C-glycosyl-flavones in barley (*Hordeum vulgare* L.). *Plant Sci.,* **41**, 1, 19–22.

McNellis, T. W. & Deng, X.-W. 1995. Light control of seedling morphogenetic pattern. *Plant Cell*, **7,** 11, 1749–1761.

Margna, U. 1977. Control at the level of substrate supply – an alternative in the regulation of phenylpropanoid accumulation in plant cells. *Phytochemistry*, **16**, 4, 419–436.

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

Margna, U. & Vainjärv, T. 1989. Kinetin effects on the formation of flavonoids in buckwheat cotyledons depending on the timing and illumination conditions of the treatment. *Proc. Estonian Acad. Sci. Biol.*, **38**, 3, 200–207.

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

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, 1395–1399.

Takeda, J. 1990. Light-induced synthesis of anthocyanin in carrot cells in suspension. *J. Exp. Bot.,* **41**, 227, 749–755.

Tohver, A. K. 1990. Effect of light intensity and quality on shikimic acid accumulation in buckwheat seedlings affected with glyphosate. *Fiziologiya Rastenij*, **37**, 4, 712–717 (in Russian).

Tohver, A., Laanest, L. & Vainjärv, T. 1995. Heterogeneity in buckwheat seedling population in relation to light- and kinetin-induced anthocyanin accumulation. *Proc. Estonian Acad. Sci. Biol.,* **44**, 3/4, 109–118.

Tohver, A., Vainjärv, T. & Laanest, L. 1996. Shikimate accumulation in glyphosate-treated buckwheat cotyledons: Effect of the preillumination of the seedlings. *Proc. Estonian Acad. Sci. Biol.*, **45**, 1/2, 44–50.

Tong, Z., Kasemir, H. & Mohr, H. 1983. Coaction of light and cytokinin in photomorphogenesis. *Planta,* **159**, 2, 136–142.

Wellmann, E. 1974. Gewebespezifische Kontrolle von Enzymen des Flavonoidstoffwechsels durch Phytochrom in Kotyledonen des Senfkeimlings (*Sinapis alba* L.). *Ber. Deutsch. Bot. Ges.*, **87**, 2, 275–279.

Wiermann, R. 1981. Secondary products and cell and tissue differentiation. In *The Biochemistry of Plants*. Vol. **7**. Acad. Press, 85–116.

VALGUSE JA KINETIINI TOIME ANTOTSÜANIINI MOODUSTUMISELE TATRAIDANDITES

Ants TOHVER, Lembe LAANEST ja Elmo PALM

On võrreldud valguse ja kinetiini poolt indutseeritud antotsüaniini akumulatsiooni ajalist käiku tatra idulehtedes. Pigmendi akumulatsiooni viitaeg oli valguse puhul neli tundi ja kinetiini mõjul pimedas seitse tundi. Valguse ja kinetiini samaaegsel rakendamisel ilmnes valguse efekt samuti neli tundi pärast valgustamise algust ja kinetiini mõju kolm tundi sellest hiljem. Idulehtede üle kümne tunni kestev töötlemine valguse või kinetiiniga enne alternatiivse mõjuri rakendamist ei kõrvaldanud viimase vastusreaktsiooni viitaega. Valguse ja kinetiini koosmõjul saavutatav antotsüaniini akumulatsiooni kiirus ületas oluliselt nende tegurite poolt üksikult indutseeritud akumulatsiooni kiiruste summa. On järeldatud, et valguse toimemehhanism ei sõltu kinetiinist, kuid kinetiini toimemehhanism on valgustundlik. Varem saadud tulemuste alusel võib arvata, et kinetiin suurendab rakkude hulka, mis on võimelised valgustamisel antotsüaniini produtseerima.