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The effect of inductive photoperiod on flower formation and phytohormones level in a long day plant *Hyoscyamus niger* L.

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Abstract

The anatomical and hormonal investigations on a long day plant *Hyoscyamus niger* L. during the time of the generative photoinduction have been conducted. The plants were grown during 75 days on a short photoperiod and then they were transferred to long day conditions. The earliest anatomical symptoms of flower initiation were noticed after four long photoperiods. The inductive photoperiod causes also a general increase in the amounts of phytohormones. During the flower evocation the intensive accumulation of cytokinins and gibberellins in leaves takes place. The post-inductive period, in which the development of flower elements happens, is characterized by changing amount of phytohormones. The content of hormonal substances is subjected to the rhytmical changes related to the periods of light and darknees in the twenty four hours' cycle.

INTRODUCTION

All the hypotheses dealing with the hormonal regulation of flowering assume the existence of florigen in plants; they differ, however, in the conception concerning the nature of this factor. Considering the impossibility of chemical identification of floral stimulus the compositness and complexity of its structure has been proposed (Salisbury, 1967; Chailakhyan, 1968). So, florigen remains a physiological concept rather than a chemical reality. Carr (1967) differentiates florigen from flower hormones introducing the idea of primary and secondary flower induction. Such an assumption is also a starting point in the present investigations. It seems that the primary induction in photoperiod — sensitive plants is connected with the phytochrome action and consist in many modification of physico-

-chemical properties of cytomembranes. The secondary induction would be connected with the stabilization of the photoinduction, mainly through the creation of an appropriate balance of endogenous hormonal substances.

The presented investigations concern the anatomical and hormonal changes occuring during the photoinduction of flowering in a long day plant. These experiments are a part of investigations the aim of which is to study the physiological mechanism of generative differentiation in plants.

MATERIAL AND METHODS

The experiments were carried out under controlled conditions in growth chambers (8 or 16 hours of light, cool-white fluorescent tubes, light intensity about 6500 lux, 23°C in light and 18°C in darkness). Seeds of black henbane (*Hyoscyamus niger* L. f. *annus*) were germinated in containers with garden soil in a greenhouse. After 10 days the seedlings were selected and exposed in growth chambers to a short (8 hours of light) photoperiod during 75 days. Then, the plants were divided into two groups: controls—kept under the short photoperiod, and induced plants — transferred into 16 hours long photoperiod.

The samples for the anatomical investigation were taken from both groups of the seedlings on the following days: a. the last day of the short photoperiod (0-day), b. 1st, c. 2nd, d. 4th, e. 5th, f. 7th, g. 10th, h. 12th and i. 15th day of long photoperiod. Apical parts (about 0.5 cm long) were fixed with uranyl nitrate and formaline (Podbielkowska, 1971) or CrAF (Jensen, 1962) and embedded in paraffin (after dehydratation in a graded series of ethanol and infiltration with toluene). Serial horizontal sections cut at 6 μm were stained with Ehrlich hematoxylin (Johansen, 1940) or azure B (Flax and Himes, 1952).

The investigations on hormonal content were conducted at the following stages: A. the last day of the short photoperiod (0-day), B. 3th, C. 5th, D. 7th, E. 10th and F. 15th day of long photoperiod. The material for hormonal analysis was taken several times during short (0_1 — 8 hours of light, 0_2 — 8 hours of darkness, 0_3 — 16 hours of darkness) or long (1_1, 2_1, 3_1, 4_1, 5_1 — 8 hours of light, 1_2, 2_2, 3_2, 4_2, 5_2 — 16 hours of light, 1_3, 2_3, 3_3, 4_3, 5_3 — 4 hours of darkness, 1_4, 2_4, 3_4, 4_4, 5_4 — 8 hours of darkness) photoperiods. All handlings during the dark-time were done in dim green safe light.

Plant growth hormones (gibberellins, cytokinins, auxins and abscisic acid-like inhibitor) were extracted from the same samples (50 g of leaves) with 80% methanol during 48 hours at +5°C. The evaporation at +35°C removed the methanol leaving the aqueous residue par-

titioned twice at pH 2.7 with equal volumes of ethyl acetate and once with ethyl ether. The aqueous phase was kept for cytokinin determination. The combined ethyl acetate-ethyl ether fraction was then partitioned three times with equal volumes of 4% sodium bicarbonate solution. The combined bicarbonate fractions were adjusted to pH 2.7 and partitioned three times with ethyl acetate and ethyl ether. The acidic ethyl acetate-ethyl ether fraction was taken to dryness *in vacuo*. The residue was redissolved in 80% methanol and divided into three parts. One part was used for the determination of gibberellins, the second for auxins and the third one for abscisic acid-like inhibitor.

Cytokinins were puriefied according to the method of Hewett and Wareing (1973). The aqueous phase at pH 2.7 was absorbed on the cation exchange column (Dowex 50-X8H^+ 50-100 mesh) which was washed with 70% methanol and distilled water. The active substances were eluted with 2 and 5 N NH_4OH. After the evaporation the residue was chromatographed on Whatmann 3MM paper (solvent system: butan-2ol-25% NH_4OH 4 : 1 v/v) and bioassayed using the soybean tissue test (Miller, 1968).

Gibberellins were partitioned chromatographically using Whatmann 3MM paper with distilled water as a solvent. The place of localization of gibberellins (zone R_F 0.7-1.0) was eluted and rechromatographed using TLC (silica gel G, solvent system: benzene-acetic acid 10 : 3 v/v). The lettuce hypocotyl test (Frankland and Wareing, 1960) was used for quantitative determination.

Auxins were chromatographed on Whatmann 3MM paper with a solvent system: isopropanol-ammonia-water 10 : 1 : 1 v/v and bioassayed by the Avena section straight growth test (Nitsch, 1956).

Abscisic acid-like inhibitor was chromatographed on Whatmann 3MM paper with distilled water and bioassayed using the wheat coleoptile test (Bentley and Housley, 1954).

RESULTS

Flower evocation (see Evans, 1971) in *Hyoscyamus* plants was induced by the transferring of the 75 days old seedlings from a short photoperiod into the long day conditions. The investigation of the events associated with the flower formation concerned both the anatomical features as well as the analysis of plant hormones.

The anatomical symptoms of flower initiation were: a. length of internodes, b. number of produced axillary meristems, c. developmental changes of these meristems.

For the establishment of some correlation between the number of inductive photoperiods and the degree of flower bud development the following stages of axillary meristem differentiation were stated (Plate I):

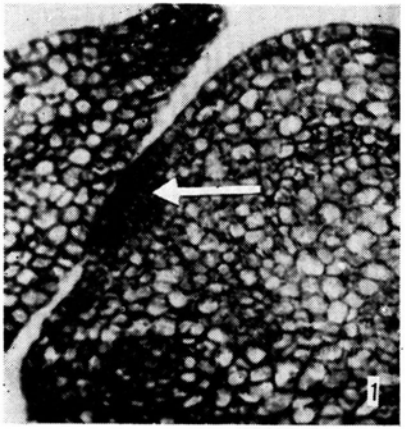
A. Axillary meristem composed of a few cells rich in RNA. No morphological differentiation among its cells were noticed (Photo 1).
B. Protuberant axillary meristem (Photo 2).
C. Axillary bud with initiated primordia of calyx and corolla (Photo 3).
D. Axillary bud with initiated primordia of calyx, corolla and stamens (Photo 4).
E. Axillary bud with all flower parts initiated (Photo 5).

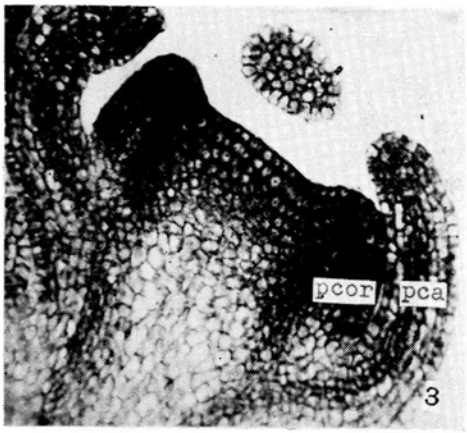
During the whole period of the experiment the control plants remained at the vegetative stage. No change in the length of their internodes was noticed (Fig. 1). The axillary meristems of these plants were produced only in the middle part of the shoot (at about the 11th node — counting from the tip of the shoot). These meristems were and remained small and poorly differentiated (Plate I).

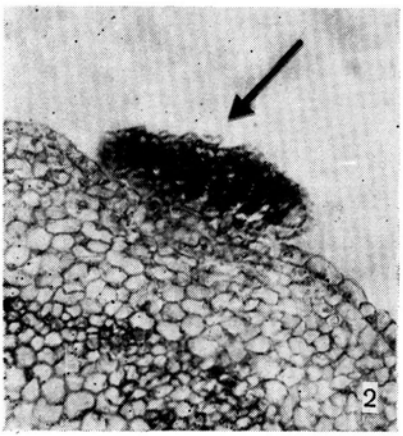
The black henbane plants transferred to the inductive photoperiod did not show any noticeably difference in comparison to the control plants during four long photoperiods: the shape of these plants, the length of their shoots and the stage of axillary meristem development were the same as in the control. After the fourth long photoperiod the gradual increase of the length of the shoot could be observed. At this very time the axillary meristems began to emerge also on the higher nodes of the plants up to the shoot apex. The gradual differentiation of the axillary meristems forming axillary and then flower buds was observed during the successive long photoperiods (Fig. 1). The high degree of the flower bud differentiation was noticed after 12 and 15 long photoperiods (Fig. 1). Taking these observations into account it is possible to suppose that the flower evocation of the investigated plants has taken place during the first four long photoperiods.

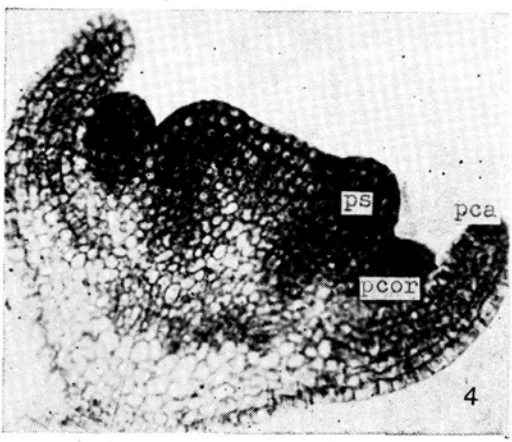
The investigations concerning the content of hormonal substances have been conducted during the last short (0-day) and 3rd, 5th, 7th, 10th and 15th long photoperiod.

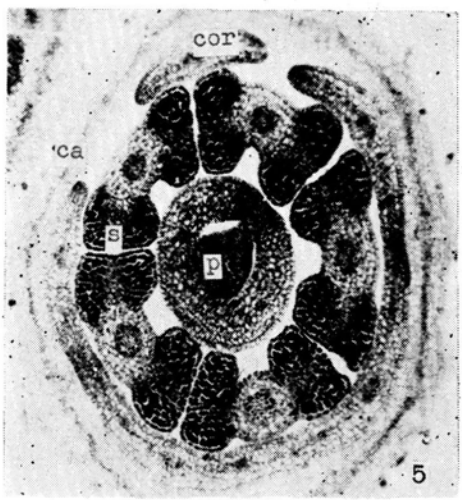
The results of the experiments on gibberellins show (Fig. 2) that in the leaves of control plants there are two groups of gibberellin-like substances localized at R_F 0.0—0.2 and R_F 0.4—0.6. During inductive photoperiods there appear two additional groups of gibberellins (R_F 0.6—0.8 and R_F 0.8—1.0). The total content of gibberellins also displayed considerable changes (Fig. 3). There are, so to say, two phases of occurrance of these substances. The first — from the beginning of inductive photoperiod until the fifth long day; during this period both the flower evocation and the successive increase of the











Successive stages of axillary differentiation in *Hyoscyamus niger* L.

Photos 1 and 2 — Transverse sections through the shoot — arrows indicating axillary meristem — stage A and B respectively.
Photos 3 and 4 — Longitudinal sections through young flower buds — stage C and D.
Photo 5 — Transverse section of flower bud — stage E
pca — calyx primordium; pcor — corolla primordium; ps — stamen primordium; ca — calyx, cor — corolla; s — stamen; p — pistillum

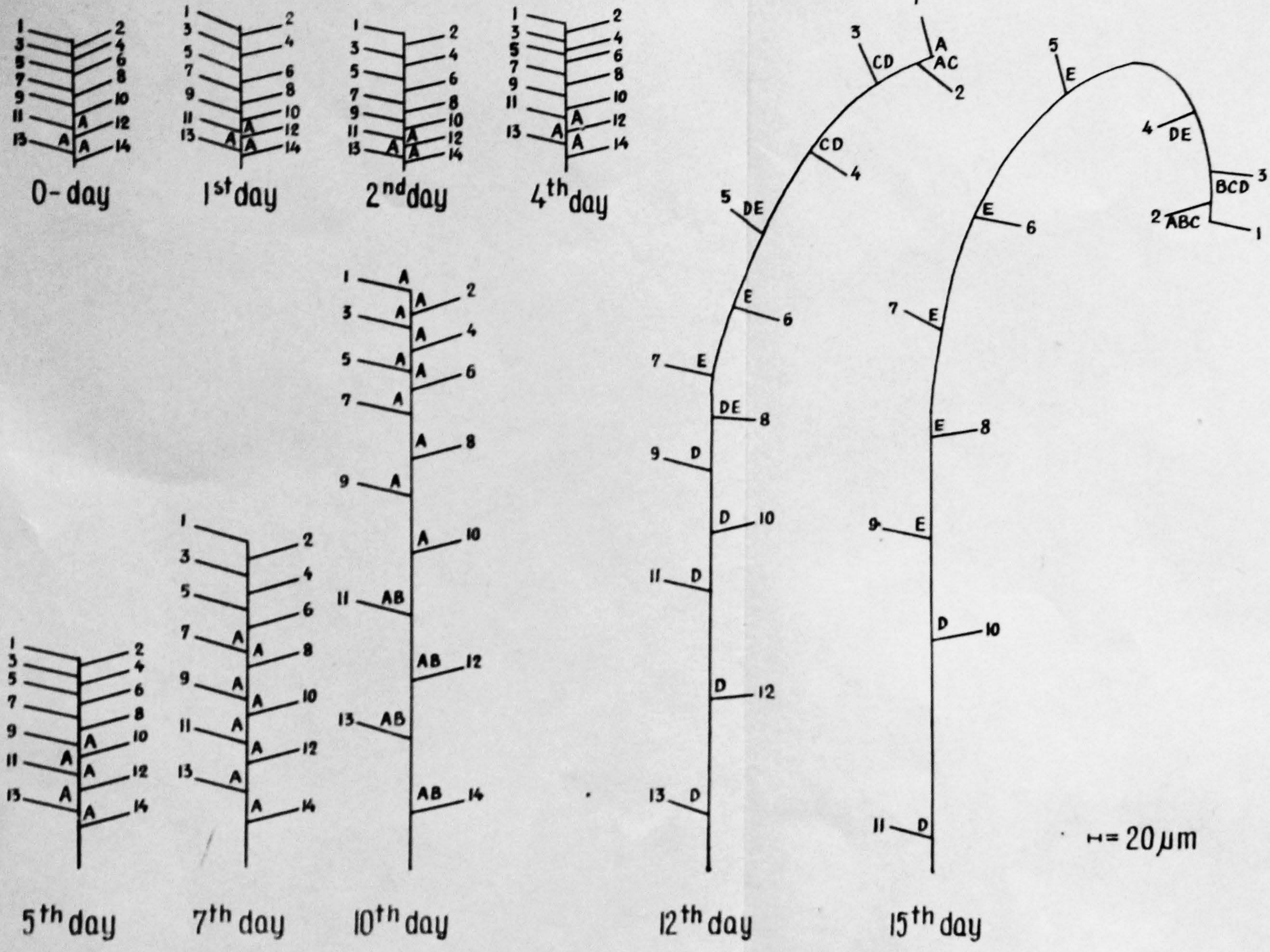


Fig. 1. Diagrammatic comparison of the length of internodes and axillary meristem differentiation between the *Hyoscyamus niger* L. plants grown under short (0-day) or long photoperiods (1—15 days)

1—14 — number of nodes counted from the apex of the shoot, A—E — stages of axillary meristem differentiation. Two or more symbols on the same node denotes individual differences among various plants of the same age

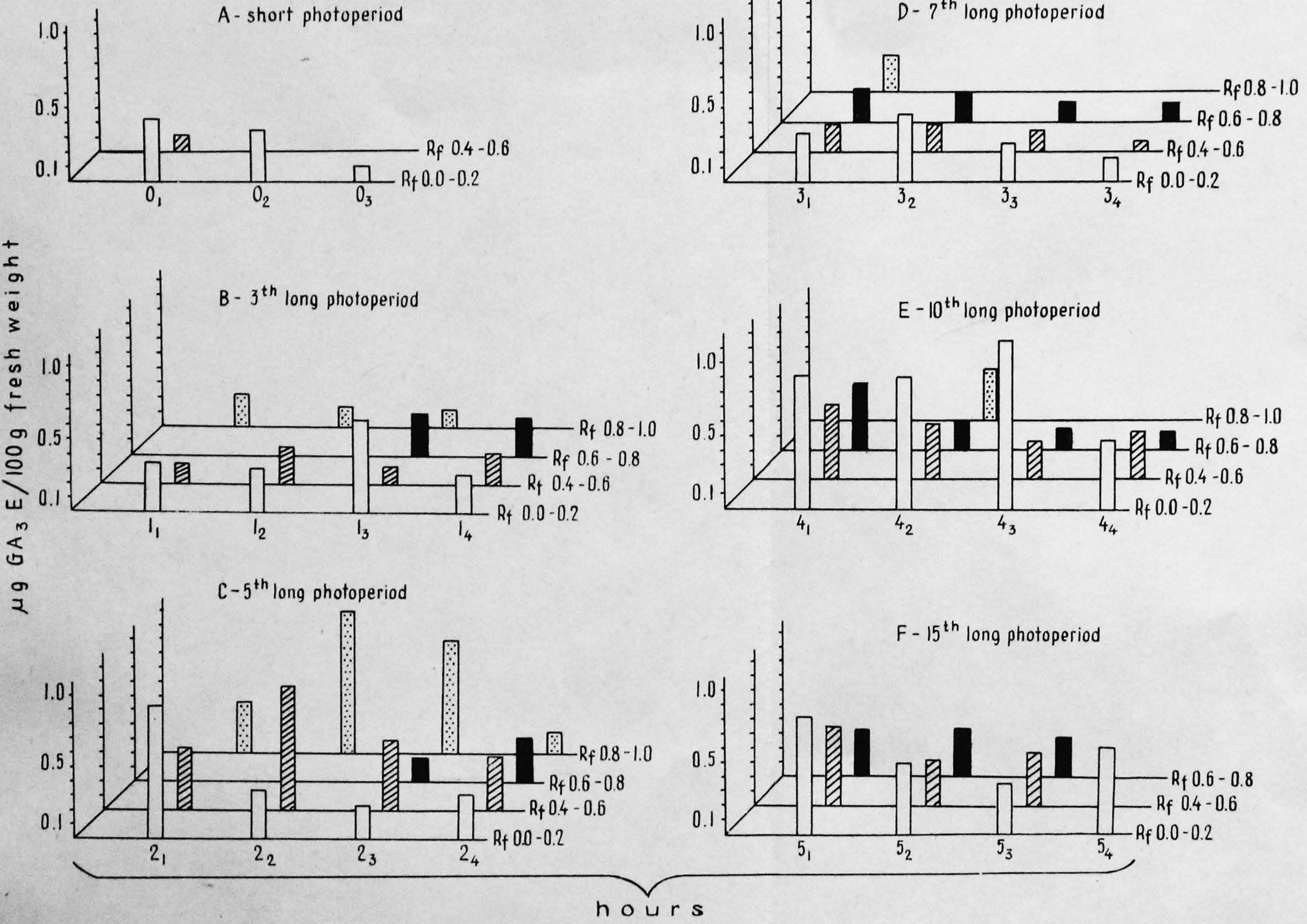


Fig. 2. Changes in the amounts of various gibberellin-like substances in leaves of *Hydscyamus niger* L. during the generative photoinduction

amouunt of gibberellins occur. The second phase — during which a stimulation of flower elements development takes place — are characterized by the changing amounts of gibberellin-like substances. The obtained results also show (Fig. 3) that within twenty-four hours' cycle there are noticeable differences in the content of gibberellins. The increased amounts of these compounds appear during a light period, whereas in darkness the decrease in the content of gibberellins is observed.

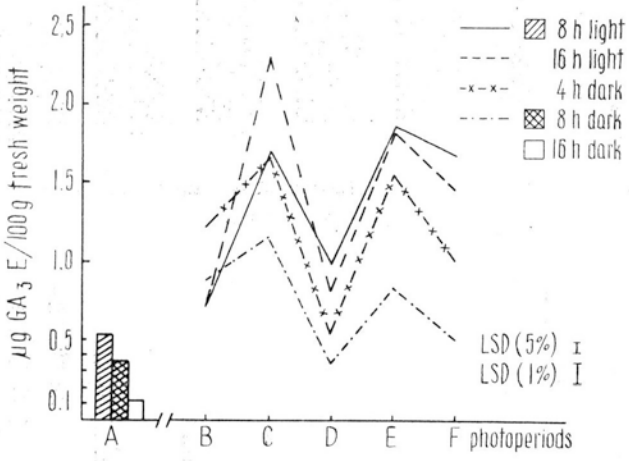


Fig. 3. Total amounts of gibberellins in leaves of *Hyoscyamus niger* L. during the generative photoinduction

The results of investigations on cytokinins show (Fig. 4) that the transferring of the plants into inductive photoperiod coincides with a rapid increase of the amounts of these substances in the leaves of black henbane plants. The highest content of cytokinins has been established during the third long day, that means in the period of reaching the state of flower evocation by plants. The later period has been characterized by a gradual drop of the content of these substances. In the leaves of black henbane, both on a short or long photoperiod, the presence of two groups of cytokinin-like substances has been stated (R_F 0.2—0.4 and R_F 0.6—0.9). The second zone displayed greater activity and its localization corresponded to zeatine ryboside. The changes in the content of cytokinins in twenty-four hours' cycle of light and darkness have been established. The content of cytokinins increased in the light phase. In the period of darkness the initial drop in the amount of these compounds had been observed, after the renewed increase of their content took place (Fig. 4).

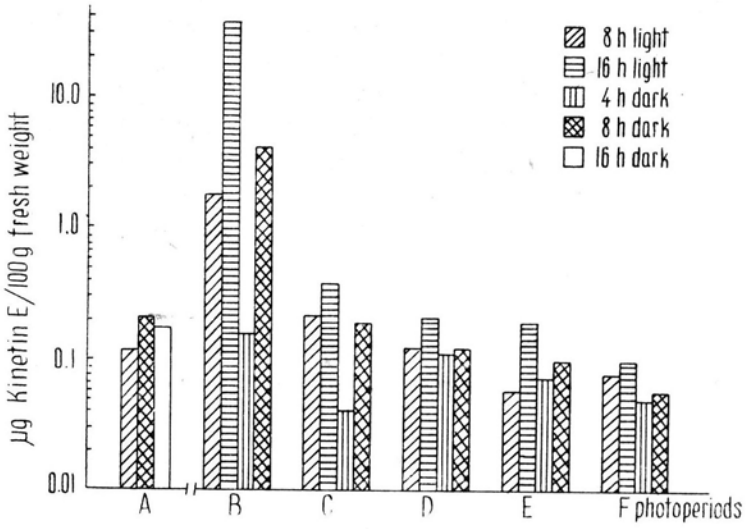


Fig. 4. Cytokinins in leaves of *Hyoscyamus niger* L. during the generative photoinduction

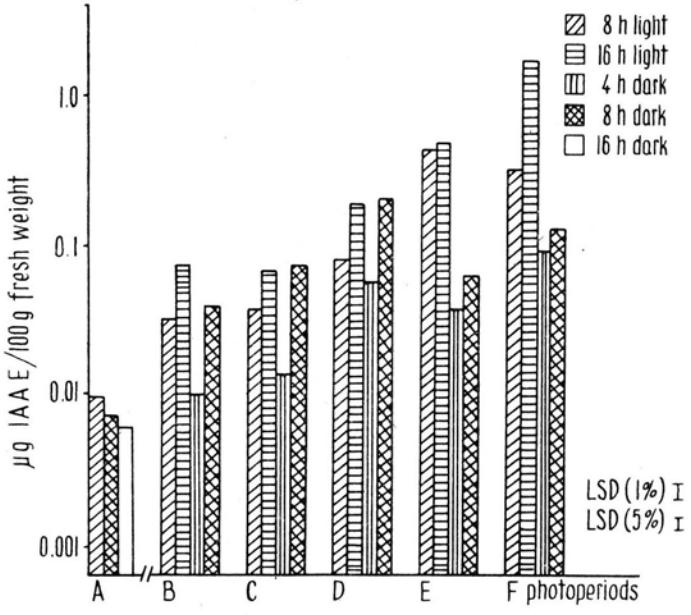


Fig. 5. Auxins in leaves of *Hyoscyamus niger* L. during the generative photoinduction

The results of the experiments on auxins show (Fig. 5) that the change from a short to a long photoperiod causes the increase in the content of auxins in the leaves of black henbane plants. The amount of auxins increased successively reaching its maximum during the 15th

long day. In the particular photoperiods the amount of auxins increased during the light phase and was next diminished in the first hours of darkness. During further hours of darkness, similarly as in the case of cytokinins, the renewed increase in the content of auxins has been observed (Fig. 5).

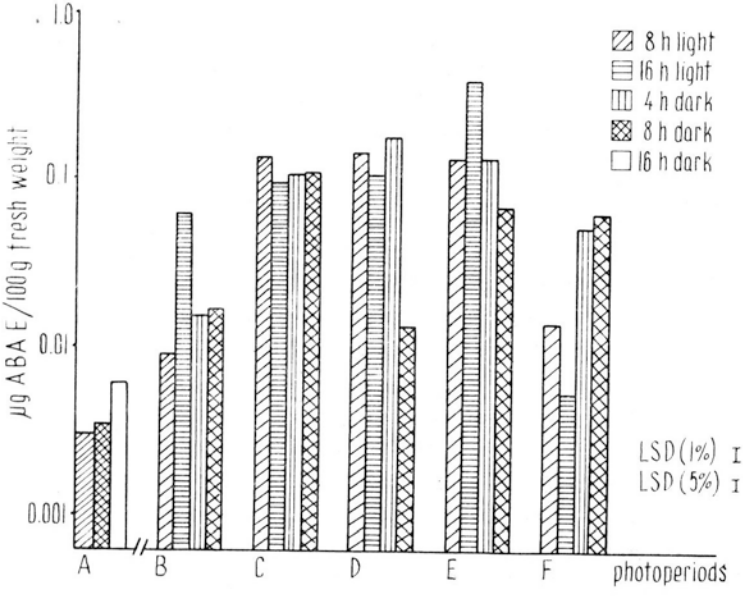


Fig. 6. Abscisic acid-like inhibitor in leaves of *Hyoscyamus niger* L. during the generative photoinduction

The results of investigations on abscisic acid-like inhibitors (Fig. 6) show that a long photoperiod causes the increase in the content of these substances. The successive increase of the content of the inhibitor is, however, observed only during the first five long photoperiods. The post-inductive period is characterized by the constant high level of abscisic acid in the leaves of *Hyoscyamus* plants.

DISCUSSION

Black henbane is a classical object in the experiments on plant photoperiodism (L a n g, 1965), that is why it was used in our investigations. The aim of the investigations was to study correlation between the flower differentiation and the content of endogenous hormonal substances in the leaves of induced plants. The plants were grown for 2.5 months under the conditions of a short (8 hours light) photo-

period. After this time the plants produced well-formed leaves, being all at the rosette stage. The only factor causing these plants to remain in the vegetative phase was the inappropriate length of days and nights. The transition of these plants to a generative stage was caused by changing the length of the photoperiod into an inductive one (Table 1).

Table 1

The effect of different photoperiods on flower formation in *Hyoscyamus niger* L. growing during 75 days under short day conditions

Number of photoperiod	Duration of light phase in 24 hours' cycle (hours)				
	8	10	13	16	24
20	0	0	30 *	100	100
40	0	0	70	100	100
60	0	0	100	100	100

* per cent of flowering plants.

For the characterization of the degree of flower differentiation the increase of internode elongation, the initiation of new axillary meristems and the gradualy differentiation of these meristems were taken into account. The increase of internode elongation is commonly known as a early symptom of flower evocation of rosette-forming plants (Lang, 1965). Choosing the axillary meristems for the anatomical observation followed from the fact that the individual flowers or flower bearing lateral shoots of black henbane are produced directly by the differentiation of the axillary meristems. However, it is known that the shoot apex of black henbane has also been changed during the generative photoinduction (Seidlova and Jurakova, 1964; Seidlova and Jurakova, 1965). Our studies have showed that the earliest symptoms of flower initiation are noticeable after four long photoperiods. This number of inductive photoperiods was mentioned as necessary for flower evocation also in the other varieties of *Hyoscyamus* plants (Lang, 1965).

The analysis of plant hormones was undertaken applying methods of extraction and fractionation which allow to determine all the groups of hormonal substances in the same sample of the plant material. It made possible to define the general hormonal situation during the particular periods of photoinduction. The obtained results bring in some new data to the understanding of the mechanism of generative photoinduction in long day plants. It showed that contrary to some earlier opinions (Lang, 1965; Chailakhyan, 1968), the amount of gibberellins increased successively only in the period of flower

evocation. At this time also new gibberellins that do not occur in plants during the non-inductive photoperiod appear in the leaves. Thus, inductive photoperiod leads to the pronounced changes in the content of endogenous gibberellins. There are numerous data in literature concerning the role of gibberellins in the flowering of long day plants (Lang, 1965, Chailakhyan, 1968). However, the opinions differ and many authors suggest that the primary effect of gibberellins action is the stimulation of the elongation of internodes. Our results seem to suggest that, at least in some plants, gibberellins may play important morphogenetic functions in flower induction. In the case of black henbane gibberellins localized on chromatograms at R_F 0.6—0.8 and R_F 0.8—1.0, that appeared only in the inductive photoperiod, may be suspected to take part in the mechanism of photoinduction.

The obtained results seem also to suggest an important role of cytokinins in the phenomena of generative photoinduction in long day plants. There are no literature data concerning the promotive effect of cytokinins on flowering of long day plants, apart from the work by Michniewicz and Kamieńska (1965) showing the influence of cytokinin on the flowering of long day plant *Arabidopsis thaliana* under non-inductive conditions. However, there are scarce and contradictory data concerning the influence of these compounds on the flowering of short day plants (Zeevaart, 1976; Krekule and Seidlova, 1977). The fact established in our experiments that the maximum of cytokinin occurrence appear in the period of flower evocation, preceding the maximum of gibberellin occurrence, strongly suggests that cytokinins and gibberellins toogether may play an important role in the induction and differentiation of flowers in long day plants.

As far as auxins and abscisic acid are concerned no distinct dependences have been found between the state of generative photoinduction and the content of these substances. The inductive photoperiod caused the successive increase in the content of auxins. It is consistant with previously obtained data (Lang, 1965; Chailakhyan, 1968). It is known, however, that a long photoperiod causes the increase in the content of auxins also in short day plants (Lang, 1965). Occasionally appearing suggestions (Addicott and Lyon, 1969) trying to treat abscisic acid as an inhibitor of flowering in long day plants on a short photoperiod, seem to be devoid of any substantial reasons. This compound cannot be any flowering inhibitor if its amount on the inductive photoperiod increases. So, the levels of auxins and abscisic acid-like inhibitor do not seem to be directly connected with the flower induction. It may be, however, that similarly as in the case of sex differentiation (Atsmon et al., 1968), the absolute amount of auxins and inhibitors in the differentiating tissues

is not so important as their proportional participation in relation to other groups of hormones.

Owing to the fact that during twenty four hours' cycle both the changes of light-darkness and photostationary state of phytochrome take place (Vince, 1972), the hormonal content was investigated at various periods of day and night. The obtained results indicate that the level of phytohormones is correlated with night and day changes of light and darkness. It suggests the participation of phytochrome in the regulation of hormonal substances level and may constitute an important manifestation of phytochrome participation in the control of plant photoperiodism.

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Wpływ indukcyjnego fotoperiodu na tworzenie kwiatów oraz poziom fitohormonów w roślinie dnia długiego Hyoscyamus niger L.*

Streszczenie

Przeprowadzono badania morfologiczno-anatomiczne pędów i merystemów pachwinowych oraz zawartości fitohormonów w liściach lulka czarnego w trakcie fotoindukcji generatywnej. Rośliny uprawiano w warunkach krótkiego fotoperiodu przez okres 75 dni, po czym poddawano je działaniu długiego — indukcyjnego fotoperiodu. Pierwsze anatomiczno-morfologiczne symptomy fotoindukcji generatywnej zauważono po upływie czterech długich fotoperiodów. Wykazano jednocześnie, że indukcyjny fotoperiod powoduje ogólne podwyższenie ilości fitohormonów. W okresie właściwej inicjacji generatywnej (pierwsze pięć długich fotoperiodów) dochodzi do wzmożonej akumulacji cytokinin i giberelin w liściach. Okres po-indukcyjny, w którym zachodzi różnicowanie się elementów kwiatowych, charakteryzuje się zmienną ilością fitochormonów. Zawartość substancji hormonalnych podlega rytmicznym zmianom związanym z występowaniem okresów światła i ciemności w cyklu dobowym.

* Praca wykonana w ramach tematu węzłowego Nr 09.7.3.1.5.