THE EFFECT OF EXOGENOUS ACETYLCHOLINE AND OTHER CHOLINERGIC AGENTS ON PHOTOPERIODIC FLOWER INDUCTION OF *PHARBITIS NIL*

HANNA ŁUKASIEWICZ-RUTKOWSKA, ANDRZEJ TRETYN, MARIUSZ CYMERSKI, JAN KOPCEWICZ

Nicholas Copernicus University,
Institute of Biology and Environmental Protection,
Department of Plant Physiology and Morphogenesis
Gagarina 9, 87-100 Toruń, Poland

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ABSTRACT

Exogenous acetylcholine (ACh), acetylcholinesterase (AChE) inhibitors, as well as agonists and antagonists of ACh receptors applied on the cotyledons of 5-day-old seedlings of *Pharbitis nil* during a 16-hour long inductive night or during a 12-hour long subinductive night, do not essentially influence the flower bud formation. Also the application of above mentioned substances to the seedlings growing under the conditions of 72 hours of darkness, 24 hours of light and then 24 hours of darkness does not influence in an essential way flowering of *P. nil*. On the other hand, applying these substances on the cotyledons of *P. nil* during 24-hour-long inductive night, preceded by 72 hours of darkness and then 24 hours of light of lowered intensity finished by 15-minute-long impulse of far red light which inhibit flowering, caused the reversion of the effect of far red light irradiation and stimulated the flowering. The obtained results suggest that endogenous system ACh/AChE could participate in the mechanism of a phytochrome controlled flowering of short day plants.

KEY WORDS: *Pharbitis nil*, flower induction, acetylcholine, acetylcholinesterase.

INTRODUCTION

One of the important environmental factors influencing the induction of flowering in plants is light. It is absorbed by a specific photoreceptor – a phytochrome, occurring in two molecular forms differing in spectral properties. The form Pr absorbs red light (660 nm) and the form Pfr – biologically active – absorbs far red light (730 nm). It is assumed that both a labile phytochrome and a stabile one participate in the control of photoperiodic induction (Thomas 1991). In plants treated with an inductive photoperiod a suitable balance of different forms of a phytochrome is established. The perception of light stimulus and the phytochrome reactions take place in the cotyledons and young leaves. The consequence of these processes is the change in the differentiation of growth apex towards a generative one. Thus, there must exist the way of information transition from leaves to apices. It is assumed that as a result of induction the flowering signal (inductor) is formed in cotyledons or leaves, and it is transported to the shoot apex (Vince-Prue 1994, O'Neil 1992).

In the 1970 Jaffe proposed that one of the links in the chain of transduction of light signal from the form Pfr of phytochrome to the photomorphogenetic response of plant may be acetylcholine (ACh). This animal neurotransmitter has been found in representatives of all major taxonomic groups of

plants (Hartmann and Gupta 1989, Tretyn and Kendrick 1991). In plants the enzymes taking part in both in synthesis (acetylcholinetransferase) and hydrolysis (acetylcholinesterase; AChE) have also been found (Hartmann and Gupta 1989, Tretyn and Kendrick 1991). There are also indirect evidences suggesting for the occurrence in the plant tissues of ACh receptors (Tretyn et al. 1992).

The common occurrence of ACh and other members of cholinergic system in plants may testify for its physiological importance. The effectiveness of cholinergic system in plants seems to be controlled by phytochrome (Jaffe 1976, Hartmann and Gupta 1989, Tretyn and Kendrick 1990). Many data point out also to the fact that ACh participate in the controlled by phytochrome transition of the plant from a vegetative phase into a generative one. In case of *Spinacia oleracea* ACh imitating the action of red light inhibited the stimulated by far red light activity of peroxidase (Penel and Greppin 1973, 1974). Under the conditions of continuous illumination ACh inhibited the flowering of *Lemna gibba* G1 and stimulated the flowering of *Lemna perpusilla*. Ladeira and others (1982) investigated the dependences existing between ACh and the flowering of *Porophyllum lanceolatum* and *Xanthium strumarium* – short day plants and *Sinapis alba*, a long day plant. The authors established the irrelevance between the increase of endogenous ACh level during an inductive dark

photoperiod and the flowering and the lack of influence of exogenous ACh and eserine on the flowering of these plants.

In present paper the attempt to define the role of acetylcholine and other compounds of cholinergic system in the generative induction of *Pharbitis nil* has been undertaken. The aim of this work was to investigate the effect of acetylcholine and the modulators of cholinergic system on the flowering of a short day plant *Pharbitis nil*.

MATERIAL AND METHODS

The investigations were conducted on 5-day-old seedlings of *Pharbitis nil* L. Chois (*Convolvulus nil* L., *Ipomoea nil* Roth., *Convolvulaceae*), of Japanese variety Violet (Morutane Co., Kyoto, Japan). *Pharbitis nil* is characterised by great sensitiveness, because it initiates flowers already after a single cycle: short day/long night. It reacts to photoperiodic induction at a very early stage of development of a seedling, showing the maximum of sensitiveness on the 5th-6th day of cultivation. Flower buds may be recognised and their number may be defined on the 10th-11th day after the induction.

In order to obtain homogeneous germination the seeds of *Pharbitis nil* were subjected to the treatment of concentrated sulphuric acid for 50 minutes. Then they were washed in warm (about 30°C), running water for 60 minutes. Washed seeds were immersed in distilled water and placed in a thermostat at 26°C for 24 hours. The seeds prepared in such a way were sown to plastic pots filled with a mixture of wet sand and perlite (1 : 2 w/w). The cultivation of plants was conducted under controlled temperature (26°C) and light conditions, in a growth chamber with a continuous light (cool white and white light, fluorescent tubes) having the intensity of 18.3 Wm⁻².

Depending on the type of an experiment the plants were cultivated under the following noninductive conditions: (i) 96 hours on continuous white light (intensity: 18.3 Wm⁻²), (ii) 72 hours in darkness and 24 hours on continuous white light of lowered intensity (intensity: 6 Wm⁻²), and (iii) 72 hours in darkness and 24 hours on white light of reduced intensity, finished with 15-minute-long impulse of far red light ($\lambda_{\max} = 730$ nm, $\lambda_{1/2} = 7$ nm, intensity, 0.87 Wm⁻²).

After four days of cultivation the plants were subjected to the following treatment: (i) 12-hour-long subinductive night, (ii) 16-hour-long inductive night, (iii) 24-hour-long inductive night.

The influence of exogenous ACh as well as modulators of cholinergic system such as: carbamylcholine (analogue of acetylcholine); nicotine and D-tubocurarine (an agonist and an antagonist of nicotine ACh receptors, respectively); muscarine and atropine (an agonist and an antagonist of muscarine ACh receptors, respectively), and eserine and neos-

tygmine (both AChE inhibitors) were investigated. The tested substances were applied on the cotyledons of *Pharbitis nil* with a sprayer or brush with soft horse hair. In all the experiments the control plants were sprayed or moistened with distilled water.

After the inductions the seedlings were cultivated for 14 days on the continuous light at 26°C. During that period they were watered with Knopp's medium. After the experiment was finished the number of flower buds was counted using the stereoscopic microscope. The flowering responses of the seedlings were expressed as: (i) average number of flower buds per plant, (ii) percentage of flowering plants (i.e. having 3 or more flower buds), (iii) percentage of plants with terminal flower buds. In every experiment two pots were used (2 x 15 plants) and experiments were repeated 3 times. The results were presented on diagrams and standard errors were calculated.

RESULTS

Effect of different light condition on flowering

The seedlings subjected to 16-hour-long period of darkness developed 6-7 axillary flower buds per plant. In case of the plant fully induced also terminal flower bud was formed. In the subinductive cycle (12 hours of darkness) the plants developed, first of all, vegetative buds and only small number of flower ones, on the average 2-3 per plant (Table 1). The extent of flowering of these plants was low, which was proved by the complete lack of terminal flower buds. The *Pharbitis nil* plants grown in cycle: 72 hours of darkness, 24 hours of light of lowered intensity, 24-hour-long inductive night – developed slightly fewer flower buds per plant, 5.1 on the average, but among other there were also fully flowering plants with terminal flower buds. The end-of-day irradiation with a 15-minute-long impulse of far red light caused the inhibition of flowering and the plants developed almost exclusively the vegetative buds, they did not bloom and did not develop terminal buds (Table 1).

Effect of ACh and cholinergic agents on flowering: 12-hour-long subinductive night

ACh and modulators of cholinergic system were applied on the plants once, before the dark period or during 12-hour-long subinductive night (Fig. 1). However, spraying the cotyledons of *Pharbitis nil* with the investigated substances both before and during the subinductive dark period had no considerable influence on flowering. What followed was only a slight increase of the average number of flower buds per plant, especially at the end of dark period when tested substances were applied during the 8th and 12th hour of darkness (Fig. 1).

TABLE 1. The effect of conditions of cultivation on flowering of *Pharbitis nil*. D – darkness, FR – far red light, WL – white light.

Conditions of cultivation	Average number of flower buds per plant	Percentage of flowering plants	Percentage of plants with terminal flower buds
Continuous WL	0	0	0
96 h WL + 12 h D	2.3 ± 0.19	0	0
96 h WL + 16 h oD	6.4 ± 0.18	100	100
72 h D + 24 h WL + 24 h D	5.1 ± 0.21	100	100
72 h D + 24 h WL + 15 min FR + 24 h WL	0.4 ± 0.27	0	0

Fig. 1. The effect of acetylcholine (10^{-5} M) and carbamylcholine (10^{-5} M) (A), eserine (10^{-4} M) and neostygmine (10^{-4} M) (B), atropine (10^{-5} M) and muscarine (10^{-6} M) (C), D-tubocurarine (10^{-5} M) and nicotine (10^{-6} M) (D) applied during 12-hour-long sub-inductive night on the flowering of *Pharbitis nil*.

In the next experiment ACh and modulators of cholinergic system were applied several times onto the same plants before and during 12-hour-long subinductive dark period (Fig. 2). The application of acetylcholine and modulators of cholinergic system during 12-hour-long subinductive night did not have any substantial effect on the flowering of *Pharbitis nil* (Fig. 2). The plants formed only more flower buds per plant in comparison with controls. The degree of flowering of these plants was however low and moreover they did not produce terminal flower buds.

16-hour-long inductive night

Cotyledons of *Pharbitis nil* were sprayed with ACh, its analogue, agonists and antagonists of acetylcholine receptors and acetylcholinesterase inhibitors, before as well as during the 16-hour-long inductive night. The obtained results have

Fig. 2. The effect of acetylcholine and cholinergic system modulators applied during 12-hour-long subinductive night on the flowering of *Pharbitis nil*. The examined substances were applied several times onto the same plant during a dark period at the following hours: 0, 4, 8, 12. They were used in the following concentrations: acetylcholine (10^{-4} M), carbamylcholine (10^{-4} M), atropine (10^{-4} M), muscarine ($5 \cdot 10^{-5}$ M), D-tubocurarine (10^{-4} M), nicotine ($5 \cdot 10^{-5}$ M), neostygmine (10^{-4} M), neostygmine (10^{-4} M) + ACh (10^{-4} M), eserine (10^{-4} M), eserine (10^{-4} M) + ACh (10^{-4} M).

Fig. 3. The effect of acetylcholine (10^{-5} M) and carbamylcholine (10^{-5} M) (A), eserine (10^{-4} M) and neostygmine (10^{-4} M) (B), atropine (10^{-5} M) and muscarine (10^{-6} M) (C), D-tubocurarine (10^{-5} M) and nicotine (10^{-6} M) (D) applied during 16-hour-long inductive night on the flowering of *Pharbitis nil*.

been presented in Fig. 3. It has been stated that the applied substances did not essentially influence the flowering of *Pharbitis nil*. These substances did not show any effects, or lowered to a slight degree the average number of flower buds per plant. Similarly as in control plants, 100% of plants under treatment started blooming and formed terminal flower buds (Fig. 3). However, a slight inhibition of *Pharbitis nil* flowering has been observed, when tested substances were applied on cotyledons at 8th hour of long inductive night. Especially distinct, inhibiting influence could be observed in case of nicotine, muscarine and when ACh was applied together with AChE inhibitors (Fig. 3).

In the next experiment (Fig. 4) ACh and modulators of cholinergic system were applied several times onto the same plants before the dark period and during the 16-hour-long inductive night. They did not influence the flowering when

Fig. 4. The effect of acetylcholine and cholinergic system modulators applied either during 16-hour-long inductive night on the flowering of *Pharbitis nil*. The examined substances were applied several times onto the same plant during a dark period at the following hours: 0, 4, 8, 12, 16. They were used in the following concentrations: acetylcholine (10^{-4} M), carbamylcholine (10^{-4} M), atropine (10^{-4} M), muscarine (5×10^{-5} M), D-tubocurarine (10^{-4} M), nicotine (5×10^{-5} M), neostygmine (10^{-4} M), neostygmine (10^{-4} M) + ACh (10^{-4} M), eserine (10^{-4} M), eserine (10^{-4} M) + ACh (10^{-4} M).

were treated before the dark period. The number of treated plants that were blooming and formed terminal buds amounted to 100% and the average number of flower buds per plant lowered only slightly. On the other hand, however, applying the tested substances during the inductive night caused the inhibition of flowering (Fig. 4). The greatest inhibition was observed in case of nicotine and muscarine, and when ACh was applied with AChE inhibitors, neostygmine and eserine. In this experiment all the applied substances caused the considerable decrease in the average number of flower buds per plant, in the percentage of plants with terminal buds as well as in percentage of flowering plants.

24-hour-long inductive night

ACh and modulators of cholinergic system were applied only once onto the cotyledons of *Pharbitis nil* during 24-hour-long inductive night (Fig. 5). Before the dark period the plants were cultivated for 72 hours in darkness and then for 24 hours on continuous white light with lowered intensity. Under these experimental conditions the control plants formed about 5 flower buds per plant, 100% from among them were blooming and formed terminal flower buds. The application of acetylcholine, agonists and antagonists of acetylcholine receptors as well as AChE inhibitors did not essentially influence the flowering of *Pharbitis nil* (Fig. 5) Only the reduced number of flower buds per plant could be observed. This effect could be most clearly observed in the 8th

hour of darkness. Nicotine, muscarine, neostygmine + ACh, eserine + ACh caused also the decrease of both percentage of flowering plants and the percentage of plants with terminal flower buds. In the remaining cases, similarly as in controls, all plants were in bloom and formed terminal flower buds (Fig. 5).

In the last experiment (Fig. 6) ACh and modulators of cholinergic system were applied on the cotyledons of *Pharbitis nil* only once during 24-hour-long inductive night. Before the inductive night the plants were kept in 72 hour in darkness, 24 hour on the continuous white light of reduced intensity and then, before transferring them into darkness (inductive night), they were irradiated for 15 minutes with far red light. End-of-day irradiation with far red light caused the complete blockade of flowering followed. The plants remained vegetative, they did not bloom and did not form terminal flower buds. On the other hand, however, the application of ACh, agonists and antagonists of acetylcholine receptors and inhibitors of AChE reversed the effect of far red irradiation (Fig. 6). All the applied substances markedly promoted the flowering of *Pharbitis nil*. The plants treated with tested substances formed flower buds. The strongest influence was observed when acetylcholine and modulators of cholinergic system were applied in the middle of the dark period. In all cases the increase in both the percentage of flowering plants and number of plants with terminal flower buds has also been observed (Fig. 6).

Fig. 5. The effect of acetylcholine (10^{-4} M) (A), eserine (10^{-4} M) and neostygmine (10^{-4} M) (B), atropine (10^{-4} M) and muscarine ($5 \cdot 10^{-5}$ M) (C), D-tubocurarine (10^{-4} M) and nicotine ($5 \cdot 10^{-5}$ M) (D) applied during 24-hour inductive night on the flowering of *Pharbitis nil*. Before the dark period the plants grew for 72 hours in darkness, and then 24 hours in continuous white light of reduced intensity.

Fig. 6. The effect of acetylcholine (10^{-4} M) (A), eserine (10^{-4} M) and neostigmine (10^{-4} M) (B), atropine (10^{-4} M) and muscarine (5×10^{-5} M) (C), D-tubocurarine (10^{-4} M) and nicotine (5×10^{-5} M) (D) applied during 24-hour inductive night on the flowering of *Pharbitis nil*. Before the dark period the plants grew for 72 hours in darkness and then 24 hours in continuous white light of reduced intensity. Before transferring the plants into darkness they were irradiated for 15 minutes with far red light.

DISCUSSION

A phytochrome is a receptor of light signal in the photoperiodic induction of flowering of a long- and short-day plants (Vince-Prue 1994). In case of *Pharbitis nil* breaking 16-hour-long inductive night with red light causes the inhibition of flowering. The action of red light is reversible, in particular, by means of far red light. In the eight hour of an inductive night this plant is especially sensitive to the impulse of red light (Vince-Prue 1994).

In the experiments during which plants were sprayed with exogenous ACh and modulators of cholinergic system we did not find any essential influence of these substances on the flowering of *Pharbitis nil* (Figs 1 and 3). Only a slight reduction of the average number of flower buds per plant was observed; it was particularly visible in the 8th hour of inductive night. However, when the examined substances were applied on the cotyledons with a soft horse hair brush every four hours during the inductive night a slight inhibition of flowering was observed (Figs 2 and 4). The higher effect was in case of ACh, especially when it was applied together with AChE inhibitors; and case of agonists of ACh receptors – nicotine and muscarine (Fig. 2). ACh, AChE inhibitors, agonists and antagonists of ACh receptors treated during 12-hour-long subinductive night were not able to stimulate flowering

(Fig. 1). However, when the tested substances were applied every 4 hours an increased number of flower buds per plant was observed (Fig. 2). So, from this part of experiments it was only possible to show a relatively slight participation of the cholinergic system in the induction of *Pharbitis nil* flowering.

Previously, we have shown that studies on photoperiodic flower induction should be conducted under the conditions enabling the functioning of only one phytochrome system – stable or labile one (Cymerski and Kopcewicz 1994, 1996). We have found that far red light applied at the end of light phase inhibits the flowering of *P. nil*. It has to be noticed, however, that far red light does not exert any influence, or the effect is only very slight, if the light phase of the photoperiod is too long, and the light is of high intensity. The most pronounced effect of far red light on flowering was obtained when *P. nil* seedlings were grown 72 hours in darkness, 24 hours on white light with lowered intensity (day) and then were treated with 24-hour-long inductive night. Under such conditions the seedlings enter the inductive 24-hour night with unfinished processes dependent on stable phytochrome (Cymerski and Kopcewicz 1996). The irradiation at the end of the day with the impulse of far red light causes the blockade of a stable phytochrome and the inhibition of flowering of *Pharbitis nil*. In such experimental condition it has been

stated that all the applied substances, particularly ACh, nicotine and muscarine were breaking the inhibition of flowering caused by far red light (Fig. 6). The greatest stimulation of flowering was recorded in the 8th hour of a dark period. So, the results of these experiments clearly suggest that ACh/AChE system is taking part in the phytochrome regulated mechanism of flower induction in *Pharbitis nil*.

We can assume that entering the first phase of the inductive 24-hour night by a plant is accompanied by maintaining a high concentration of endogenous ACh. This hypothesis is confirmed by two observations. Exogenous application of ACh, in all the tested experimental variations, has never caused the inhibition of flowering. On the other hand, the irradiation of *Pharbitis nil* seedlings at the end of the day with far red light, which induced the increase of AChE activity and decrease of ACh level, inhibited flowering. This inhibiting effect of far red light could be reversed through the application of exogenous acetylcholine.

It may be also assumed that the system ACh/AChE plays an important role in the mechanism of night-break-reactions. Irradiating the seedlings of *Pharbitis nil* in the 8th hour of the inductive night with red light results in the complete inhibition of flowering. As it has been established, in this period the highest level of ACh during the whole inductive night is recorded (Łukasiewicz-Rutkowska, unpublished data). The irradiation of plants with red light, which inhibits flowering, is probably accompanied by a additional increase of ACh concentration in the cotyledons, which is caused by the lowering of AChE activity. So, the inhibition of flowering may be a result of excessive increase of ACh level in the cotyledons. This phenomenon was confirmed by investigations in which exogenous acetylcholine, its agonists and antagonists were applied on the cotyledons of *Pharbitis nil*. In all these cases the maximum of flowering inhibition was observed in the middle of an inductive night (Figs 2 and 5).

In what way ACh influences the process of flowering induction and is taking part in night-break reactions? It may be only supposed that this substance initiating certain links in the chain of transduction of the phytochrome signal, stimulates the changes of membrane permeability for ions, which may have an important influence on the investigated process (Kandeler 1972, Greppin et al. 1973, Oota and Hoshino 1974, Greppin and Horowitz 1975, Hoshino 1979; Tretyn et al. 1990, 1992).

Generally, ACh participates in the regulation of many physiological and metabolic reactions of plants imitating the action of red light in the processes of growth and development mediated by a phytochrome (Tretyn and Kendrick 1991). It seems that ACh may also participate in the controlled by a phytochrome transition of plants from vegetative to a generative phase (Tretyn and Kendrick 1991). So far only few works have been published on this subject. Oota and Hoshino (Oota and Hoshino 1974, Oota 1977, Hoshino 1979) reported on the influence of cholinergic substances on the flowering of water duckweed. These authors showed that under the conditions of continuous illumination ACh inhibited the flowering of *Lemna gibba* G3 and stimulated the flowering of *Lemna perpusilla*. On the other hand, Ladeira and others (1982) established the lack of influence of exogenous ACh and eserine on the flowering of *Xanthium strumarium*, *Porophyllum lanceolatum* and *Sinapis alba*.

The obtained in this paper results suggests that the endogenous system ACh/AChE, could participate in a phytochrome-controlled flowering of short day plant *Pharbitis nil*. However, it is still difficult to decide whether ACh plays the

function of universal transmitter of the phytochrome signal also in other photoperiodic plants. In order to solve this problem further investigations covering both short and long day plants are necessary.

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WPŁYW EGZOGENNEJ ACETYLOCHOLINY I ZWIĄZKÓW CHOLINERGICZNYCH NA FOTOPERIODYCZNĄ INDUKCJĘ KWITNIENIA *PHARBITIS NIL*

STRESZCZENIE

Egzogenna acetylocholina (ACh), inhibitory acetylocholinoesterazy (AChE) oraz agoniści i antagoniści receptorów ACh podawane na liścienie 5-dniowych siewek *Pharbitis nil* w czasie 12-godzinnej podindukcyjnej, jak i 16-godzinnej indukcyjnej nocy nie wpływały w istotny sposób na przebieg indukcji kwitnienia. Również podawanie wymienionych substancji na liścienie siewek rosnących w warunkach: 72 godziny ciemności, 24 godziny światła, 24 godziny ciemności nie wpływało w istotny sposób na kwitnienie *P. nil*. Z drugiej strony podawanie omawianych substancji w czasie 24-godzinnej nocy indukcyjnej, poprzedzonej 72 godzinami ciemności, 24 godzinami światła (o obniżonej intensywności) zakończonymi 15-minutowym impulsem światła dalekiej czerwieni powodowało odwrócenie efektu końcowodniowego naświetlania daleką czerwienią (hamujące zakwitanie) i stymulowało kwitnienie *P. nil*. Uzyskane wyniki sugerują, że endogenne system acetylocholina/acetylocholinoesteraza może brać udział w kontrolowanej przez fitochrom indukcji kwitnienia roślin dnia krótkiego.

SŁOWA KLUCZOWE: *Pharbitis nil*, indukcja kwitnienia, acetylocholina, acetylocholinoesteraza.