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Flower induction in Norflurazon-treated *Pharbitis nil*: photo-induction of photoperiodic sensitivity in seedlings grown *in vitro* and daylength sensitivity in partly bleached potted plants

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SUMMARY

Photo-induction of photoperiodic sensitivity in seedlings of the short-day plant *Pharbitis nil* Choisy was unaffected by Norflurazon when grown under sterile conditions on a medium containing sucrose. Photoperiodic sensitivity was induced by 6 h light or by intermittent illumination. This is discussed in relation to results reported elsewhere on photo-induction of the capacity for dark reversion of the far-red absorbing form of phytochrome. Application of Norflurazon to the tips of potted plants grown in the light caused bleaching of the youngest leaves and internodes and stimulated flowering at a day length of 11 h, which normally results in weak photoperiodic induction.

Key-words: flower-induction, heterotrophic culture, Norflurazon, phytochrome action, short-day photoperiodism.

INTRODUCTION

Photoperiodicity in the short-day plant *Pharbitis nil* is controlled by phytochrome. Three phytochrome-dependent processes have been discerned. The first is a preparatory process which makes seedlings grown in the dark responsive to flower induction by an inductive dark period (Marushige & Takimoto 1967). Friend (1975) found that the preparatory process was phytochrome-dependent, that it required more than 12 h for completion and that the far-red absorbing form of phytochrome (Pfr) involved was stable. The second is a process which proceeds during the inductive dark period. This dark period must exceed a critical length for induction to occur. The third process becomes discernable at a critical phase during the inductive dark period when flower induction is counteracted by interruption with a short period of light (the night break effect). The second process depends on stable Pfr (far-red absorbing form of phytochrome) (Takimoto & Saji 1984), but the night break effect, which is also Pfr dependent (Fredericq 1964), is predicted to be controlled by a short-lived Pfr species. The stable Pfr of the second process and the Pfr of the night break effect have an opposite effect on flowering, which has led to the proposal that there are at

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Abbreviations: Norflurazon = 4-chloro-5-(methylamino)-2-(α,α,α,-trifluoro-*m*-roly-3(2H)pyridazinone; P = probability that a discussed difference is larger than zero; Pfr = far-red absorbing form of phytochrome; SEM = standard error of the mean.

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least two species of phytochrome (Pfr) operative in flower induction (Rombach *et al.* 1982). This proposal was extended by Takimoto & Saji (1984) and called the two phytochrome pool theory.

Investigations on phytochrome in illuminated *Pharbitis* seedlings showed the presence of stable Pfr (Heim *et al.* 1981, Brockman & Schaefer 1982, Rombach *et al.* 1982). Rapid dark-reverting Pfr was observed by Rombach (1986) in *Pharbitis* seedlings de-etiolated by white light. It was concluded that the capacity for rapid dark-reversion of Pfr was induced by a light treatment period of 6 h or longer, or an equivalent cyclic irradiation schedule. Since the aforementioned preparatory process of induction of photoperiodic sensitivity is also induced by light, it is possible that photoperiodic induction of flowering depends on the presence of this rapid dark-reverting Pfr. If this is the case, then photo-induction of photoperiodic sensitivity would take at least the same time as that for induction of the capacity for rapid dark-reversion of Pfr. The present experiments address this possibility.

In the experiments on photo-induction of the capacity for rapid dark-reversion of Pfr (Rombach 1986) the seedlings of *Pharbitis nil* were treated with Norflurazon. This was necessary since the phytochrome measurements required plants devoid of chlorophyll. Norflurazon is a herbicide which blocks carotenoid synthesis (Eder 1979). Photomorphogenetic responses are generally unaffected by Norflurazon (Jabben & Deitzer 1979), although sometimes they are promoted (Widell 1983). In the absence of carotenoids, the initial products of photoconversion of protochlorophyll are photobleached. However, as Norflurazon itself has been reported to have a stimulatory effect on flower induction in *Lemna gibba* (Cleland 1984), time dependence of photo-induction of photoperiodic sensitivity was studied in Norflurazon-treated seedlings. The cotyledons of seedlings grown in darkness have been used for phytochrome measurements in previous studies (Rombach 1986). The seeds contain sufficient food reserve for unfolding and growth of the cotyledons enabling phytochrome measurement (Rombach 1986) and for photoperiodic induction (Marushige & Takimoto 1967, Friend 1975), but for the formation of flower buds that are large enough to be distinguished from vegetative buds, the plants have to be sustained in growth for at least 2 weeks after photoperiodic induction. Seedlings treated with Norflurazon during germination die within 14 days, due to lack of photosynthesis, unless they are fed with a carbohydrate source. In the present experiments the plants were grown in sterile culture on a medium containing sucrose. The effect of sucrose has been investigated by Takimoto (1960), who observed that some flowering (15%) occurs in continuous darkness but that a normal short-day treatment is required for maximum flowering.

Before the experiments on photo-induction of photoperiodic sensitivity were conducted, we tested whether the system of photoperiodic induction of flowering, as it exists in *Pharbitis nil*, stayed intact after Norflurazon application to the apex and youngest leaves. Potted plants were used in these experiments and only the upper part of the shoot was bleached. The cotyledons and the basal 2 leaves remained green and provided the upper part of the shoot with photosynthate.

MATERIALS AND METHODS

Experiments with plants in sterile culture

Seeds of *Pharbitis nil* Choisy c.v. Violet, purchased from Marutane, Kyoto, Japan, were scarificated for 40 min in concentrated H_2SO_4, washed for 2 h in running water and

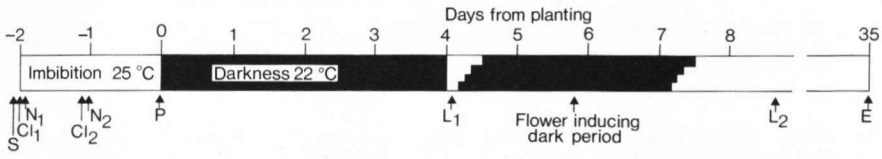

Fig. 1. Diagram illustrating the treatment schedule of the plants *in vitro*. The temperature was 22°C, unless otherwise indicated. S = 40 min scarification in 96% H_2SO_4; Cl_1 = 30 min sterilization by 15 g l^{-1} NaClO; N_1 = 24 h imbibition in 5×10^{-5} M Norflurazon; Cl_2 = 10 min sterilization by 1 g l^{-1} NaClO; N_2 = 24 h imbibition in 5×10^{-5} M Norflurzon; P = planting in culture medium; L_1 = preparatory white light treatment (9 W m^{-2}); L_2 = continuous white light (9 W m^{-2}); E = evaluation of flowering.

sterilized for 30 min in 15 g l^{-1} NaClO. The seeds were then incubated for 48 h in a sterilized solution of 5×10^{-5} M Norflurazon at 25°C in darkness. During the first 24 h the seeds imbibed and the seed coats burst. The seeds were then sterilized again (10 min 1 g l^{-1} NaClO) and transferred to a fresh Norflurazon solution. The water controls, without Norflurazon, received the same treatment. After a further 24 h the radicles protruded. The seed coats were peeled off under sterile conditions, otherwise they prevented unfolding of the cotyledons. The seeds were then placed in 26-mm diameter glass tubes containing glass beads and 10 ml of a modified Hoagland medium containing macro- and micro- elements (Rombach 1976) and 30 g l^{-1} sucrose. The medium was sterilized by autoclaving. After 4 days the culture tubes containing the plants were exposed to preparatory light treatments of different lengths, followed by an inductive dark period of 72 h. A diagram of the experimental schedule is given in Fig. 1. After the inductive dark period all plants were exposed to continuous white light (9 W m^{-2} fluorescent light TL 33 Philips, Eindhoven) for 14 days. They were subsequently examined for flowering under a stereomicroscope. Sections of buds with flower primordia showed that they were larger and differed in form from vegative buds, which remained small and dormant. The size and form of the buds were used as criteria for flower induction.

Experiments with potted plants

Origin and scarification treatment of the seeds was the same as described above. After a 2-h washing in running water the seeds were incubated for 24 h at 28°C in water, arranged on moist coarse sand and germinated at 21°C in darkness for 24 h. Seeds with protruding radicles were then planted in pots and exposed to continuous white light (9 W m^{-2} fluorescent light TL 33 Philips, Eindhoven). When the primary leaves were full-grown, the apices were submerged for 30 s in a solution of 5×10^{-5} M Norflurazon and 80 mg l^{-1} Tween 80 or in a control solution consisting of 80 mg l^{-1} Tween 80 in water. When the oldest leaf of these pretreated apices was full-grown, the plants received a single dark period of varying duration. Flowering was evaluated under a stereomicroscope after 14 days.

RESULTS

Experiments with potted plants

The lowest of the leaves which developed from the apices submerged in Norflurazon was fully expanded within 4 days. This leaf and the shoot above were completely white. In the subsequent days the midrib region of these leaves exhibited reversion of bleaching at the base and became pale green. After about 1 week the mesophyll of these leaves became

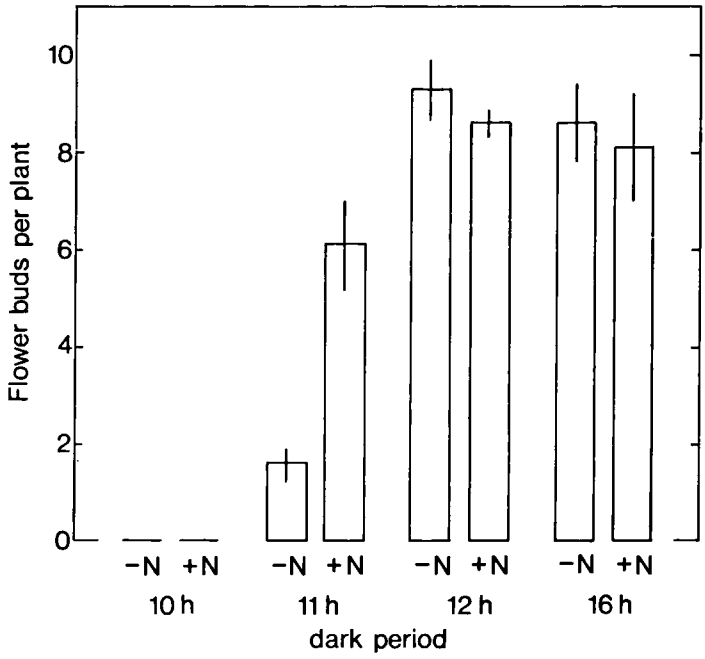

Fig. 2. Number of flower buds per plant ± SEM as a function of the length of a single dark period in plants treated with (+) or without (−) Norflurazon (N) at the apex 4 days before the dark period. Plants were in continuous light before and after the single dark period. The mean total number of buds per plant (vegetative and floral) was 18·2 ± 0·4 and the differences between the treatments in the total number of buds were insignificant.

necrotic, which could be prevented by keeping the plants at low irradiance. The Norflurazon-treated apex remained small; the leaf veins were bleached and in some plants the tip of the shoot died. The plant parts below the treated apex remained green. Flowering occurred in Norflurazon-treated and in control plants after a single dark period longer than 10 h. No flowering was observed in the axils of the leaves below those treated with Norflurazon or the control solution. A Norflurazon-stimulated flowering was observed repeatedly when induction was weak (a single dark period of 11 h). Figure 2 gives the results of an experiment in which all apices were still growing at the time of examination and the Norflurazon-treated plants developed the same total number of buds (vegetative and floral) as the control plants.

Experiments with plants in sterile culture

The growth of the plants in sterile culture was less uniform and slower than in soil. During the preparatory light treatment the controls were yellow or showed some greening while the Norflurazon-treated plants were white. During the 14 days continuous white light, after the 72 h inductive dark period, the Norflurazon-treated plants recovered slowly from bleaching (the culture medium of the Norflurazon-treated plants contained no Norflurazon). After 35 days, necrosis of the leaves was often observed. Nevertheless, Norflurazon treatment had no effect on the total number of buds per plant (floral and vegetative) 35 days after planting, except in Treatment 5 (Table 1) where the number of buds was significantly lower than in the Norflurazon-free controls($P > 97{\cdot}5\%$). The total number of buds was significantly increased by cyclic illuminations (Treatment 6).

Table 1. Flower buds and total buds (vegetative and floral) expressed as a mean number per plant ± SEM, as a function of the length of the white light period preceding the inductive dark period of 72 h or after cyclic treatments, as indicated. Plants were grown in sterile culture. The treatment was according to the scheme in Fig. 1

Treatment	Without Norflurazon			With Norflurazon		
	(*n*)	Total buds	Flower buds	(*n*)	Total buds	Flower buds
1. no light	(33)	5·4 ± 0·2	0·12 ± 0·07	(9)	5·2 ± 0·6	0·0
2. 4 h L	(58)	4·8 ± 1·2	0·12 ± 0·04	(56)	4·8 ± 0·1	0·25 ± 0·08
3. 6 h L	(22)	5·0 ± 0·3	0·82 ± 0·28	(25)	5·2 ± 0·2	0·76 ± 0·30
4. 8 h L	(17)	5·4 ± 0·2	1·41 ± 0·38	(22)	5·1 ± 0·2	0·91 ± 0·29
5. 12 h L	(17)	5·9 ± 0·3	1·00 ± 0·37	(14)	4·8 ± 0·3	0·36 ± 0·17
6. 3 × (8 h L–16 h D)	(14)	6·7 ± 0·4	2·90 ± 0·70	(7)	6·2 ± 0·3	2·50 ± 0·60
7. 24 × (5 min L–175 min D)16 h D	(7)	6·0 ± 0·2	2·60 ± 0·80	(10)	5·6 ± 0·5	2·70 ± 0·60

(*n*) = number of plants per treatment,
L = white light,
D = darkness.

For the number of flower buds, Norflurazon treatment resulted in an increase ($P>$ 90%) in light Treatment 2 (Table 1). We assume that the reduction by Norflurazon in light Treatment 5 was caused by the poor condition of the Norflurazon-treated plants for this experiment; evidence to support this assumption is that the total number of buds was also lower in the Norflurazon-treated cultures. In general, in these experiments Norflurazon had almost no effect on flowering. The effects of the light pretreatments were more pronounced: a pretreatment period of 4 h light slightly increased photoperiodic sensitivity when Norflurazon was present ($P>90\%$) but 6 h of light were required for a marked increase (the difference between light Treatments 2 and 3 was real with $P>97{\cdot}5\%$ for non-Norflurazon-treated and with $P>90\%$ for Norflurazon treated plants).

DISCUSSION

The experiments with partly bleached potted plants show that Norflurazon applied to the apex and youngest leaves does not interfere with photoperiodic flower induction. Norflurazon had a stimulatory effect on flowering at a dark period of 11 h, when photoperiodic induction is weak and a low level of flowering occurred in the absence of Norflurazon. A similar effect has been demonstrated by Ogawa & King (1979a) for the cytokinin benzyladenine.

The experiments on induction of photoperiodic sensitivity, carried out with seedlings grown *in vitro* on a medium containing sucrose, show that sucrose could not replace the light effect. However, a low level of flowering (2%) was obtained without induction by light (Treatment 1 in Table 1). Some escape from inhibition of flowering in plants kept under non-inductive conditions has often been reported, especially when the plants were grown under unusual conditions (e.g. complete darkness, Takimoto 1960).

Norflurazon had almost no effect on the induction of photoperiodic sensitivity. This was unexpected since it increased flowering at weak photoperiodic induction (Fig. 2), and Ogawa & King (1979b) have shown that benzyladenine, which has a similar effect to Norflurazon on photoperiodic induction, reduced the light period required for induction of photoperiodic sensitivity to 5 min.

Photoperiodic sensitivity was induced by a light treatment period of 6 h (Table 1). An increase in the length of the light period to 8 h or 12 h had no significant effect on flower induction after a 72-h dark period. Marushige & Takimoto (1967) have found, with potted plants, that 16 h light was required for induction of photoperiodic sensitivity, 8 h was insufficient. This does not disagree with our results since the inductive dark period of 16 h, which they used, was much less effective than the 72-h dark period used here (Saji *et al.* 1984).

Flowering at a level to that obtained by three cycles of 8 h light and 16 h darkness (Treatment 6 in Table 1) was obtained by substituting the light period with intermittent illumination of 5 min light every 3 h (Treatment 7). That brief illuminations can replace longer light periods is in agreement with the observations of Friend (1975) who has found that photoperiodic sensitivity of potted plants could be induced by two illuminations of 5 min red light when they were separated by a 24-h dark period, a 12-h dark period was ineffective. In Friends's experiments flowering was induced by a second dark period of 24 h after the second 5 min of red light. This second dark period can be considered to be the inductive dark period which is initiated by the light-off signal which has to be more than 12 h after the beginning of the first 5-min illumination. In our Treatment 4 (shown in Table 1) the light-off signal is at the end of the 6 h light period. We think that the much

shorter interval of time required in our experiments, from the beginning of the light period which induces photoperiodic sensitivity to the light-off signal initiating the inductive dark period, is caused by Pfr being more active in the light than in the dark. Evidence for this was found earlier in growth stimulation of *Lemma* (Rombach 1976).

The report by Rombach (1986) on phytochrome in *Pharbitis nil* showed that rapid dark-reversion of Pfr occurred in plants de-etiolated by a single light period of at least 6 h or by intermittent illumination. The *in vitro* experiments reported here show that sensitivity to photoperiodic induction of flowering develops under the same conditions. If we assume that the processes of flower induction in the *in vitro* cultures used here, and in potted Norflurazon-treated seedlings used previously for phytochrom measurements, proceed at the same rate, the unstable Pfr (= rapidly dark-reverting Pfr) could be involved in photoperiodic flower induction. We consider this Pfr to be the most likely candidate for the Pfr effective in the night-break effect on flowering.

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