Response of *Lemna perpusilla* to Periodic Transfer to Distilled Water[1]

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ABSTRACT

The flowering of *Lemna perpusilla* grown on half-strength Hutner's medium with sucrose under inductive photoperiods is inhibited in a periodic manner by daily transfers to water for short periods of time. The phase of maximal inhibition of flowering caused by water treatment is about 1 to 2 hours after the time of maximal sensitivity to light pulses. The rhythm of sensitivity to water treatments does not persist under continuous blue light. Supplementing the water with either $Ca(NO_3)_2$ or K_2HPO_4 partially reverses the inhibition of flowering, with the first salt being more effective. Supplementation with NH_4NO_3 or $MgSO_4$ increases the inhibition. The water effect on flowering is not observed in plants grown on half-strength Hutner's medium without sucrose. The water treatments may act by removing or destroying a crucial precursor for photoperiodic induction, with the other conditions modifying permeability. The system provides a new technique for investigating the mechanism of photoperiodic induction.

It has been well documented in the last few years that circadian rhythms are involved in the process of photoperiodic time measurement (1–5, 7, 15, 16). The supporting evidence consists mainly of good correlations between overt circadian rhythms and the photoperiodic responsiveness of the organism. However, a further step necessary to understanding the time measurement process is to determine specifically which metabolic oscillations interact with light-dark cycles. *Lemna perpusilla* is a convenient organism in which to study this problem since it is a short day plant (10), and its photoperiodic time measurement appears to be controlled by a circadian rhythm (7) which can be correlated to an overt circadian rhythm of CO_2 output (11). In addition, the composition of the defined medium on which the plant is grown modifies or controls its flowering response to photoperiod (6, 10, 13). This paper reports the effects of periodic transfer to distilled water on photoperiodically controlled flowering in *L. perpusilla*.

MATERIALS AND METHODS

Vegetative stocks of *L. perpusilla* strain 6746 were cultured on half-strength Hutner's medium supplemented with 30 mM (1%) sucrose, under continuous illumination of cool white fluorescent lamps (150 ft-c) with the air temperature 24 to 26 C (10).

Experimental cultures were started with single three-frond

colonies from stock cultures 10 to 12 days old and were grown on 30 ml of medium in 25- × 150-mm tubes capped with foam plugs. They were placed in growth chambers for 7 days under 8-hr photoperiods of about 500 ft-c of cool white fluorescent and incandescent light at an air temperature of 25 ± 0.5 C. Manipulations during the dark period were done under dim green light (green fluorescent tube behind one 3-mm thickness each of Rohm and Haas blue 2045 and amber 2451 Plexiglas). Blue light was obtained by filtering the light of standard blue fluorescent tubes through 3 mm of blue 2045 Plexiglas (8).

The fronds were transferred to sterile twice distilled water in 25- × 150-mm tubes with a spatula for the experimental period and were then transferred back to fresh medium. Sterilization of the spatula was accomplished by flaming with alcohol. To protect the plants from the flame light, they were placed in a light-tight wooden box located inside the growth chamber. Determination of flowering intensity was done on day 7 of the experiment by methods described earlier (6–10). Statistical analysis—analysis of variance and Duncan's new multiple range test—was applied according to the methods of Steel and Torrie (21).

RESULTS

Effect of Periodic Water Treatments under 8-hr Photoperiods. The purpose of the preliminary experiment described below was to determine whether the nutrient medium is required at all times during the 24-hr day for short days to elicit flowering. Figure 1 graphically describes the procedure of the experiment and summarizes the results. The experimental plants grown on full nutrient medium (half-strength Hutner's + sucrose) and 8-hr photoperiods were transferred to water for 8 hr during each of the consecutive 4 short days starting with the 1st day under 8-hr photoperiods. This transfer to water was done at different times of the day for different cultures. The cultures were then grown for another 3 days under the same light conditions after which they were dissected for determination of frond and flower number. Transfer to water slightly reduced the growth of the plants as expressed in frond number. However, complete inhibition of flowering occurred only when the water was experienced during the last 8 hr of the dark period. Water treatment during the light or the first half of the dark period slightly reduced the flowering percentage, probably through the reduction in frond number.

The next objective was to determine the time of floral inhibition by water transfer more accurately and to correlate that time with the light-sensitive phase (the time at which short light pulses maximally inhibit flowering). The same procedure was followed as in the preliminary experiments except that the plants were kept on water for either 4, 3, 2, or 1 hr at different times for the initial four consecutive cycles. Another group of plants experienced light pulses of 10 min at different times for either two or four cycles. Figure 2 summarizes the flowering intensity after 7 short days. There was no significant difference in frond number between control (in which the plants were transferred to half-strength
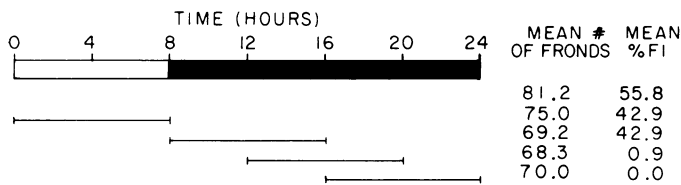
FIG. 1. Effect of transfers to water on growth and flowering of *L. perpusilla*. The plants were transferred to twice distilled water for 8 hr during each of the initial four consecutive cycles under short photoperiods. The times of water treatments are indicated by the horizontal lines. Empty and full bars indicate the light and dark periods, respectively. The stock and experimental cultures were grown on half-strength Hutner's medium supplemented with 30 mM sucrose. Plants were dissected after a total of 7 days under light-dark cycles of 8 hr light and 16 hr dark, and each number is a mean of five cultures. Time 0 is the time when the lights were turned on.
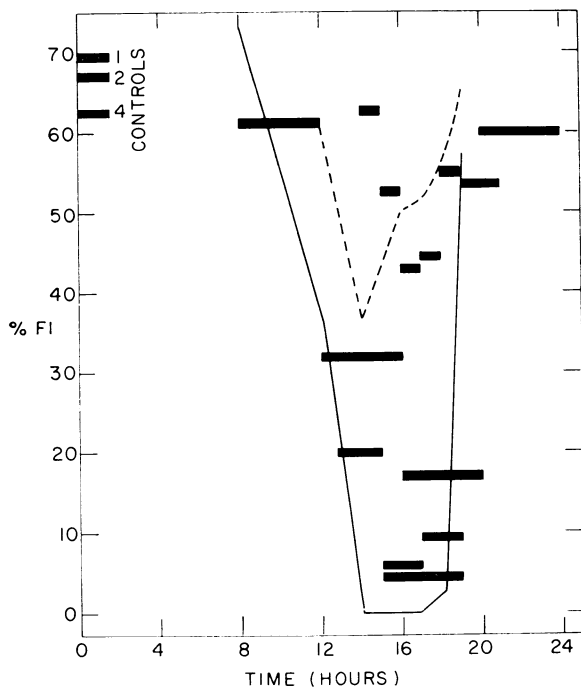

FIG. 2. Flowering response of *L. perpusilla* periodically transferred to water for 1, 2, or 4 hr during different phases of the 16-hr dark period. Each treatment was repeated for the initial four consecutive cycles. The length of each bar indicates the duration of the water treatment, and its height indicates the level of flowering by the end of 7 days of growth. The bars at upper left indicate the percentage of flowering in control cultures for the 1-, 2-, and 4-hr treatments. Solid lines represent the flowering response of cultures exposed to 10 min of white light (500 ft-c) at different times during the initial four consecutive dark periods; the broken line represents the effect of such treatment for only two consecutive cycles. Standard deviations, calculated for each experiment by the analysis of variance, ranged between 2.6 and 7.1.
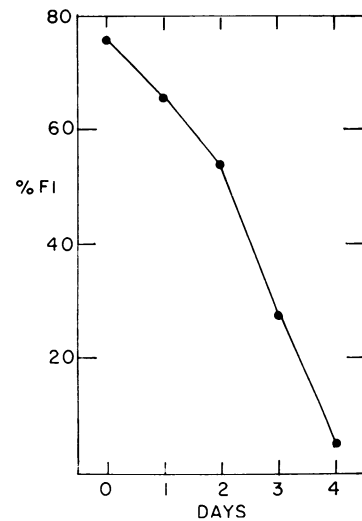

FIG. 3. The kinetics of flowering intensity with increasing numbers of cycles of water treatments. Plants were transferred to twice distilled water for 4 hr (15–19 hr after the light was turned on) for either one, two, three, or four consecutive cycles under 8-hr photoperiods. Standard deviation ± 5.4.
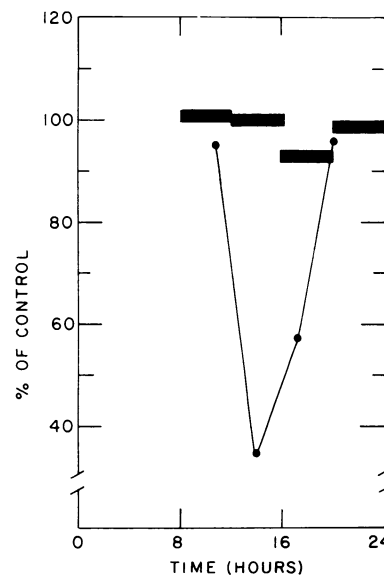

FIG. 4. Effect of periodic water transfers and light pulses on flowering intensity of plants grown on half-strength Hutner's medium without sucrose. Plants were transferred to the water (dark bars) for 4 hr during the initial four consecutive cycles in 8-hr photoperiods. Solid points represent the flowering response of cultures exposed to 10 min of white light (500 ft-c). Flowering evaluation was done after a total of 8 days in the same photoperiod. Standard deviations ranged between 4.5 and 4.9.

Hutner's) and experimental plants. The figure gives the value for 4-, 2-, and 1-hr water treatments since the 3-hr treatment gave similar results. Maximal inhibition of floral development occurred when the plants were transferred to water 15 to 19 hr after the lights were turned on with the 16th to 18th hr being the most sensitive time. An experiment in which the 4-hr water treatment was provided by a different method—the plants remained in one container and only the medium was changed—gave similar results, confirming the idea that the water effect is genuine and not a result of complex systematic errors.

The results of a kinetic experiment are summarized in Figure

3. A minimum of 2 consecutive days of repeated transferring to water was required for a significant reduction in flowering intensity. Additional days of treatment resulted in further declines of flowering percentage.

Effect of Periodic Water Transfer under Continuous Blue Light. To determine whether alternations between states of maximal and minimal sensitivity to water persist under constant conditions, the plants were grown under continuous blue light (8, 9) for 7 days and were transferred to water for a period of either 3 or 4 hr at different times during the initial 4 days. They were previously grown under either continuous fluorescent and

incandescent light (500 ft-c) or under light-dark cycles of 16 hr light and 8 hr dark for 4 days. The transfer to blue light was either at the end of the light period or at the end of the dark period. In all of these experiments the flowering level of the water-treated plants was significantly lower than the roughly 40% level of the controls, but the differences in flowering percentage between plants treated with water at different times were not significant.

Components of the Medium

Sucrose and NH_4^+. The inhibition of flowering by transfer to water was a function of the presence of sucrose. Plants grown on half-strength Hutner's without sucrose for about 2 weeks were not affected at all at any time by the water transfer, in spite of the fact that light pulses were still highly effective (Fig. 4). Nor did any inhibition of flowering occur when plants grown without sucrose were periodically transferred to 30 mM sucrose solution.

In further experiments, plants were grown on a modified half-strength Hutner's medium in which the NH_4NO_3 was replaced either by KNO_3 or NH_4Cl on a molar basis or by 0.01% (w/v) tryptone plus 0.06% (w/v) yeast extract ("NO_3" + S, "NH_4" + S and "Tryp + Y" + S respectively, Table I). With ammonium ions alone as the nitrogen source in the growth medium, there was a significant reduction of flowering after 4-hr water treatment, as compared with medium supplemented with nitrate or tryptone and yeast extract. Nevertheless, the presence of ammonium ions was not a prerequisite for the appearance of the water effect.

Parallel tests for the light sensitivity of plants grown on media with different nitrogen sources further showed that there was substantially less flowering on "NH_4" + S medium compared to "NO_3" + S medium and ½ H + S after treatment with light pulses for three cycles at the most sensitive phase.

Macronutrients. Cultures were grown as usual in half-strength Hutner's + sucrose. The plants were then transferred as described above either to twice distilled water (water control) or to half-strength Hutner's + sucrose (medium control) or to water supplemented with one of the following salts, normally present in

Table I. *Effect of Nitrogen Source and 4-hr Water Treatments on Flowering of L. perpusilla*

½ H + S: Half-strength Hutner's medium supplemented with 1% sucrose. "NO_3" + S: A modified half-strength Hutner's medium in which NH_4NO_3 was replaced by KNO_3 on a molar basis and supplemented with 1% sucrose. "NH_4" + S: As above except that NH_4NO_3 was replaced by NH_4Cl on a molar basis. "Tryp + Y" + S: NH_4NO_3 was replaced by 0.01% (w/v) tryptone and 0.06% (w/v) yeast extract. Stocks: Growth medium of stock plants during the last week before the experiment started. Time: Hours after the lights were turned on a light-dark cycle of 8 hr light and 16 hr dark. Values marked with two asterisks are significantly (1% level) lower than values for water treatment on other media. Each water value is significantly lower than its corresponding control. Values are means of five replicates.

Growth Medium		Percentage Flowering (Fl%)		Time
Stocks	Experimental	Water	Control	
½ H + S	½ H + S	44.5	77.4	16–20
"NO_3" + S	"NO_3" + S	50.1	78.3	16–20
½ H + S	"NH_4" + S	27.0**	74.2	16–20
½ H + Tryp + Y + S	"Tryp + Y" + S	62.1	86.0	15–19
½ H + Tryp + Y + S	"NO_3" + S	64.7	84.8	15–19
½ H + Tryp + Y + S	"NH_4" + S	40.8**	74.3	15–19

Table II. *Effect of Repeated 4-hr Treatments with Solutions of Macronutrient Salts on Flowering of L. perpusilla*

Water control: The percentage of flowering in plants transferred to twice distilled water at the same time for the same duration. Medium control: The plants were transferred instead to half-strength Hutner's + sucrose. Time: Hours after the lights of the 8-hr photoperiod were turned on. Values marked with two asterisks are significantly different from the water control at the 1% level. Values are means of five replicates.

Macronutrient	Concn	pH	Percentage Flowering (Fl%)			Time
			Experimental	Control		
				Water	Medium	
	mM					
a. Macronutrient salts in Hutner's medium						
Ca(NO$_3$)$_2$-4H$_2$O	0.75	6.4	25.9**	4.7	68.3	15–19
	0.75	9.8	52.8**	15.2	66.3	16–20
K$_2$HPO$_4$	1.15	6.4	7.4	4.7	68.3	15–19
	1.15	7.3	28.7**	4.7	68.3	15–19
	1.15	7.3	40.5**	15.2	66.3	16–20
NH$_4$NO$_3$	1.25	6.4	0.0	4.7	68.2	15–19
	1.25	6.4	1.3**	15.2	66.7	16–20
	1.25	6.4	3.9**	49.6	82.4	15–19
MgSO$_4$	1.00	6.4	0.0**	15.2	66.7	16–20
	1.00	6.4	11.2**	49.6	82.4	15–19
b. Test for the active ions in a—all at pH 6.4						
NH$_4$NO$_3$	1.25		3.9**	49.6	82.4	15–19
KNO$_3$	1.25		60.2	57.9	84.0	15–18
NH$_4$Cl	1.25		36.1**	49.6	82.4	15–19
MgSO$_4$	1.00		11.2**	49.6	82.4	15–19
MgCl	1.00		4.0**	49.6	82.4	15–19
K$_2$SO$_4$	1.00		6.6**	49.6	82.4	15–19
K$_2$HPO$_4$	1.15		62.7	57.9	84.0	15–18
KCl	0.92		37.7	37.2	70.4	15–19
Ca(NO$_3$)$_2$	0.75		72.5**	49.6	82.4	15–19
CaCl$_2$	0.75		73.3**	49.6	82.4	15–19

Hutner's medium: Ca(NO$_3$)$_2$, K$_2$HPO$_4$, NH$_4$NO$_3$, and MgSO$_4$. The pH of each solution was either adjusted to pH 6.4 (as normally used in the growth medium) with HCl or KOH, or was left at its original level (Table IIa). In no case was there a significant difference in frond number. As Table IIa demonstrates, Ca(NO$_3$)$_2$ was the most effective salt in promoting flowering. K$_2$HPO$_4$ was effective at its original pH 7.3; however, at pH 6.4 there was no significant promotion of flowering percentage. Both macronutrients resulted in flowering percentage significantly below the medium control. Supplementing the water with either NH$_4$NO$_3$ or MgSO$_4$ had a significant inhibitory effect on flowering compared to the water control.

In order to identify the active ions responsible for either the promotion or inhibition of flowering, the plants were transferred to solutions of different salts (Table IIb), the pH of which was adjusted to 6.4, for a period of 3 to 4 hr at the most sensitive time. The results in Table IIb indicate that NH_4^+, Mg^{+2}, and SO_4^{-2} were the only ions active in inhibition of flowering, with the last two being more effective; Ca^{2+} was the only ion effective in promotion of flowering percentage to the level of the medium control.

DISCUSSION

The experiments indicate that the flowering of *L. perpusilla* grown under inductive photoperiods on half-strength Hutner's

medium with sucrose can be strongly inhibited by periodic transfers to water. The inhibition of flowering by water transfer is mainly via its effect on photoperiodic induction rather than on development as a whole, since 2 days of repeated treatments were enough to give significant results.

The phase of maximal inhibition of flowering caused by water coincided only partially with the phase of sensitivity to light pulses. Water was most effective 16 to 18 hr after the light was turned on (under 8-hr photoperiods), which is about 1 to 2 hr after the phase of maximal sensitivity to light signals (Fig. 2, compare 1-hr water treatments with 2 days of light pulses). This suggests that water does not mimic the pigment conversion process itself, but rather the subsequent reaction of the converted pigment (probably phytochrome), which in turn inhibits photoperiodic induction (14).

The fact previously reported (8, 9), that the short day plant *L. perpusilla* flowers under constant blue light could have been explained, in terms of circadian rhythmicity, by the assumption that the rhythm persists undisturbed but that the active phytochrome form is kept at an intermediate level such that inhibition of the inductive phase does not occur. However, the results reported here indicate that the rhythm of sensitivity to water treatment—so evident under light-dark cycles—does not persist under continuous blue light. The lack of rhythmical response under blue light may be due to fast damping of the circadian oscillator or to increasing asynchrony in the population. If there is a constant increase of an asynchronous, out-of-phase, rhythmic population under blue light, then one can explain the results by assuming that the same periodic interaction of pigment and substrate occurs, as in light-dark conditions, but at all times of the day; therefore, the outcome of the water treatment is a slight inhibition of flowering irrespective of the time of application.

Another question concerns the role of the medium in potentiating the water effect. Upon elimination of sucrose from the growth medium, the water effect of flower inhibition disappeared. The action of sucrose reported here is probably closely related to the sucrose effect reported by Posner (19, 20), in which dilution of the macronutrients in sucrose-supplemented medium caused inhibition of flowering of *L. perpusilla*. This inhibition was not observed in cultures without sucrose and could be overcome by increasing the concentration of calcium and phosphate ions, with the last one being more effective. In the present case, Ca^{2+} and PO_4^{3-} were the only ion supplements that succeeded in overcoming the flowering inhibition by water, suggesting a similar mechanism. The similarity goes even further when one considers the dependence of the water effect on the presence of ammonium ions, since Hillman and Posner (in preparation) found that the sugar inhibition of flowering disappears when the plants are grown on ammonium-free medium.

With respect to the sucrose effect, however, it should be noted that at the end of the 7-day growth period plants on half-strength Hutner's medium without sucrose have produced about one-third the number of fronds of plants on medium supplemented with sucrose. Therefore, the validity of comparing equivalent times of treatments in these two different growth conditions is dubious, at least in the absence of information on the effect of multiplication rate on the rate of photoperiodic induction. Similarly, the more pronounced effect of water transfers in Hutner's medium (with sucrose) modified to provide ammonia as the only nitrogen source may not be due to a special interaction between water and ammonia but rather to the generally poorer flowering observed in plants grown on this medium. The flowering of plants on "NH4" + S medium under inductive photoperiods was consistently lower than that of plants on medium supplemented with nitrate or tryptone plus yeast extract.

Finally, one may speculate as to the physiological nature of the dark period processes with which water treatments interact. One hypothesis might propose the existence of changes in permea-

bility which affect the plant's capacity to absorb or to leak substances from and to the outside medium. However, it is more likely that the rhythm is in metabolic reactions. One may assume, as basically suggested by Pittendrigh and Minis (17, 18), that light-dark cycles entrain oscillation in metabolic activities, and that the coincidence of particular concentrations of, for example, an enzyme and a substrate is crucial for photoperiodic induction. Water treatments might cause the removal or destruction of a substrate or intermediate, thus imitating the action of the converted pigment brought about by light signals. In such a scheme, the effectiveness of the water treatment may be dependent on the permeability of the plants, which in turn may be affected by aspects of the growth conditions such as the presence of sucrose, ammonium, calcium or phosphate ions (12). This idea is supported by the known fact that under mist or rain both organic and inorganic substances leach out of plants (22). It was further demonstrated by Tukey *et al.* (23) that the coincidence of leaching with inductive conditions in *Pharbitis nil* and *Chrysanthemum morifolium* inhibited flowering.

The response to periodic water treatment provides a new technique for further analysis of these interactions.

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