ON THE INTERACTION OF KINETIN AND PHYTOCHROME IN LEMNA MINOR GROWING IN THE DARK

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SUMMARY

The multiplication rate and the rate of frond expansion of *Lemna minor* L. decline to a low level after transfer from continuous light to continuous darkness. The time course of this decline was measured. Conditions during the light period which were favourable for synthetic processes retard the decline. A retardation was also effectuated by kinetin during darkness. After 3 weeks in darkness the growth level of kinetin and non-kinetin cultures is about the same. With 5 minutes red light every 24 hours, in the presence of kinetin, a much higher growth rate is maintained.

The effect of kinetin on growth in darkness is not substitutive for, but synergestic with that of short time illuminations.

1. INTRODUCTION

HILLMAN (1957) observed that *Lemna minor* could be grown on a sucrose medium if the cultures received light at least once in 3 or 4 days during 10 minutes. The effect was considered as a phytochrome effect because a strong reduction was obtained if far red followed the red radiation. Kinetin added to cultures which had been in darkness for 5 days gave a growth response similar to that after brief illumination. HILLMAN described this effect as a "partial satisfaction by kinetin of the non-photosynthetic light requirement of *L. minor*".

ROMBACH (1961), growing *L. minor* on a sucrose medium supplemented with casein hydrolysate, observed a growth stimulation by kinetin if the growth rate was measured over a dark period of 10 days. This effect depended very much on the light intensity the plants received before. If plants were used from cultures growing previously for 6 days in darkness or at a very low light intensity and growth was measured over a dark period of 21 days, hardly any stimulation of the growth rate by kinetin was found. If, however, a very low light intensity (0.3 erg/cm^{2}sec) was maintained during this period, the growth rate was much increased over that of cultures without kinetin. It was concluded that kinetin and light had a synergistic effect at very low light intensities.

TASSERON-DE JONG (1968) and TASSERON-DE JONG & VELDSTRA (1971), however, found a growth stimulation by benzylaminopurine (BAP) in darkness irrespective of the light intensity the plants received before transfer to darkness. This stimulation lasted for 7 days after kinetin addition, thereafter the multiplication rate (MR) declined to the same value as that in cultures growing without

kinetin. A temporary stimulation of growth in darkness was also induced if kinetin was added to cultures growing for several weeks in darkness without BAP.

These observations led TASSERON-DE JONG & VELDSTRA (1971) to conclude that cytokinin substituted for the non-photosynthetic light requirement. This was in agreement with the results of HILLMAN (1957), but was not in agreement with those of ROMBACH (1961). TASSERON-DE JONG & VELDSTRA (1971) ascribed the decline of growth rate in darkness 7 days after the application of BAP to "a rapid depletion of growth potential in darkness in the presence of BAP", because after 3 weeks in darkness the cultures on a BAP medium had died.

In this paper evidence is presented that the decline in growth rate in darkness on a kinetin medium supplemented with casein hydrolysate, as used by ROMBACH (1961), was not due to a decline in the general condition of health, but to the absence of a non-photosynthetic light factor for growth, phytochrome P_{fr}.

2. MATERIAL AND METHODS

The strain of *L. minor*, introduced in our laboratory by Dr W. Lindeman, was grown in sterile culture with mineral salts, 15 g sucrose, 0.26 g casein hydrolysate and 0.005 g tryptophane per litre medium. Iron was given as a complex salt with ethylene diamino tetra acetic acid. Kinetin was used in a concentration of 3×10^{-6}M. Temperature was 23 °C. Casein hydrolysate with tryptophane, the iron complex and the kinetin solution were autoclaved separately. On this medium growth was light intensity saturated at 20000 ergs/cm²sec, with a multiplication rate MR = 0.16.

The multiplication rate was calculated according to the formula for exponential growth $MR = 1/t \log Nt/No$;

t = time in days; No and Nt are the numbers of fronds at the beginning and at the end of the experimental period.

The multiplication rate in absolute darkness was measured by harvesting groups of 10 cultures after different intervals. Frond expansion and multiplication rate in darkness interrupted by safelight were measured in cultures photographed at different intervals of time against green safelight. Frond expansion proceeds linearly with time from the moment the fronds are visible until the final size is reached. During photography (30 seconds) the intensity of the safelight at the level of the plants was 25 ergs/cm²sec. The safelight consisted of a wavelengthband with its peak at 510 nm and a half width value of 30 nm. Red light (2100 ergs/cm²sec) was obtained from fluorescent lamps combined with a filter consisting of 1 cm of an aqueous solution of 30 g/l $CuSO_4$ and 3 mm red plexiglass 501 Röhm and Haas; far red (16500 ergs/cm²sec) was obtained from incandescent lamps in combination with a filter consisting of 5 cm water and 2.5 mm RG 9 glass from Schott (Mainz).

3. RESULTS

3.1. The time course of multiplication rate in darkness

The time course of the multiplication rate in continuous darkness after transfer from continuous light is given in *fig. 1*. In the cultures without kinetin as well as in the cultures with kinetin the multiplication rate declines to a low level after the first 24 hours in darkness. Decline is faster in the cultures without kinetin. The development of new primordia as well as the expansion of existing ones was affected by darkness, because the number of primordial fronds per existing frond did not increase during darkness (*fig. 1* scale on the right).

Conditions during the light period influenced the growth rate during the first days of darkness. Light intensity and temperature (*table 1*), and the presence or absence of casein hydrolysate (*table 2*) and kinetin (*table 3*) in the medium were such conditions.

Considering the decline in multiplication rate, it must be inferred, however, that this decline does invalidate the method of calculating the multiplication rate because this is only correct if during the interval of time between two harvests the multiplication rate is constant. This was not the case during the first two weeks of darkness. By reducing the length of the time interval between measurements this error could be minimized. To this end the cultures were photographed at regular intervals of time, the fronds were counted from the photographs,

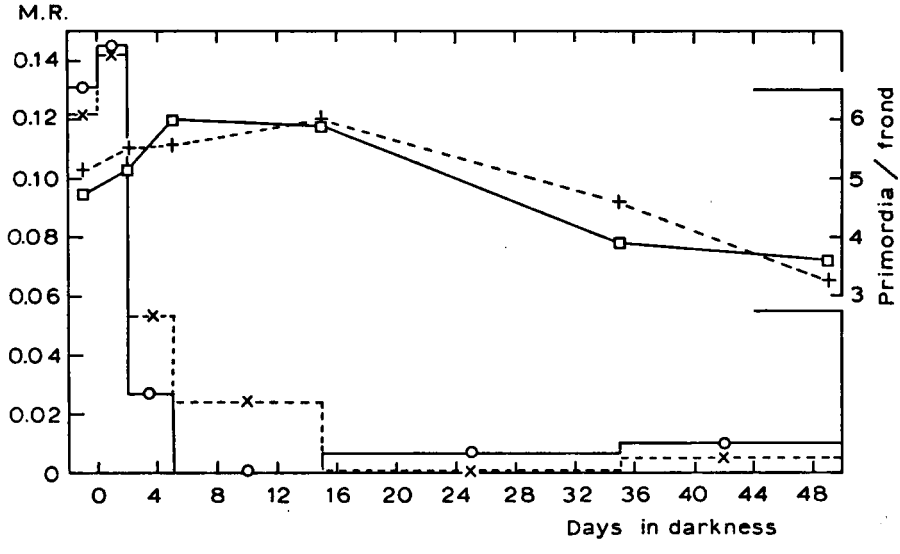

Fig. 1. Multiplication rate of *Lemna minor* in darkness after 5 days at 17000 ergs/cm²sec fluorescent light: —0: without kinetin, – –x: with kinetin. Number of primordial fronds in the two reproductive pockets belonging to an adult frond: —□: without kinetin – –+: with kinetin. Before day 0 the plants were in the light.

Table 1. The influence of light intensity (fluorescent light TL 33) at two temperatures on growth during the subsequent period in darkness. Temperature in darkness was 23 °C. Plants were transferred to fresh medium at the beginning of the dark period. Standard deviation of the means $\bar{\sigma} < 0.005$.

Condition during light period	MR during light period	MR during 1st week in darkness	MR during 2nd week in darkness
No kinetin in medium			
60000 ergs/cm²sec			
17.5 °C	0.130	0.040	0.021
25 °C	0.213	0.022	0.016
3000 ergs/cm²sec			
17.5 °C	0.079	0.035	0.019
25 °C	0.135	0.028	0.014
With 3×10^{-6}M kinetin			
60000 ergs/cm²sec			
17.5 °C	0.110	0.095	0.028
25 °C	0.200	0.054	0.027
3000 ergs/cm²sec			
17.5 °C	0.068	0.077	0.026
25 °C	0.130	0.051	0.016

Table 2. Influence of casein hydrolysate, 260 mg/L, during the light period and during the dark period on growth rate during darkness. The medium contained 3.10^{-6}M kinetin. During the light period, the plants received fluorescent light TL 33, 16 hours per day at 20000 ergs/cm²sec.
Temperature in all series 20 °C. Standard deviation of the means $\bar{\sigma} < 0.005$, − Cas = without casein hydrolysate, + Cas = with casein hydrolysate.

Condition in light	MR in light	Condition in darkness	MR in darkness	
			first week	second week
− Cas	0.11	− Cas	0.050	0.025
		+ Cas	0.057	0.037
+ Cas	0.14	− Cas	0.068	0.035
		+ Cas	0.075	0.038

Table 3. Influence of 3.10^{-6}M kinetin during the light period and during the first week in darkness on growth during this dark period.
Temperature in light 20 °C, in darkness 25 °C. Light : 16 hours/day, as 20000 ergs/cm²sec fluorescent light TL 33. Standard deviation of the means $\bar{\sigma} < 0.003$.
− K = without kinetin, + K = with kinetin in the medium.

Condition in light	MR in light	Conditions in darkness	MR during first week in darkness
− K	0.15	− K	0.029
		+ K	0.066
+ K	0.14	− K	0.054
		+ K	0.090

and multiplication rates were determined over the time intervals between the photographs. In *fig. 2* the multiplication rates of cultures growing on a kinetin medium during 26 days in darkness are shown (darkness was interrupted by safelight during photography, see under Methods).

The time course of multiplication rate in *fig. 2* is essentially the same as in *fig. 1*. The slightly higher growth rates in the photographed cultures may be due to the influence of the safelight. After 7 weeks of absolute darkness the cultures of

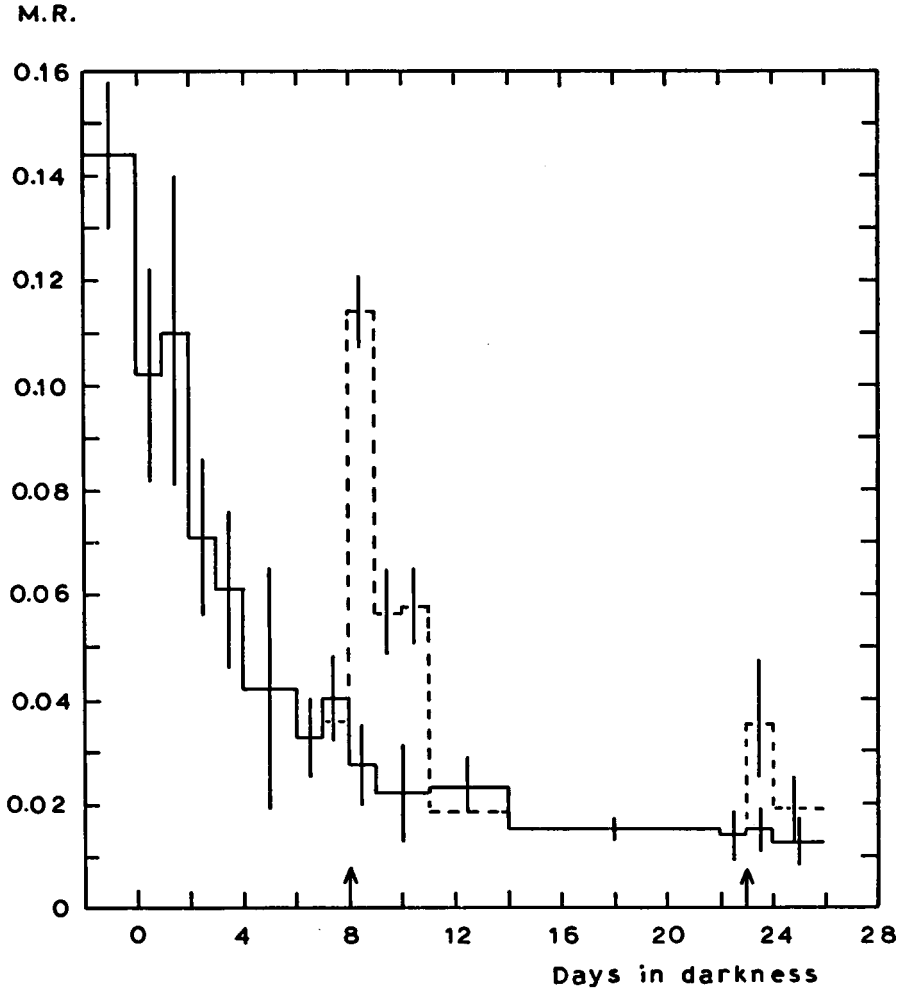

Fig. 2. Multiplication rate of *Lemna minor* in darkness in the presence of kinetin, measured from photographs. Before day 0 the plants were in continuous light of 17000 ergs/cm²sec. – –: MR of cultures receiving red light during 5 minutes at the times indicated by arrows. Vertical bars indicate the double standard deviation of the mean.

fig. 1 showed 8 % dead fronds, as is usual in cultures of that age. In the experiments of *fig. 2* at the end of the experiment all fronds were producing new offspring. The only indication that growth potential was reduced was that several old fronds produced new fronds at one side only, and that stimulation by 5 minutes red after 22 days of darkness was only 25% of that after 8 days of darkness.

The time course of frond expansion in darkness interrupted by the safelight used for photographing the plants is presented in *fig. 3*. A gradual reduction of the growth rate could be observed also here during the first two weeks of darkness.

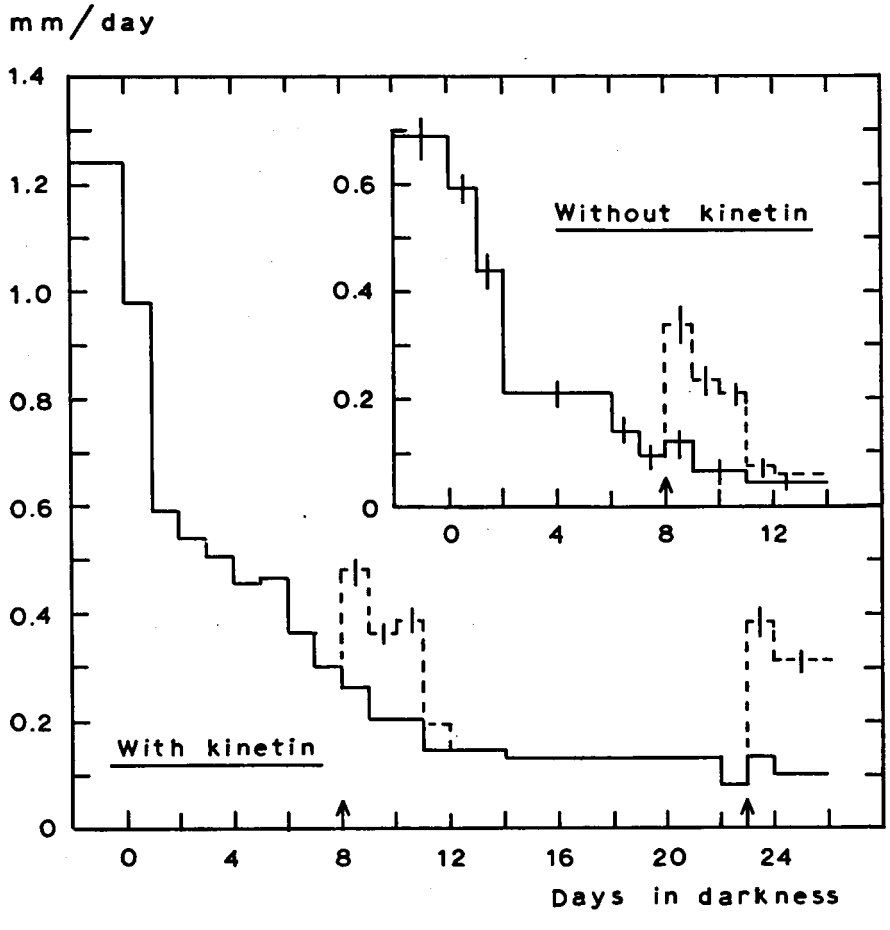

Fig. 3. Expansion rate of half-grown fronds during a prolonged period of darkness in the presence or in the absence of kinetin. Vertical bars indicate the double standard deviation of the mean. —: cultures only received safelight during photography. – –: cultures received 5 minutes red light at the time of the arrow.

In the presence of kinetin this reduction was retarded. Stimulation of frond expansion by 5 minutes red light increased as the plants were longer in the dark.

3.2. The effect of brief illuminations on the growth rate during prolonged darkness

Multiplication rate and rate of frond expansion in cultures growing in darkness are both controlled by phytochrome P_{fr} (ROMBACH 1965). This is shown in *table 4* for the multiplication rate of plants receiving an illumination once every day. Under this condition the same growth rate was maintained for an indefinite time if the plants were transferred to a fresh medium every three weeks. The effect of the red light treatment was almost completely suppressed if the red illumination was followed by far-red radiation.

Table 4. The effect of brief red illuminations (5 minutes) and red (5 minutes) followed by far red (15 minutes) radiations given once a day during a period of 21 days on the multiplication rate of cultures with and without kinetin in the medium. Standard deviation of the means $\bar{\sigma} < 0.003$.

Condition	Multiplication rate		
	red	red–far red	darkness
– kinetin	0.039	0.019	0.020
+ kinetin	0.065	0.038	0.031

The effect of a single illumination on the rate of frond expansion in the presence of kinetin during three subsequent days was studied with separate groups of cultures. The effect was determined by subtracting the growth rate in darkness from the growth rate after the light stimulus.

The effect of a single illumination on frond expansion increases as the dark period proceeds (*fig. 4*), and could be reduced by far red irradiation after the red light exposure (*fig. 4*).

In cultures without kinetin the effect of an illumination after eight days of darkness was as strong as in the presence of kinetin (*fig. 3*).

3.3. The effect of kinetin on cultures grown previously in darkness without kinetin

The observation of TASSERON-DE JONG & VELDSTRA (1971) on stimulation of growth by cytokinin in cultures which had been in darkness for some time could be confirmed.

Cultures, grown first in the light, were divided into four groups. One group remained without kinetin during the whole dark period, a second group received kinetin before transfer to darkness, a third group received kinetin after seven days of darkness, and the fourth group received kinetin after 14 days of darkness. The only light used was that of a blue burning gas flame used for sterilisation when kinetin was added to the cultures; the kinetin controls (second group) were ex-

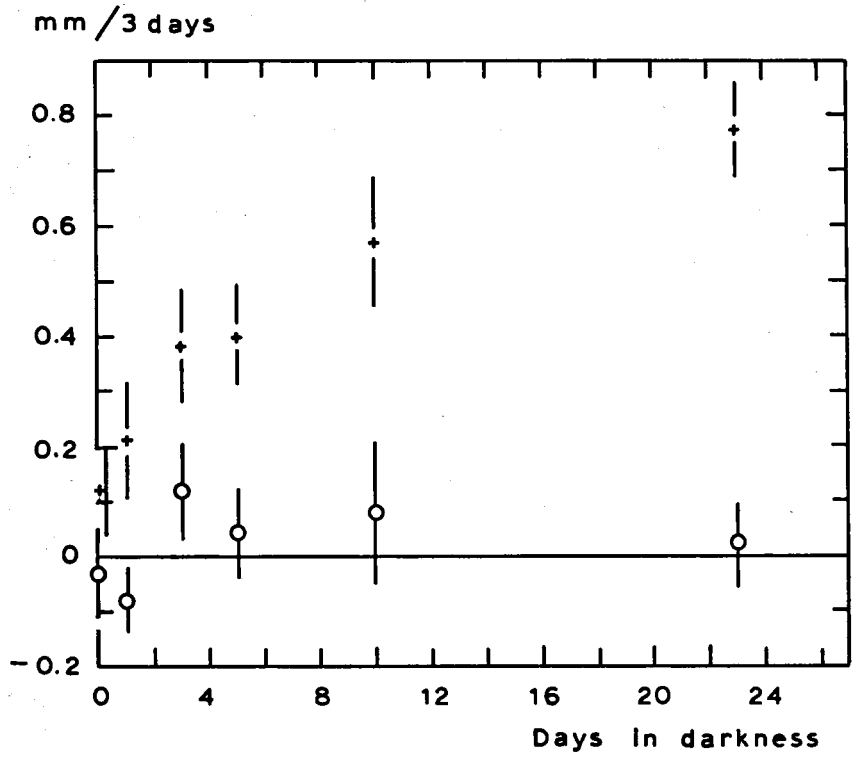

Fig. 4. The influence of the length of the preceding dark period on the effect of an exposure to 5 minutes red light (+) or to 5 minutes red followed by 15 minutes far red (o) on frond expansion. The effect is expressed as the difference in increase in length during 3 subsequent days between irradiated and non-irradiated cultures. The length of the vertical bars indicate the double standard deviation of the mean.

posed to the same treatment. Each week 10 cultures of each group were harvested. The growth, expressed as increment in logarithm of the number of fronds after different times in darkness, is given in *table 5*. In *table 6* the apparent multiplication rate in darkness from week to week is given. These values are derived from those in *table 5* by substraction of the values of the increment of the preceding time from the value after the week in consideration. For example, the multiplication rate for treatment (B) over the time interval from 25/8–1/9 is obtained by subtracting the value for the —K treatment over the time interval from 11/8–25/8 (*table 5*) from the value of the (B) group over the time interval from 11/8–1/9 and dividing the outcome by 7, the number of days of the period in consideration.

Table 6 shows that kinetin caused an increase in multiplication rate in darkness. This effect decreased as the dark period before application increased. There was a difference in size of the mature fronds also. Kinetin caused an increase;

Table 5. The increment in the logarithm of the number of fronds during succeeding weeks in darkness of *L. minor* grown previously without kinetin at a light intensity of 10000 ergs/cm²sec white fluorescent light and a temperature of 23 °C. During that light period MR was 0.120 ± 0.004. The + K cultures received 3 × 10^{-6}M kinetin either before transfer to darkness, or after 7 days of darkness (A) or after 14 days of darkness (B), while the − K cultures received no kinetin during the whole experimental period.

Treatment, and date of kinetin addition	MR × (time in days) × 1000			
	11–18 aug.	11–25 aug.	11/8–1 sept.	11/8–8 sept.
+ K 11/8	439 ± 16	549 ± 14	694 ± 19	789 ± 32
− K	200 ± 21	259 ± 16	387 ± 8	553 ± 12
+ K 18/8 (A)		410 ± 14	565 ± 15	671 ± 19
+ K 25/8 (B)			471 ± 18	670 ± 18

Table 6. Multiplication rate (MR) in darkness of *L. minor* treated with kinetin after different periods of darkness. The values are derived from those in *table 5*. The stimulation of MR, expressed as the difference between the MR of cultures treated with kinetin and the MR of cultures without kinetin during a period of two weeks after the kinetin application, is given in the last column.

Treatment, and date of kinetin addition	MR × 1000				Kinetin stimulation over 14 days
	11/8–18/8	18/8–25/8	25/8–1/9	1/9–8/9	
+ K 11/8	63.0 ± 2.3	15.8 ± 3.0	21.0 ± 3.3	13.6 ± 5.1	41.8 ± 6.1
− K	28.6 ± 3.1	8.4 ± 3.8	18.3 ± 2.4	23.7 ± 21.	
+ K 18/8 (A)		29.8 ± 3.5	22.4 ± 2.9	15.2 ± 3.4	25.5 ± 6.3
+ K 25/8 (B)			30.6 ± 3.4	28.4 ± 3.7	17.0 ± 5.8

however, this effect decreased as kinetin was applied later during the dark period.

After three weeks of darkness a few dead fronds appeared in the kinetin control group. This amounted to 5% of the total frond number at the end of the experiment. Unexpectedly, these were not the oldest fronds, but fronds grown in darkness.

A comparision of the data in *table 6* with those in *table 4* shows that kinetin, supplied after 14 days of darkness, stimulated growth rate temporarily as much as a short illumination did. In the presence of kinetin, however, stimulation by light was much greater.

4. DISCUSSION

Probably two factors are involved in the decline of the growth rate of *Lemna minor* in darkness. The experiments on the influence of light intensity and composition of the nutrient medium have indicated that exhaustion of specific substances accumulated by the fronds during the light period may be one factor.

The other factor might be described as a decrease in growth potential, but is

more likely an increase in growth inhibition, comparable to dormancy. Temporarily the cultures could be relieved from this growth inhibition by the addition of kinetin. VAN OVERBEEK *et al.* (1967) found a similar effect of BAP on the growth inhibition by abscissic acid in *Lemna minor* grown in light.

The growth inhibition induced by darkness could be removed permanently by the action of phytochrome P_{fr} and kinetin together. In the absence of kinetin P_{fr} has only little effect (*table 4*), whereas in the absence of P_{fr} kinetin only has a temporary effect. So the second factor responsible for the decline in growth in darkness seems to consist of two components: the cytokinin component and the phytochrome component. For the interaction of these components the term "synergism" can better be used than "substitution".

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