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Photophysiology of turion formation and germination in *Spirodela polyrhiza*

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Abstract

Standardized laboratory techniques for the vegetative growth of the duckweed *Spirodela polyrhiza* (*Lemnaceae*), and for formation as well as germination of their turions were described. Increasing photon fluence rates of blue or red light increased the yield of turions. A specific stimulating effect of blue light was demonstrated under autotrophic but not under mixotrophic conditions. Therefore the spectral composition of light is not important in mixotrophic formation of turions whereas in autotrophic formation light sources with a higher portion of blue light are recommended. Dark-grown (etiolated) turions showed accelerated germination and higher germination percentage in comparison with light-grown turions after induction by a single red light pulse. This difference was overcome in continuous red light by speeding up the germination response of light-grown turions. Use of Petri dishes (8 cm^3 nutrient solution) instead of Erlenmeyer flasks (50 cm^3 nutrient solution) retarded germination response. Especially for long term experiments the use of Erlenmeyer flasks is recommended. Storage of turions for 72 h at 25 °C following at 5 °C in darkness after-ripening resulted in a decreased lag phase of the light-induced germination both after induction by a single light pulse and in continuous light.

Additional key words: blue light receptor, duckweed, *Lemnaceae*, phytochrome.

Introduction

Out of 34 species of the family *Lemnaceae* (duckweeds) 15 species form turions (hibernacles, winterbuds) or turion-like fronds. Those of the greater duckweed *Spirodela polyrhiza* belong to the "true turions" because they are morphologically

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Abbreviations: c - continuous; B - blue light; D - darkness; D-turions - dark-grown (etiolated) turions; FR - far red light; L-turions - light-grown turions; R - red light; W - "white" light.
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different from vegetative fronds and do not grow any further. These resting fronds overcome unfavourable seasons by sinking to the bottom of the ponds or lakes and start germination under favourable conditions (Landolt 1986, Landolt and Kandeler 1987). In the natural aquatic environment of the temperate zone turions become dormant immediately after their formation in order to avoid germination before winter. With respect to formation, dormancy and germination they are equivalent to seeds. However, in contrast to seeds they are vegetative organs. This makes turions an attractive experimental system for studying the influence of various environmental conditions on turion formation or germination (Lacor 1969, Malek and Cossins 1983, Newton *et al.* 1978, Perry 1968, Sibasaki and Oda 1979, Smart and Trewavas 1983, Smart *et al.* 1987).

Turion formation can be experimentally induced in *S. polyrhiza* by nitrate, sulphate and phosphate limitation, however, with different degrees of dormancy (Appenroth *et al.* 1989b). Phosphate-limited conditions caused the best morphological and physiological homogeneity, a very high turion yield, and strict requirement of light induction for germination (Appenroth *et al.* 1989a, b). Turions with very different properties can be obtained by changing experimental conditions during formation or after-ripening, *e.g.*, light conditions or nitrate availability (Appenroth *et al.* 1989b, 1992). Moreover, climate conditions during turion formation (Das and Gopal 1969) determine their physiological properties. Turion formation also varied in different clonal strains (Perry 1968).

Under heterotrophic conditions in darkness (D), *S. polyrhiza* forms etiolated turions (D-turion; Appenroth *et al.* 1989a). Because of the germination kinetics (Appenroth *et al.* 1989b) D-turions were used in most experiments investigating the influence of calcium ions (Appenroth *et al.* 1994), nitrate (Appenroth 1994), and other environmental factors (Xyländer *et al.* 1993, Appenroth *et al.* 1993). In these germinating turions, which were originally chosen by Augsten *et al.* (1988) as a model system, phytochrome was demonstrated to be the photoreceptor mediating the germination response (Appenroth and Augsten 1990a). The degree of this response, however, depended very much on various other conditions, *e.g.* density of turions in the germination flasks (Appenroth *et al.* 1990b). Therefore, standardization of the experimental conditions was regarded as a prerequisite for a more detailed further analysis of the system.

The present paper describes standardized experimental procedures for the formation and germination of turions in *S. polyrhiza*. Besides the description of experimental conditions for turion formation and germination the following questions were addressed in the present paper: (*1*) Is there a specific effect of blue light on the formation of turions? (*2*) Does germination kinetics depend on the type of irradiation, *i.e.* pulse irradiation *versus* continuous irradiation? (*3*) Does the type of germination vessels affect the germination characteristics? (*4*) Is the rate of germination influenced by a storage in the dark at 25 °C before light treatment?

Materials and methods

Plant material and nutrient media: Vegetative fronds of *Spirodela polyrhiza* (L.) Schleiden [*S. polyrhiza* and *S. polyrrhiza* were assumed to be synonyms, but the spelling *polyrhiza* has to be preferred because von Linné (1771) used this spelling] were collected by F. Jungnickel in 1969 from a dead side arm of the river Saale near Jena. Several fronds were cultivated in a mineral medium until some turions were formed. Turions were sterilized by bathing for 4 min in 1.7 % (m/v) KJ-J_2 solution containing Tween 20 (0.1 % m/v). Sterilized turions were transferred into glucose-

Table 1. Light sources and filter systems for formation and germination of turions of *S. polyrhiza*.

Application	Lamps	Filter	Fluence rate [W m^{-2}]	Characteristics
1. cW	FT LS 65 white 20[1] or TL-D 36 W/93[2]	without	8 - 10	Cultivation of vegetative fronds
2. cR	FT TL 18 W/15[2]	red plexiglas[3] 501, 3 mm	2.1	λ_{max} = 658 nm hbw = 25 nm
3. cB	FT TL 18 W/18[2]	blue plexiglas[3] 627, 3 mm	3.1	λ_{max} = 450 nm hbw = 45 nm sharp Hg-line at 435 nm
4. R pulse	HL 24 V/125 W	interference filter[4]	30	irrad. area 14 cm^2 heat abs. - 3 cm layer of 1 M Na_2CrO_4 λ_{max} = 656 nm, hbw = 22 nm
5. FR pulse	HL 24 V/125 W	cut-off filter RG 9[5]	80	irrad. area 14 cm^2 heat abs. - 3 cm layer of 1 M Na_2CrO_4 > 710 nm
6. R pulse	HL 24 V/150 W use of 4 slide projectors	interference filter[4]	30	irrad. area 120 cm^2 heat abs. - 3 cm layer of 1 M Na_2CrO_4 λ_{max} = 656 nm, hbw = 22 nm
7. FR pulse	HL 24 V/150 W use of 4 slide projectors	cut-off filter RG 9[5]	40	irrad. area 120 cm^2 heat abs. - 3 cm layer of 1 M Na_2CrO_4 > 710 nm
8. dim green	HL 6 V/15 W	interference filter[4]	<0.001	λ_{max} = 525 nm, hbw = 7 nm
9. dim green	FT TL 18 W/17[2]	plexiglas[3] 303.3 + 627.3 mm	<0.001	λ_{max} = 521 nm, hbw = 22 nm

FT - fluorecsence tubes; HL - halogen lamp; hbw - half band width; [1] - *NARVA*, Branderbisdorf, Germany; [2] - *Philips*, Eindhoven, Netherlands; [3] - *Röhm & Haas*, Darmstadt, Germany; [4] - *Carl Zeiss*, Jena, Germany; [5] - *Schott*, Jena, Germany

containing medium. Following germination, vegetative fronds were tested on bacterium and fungus agar. One of these fronds were further cultivated in a sterile nutrient medium establishing the axenic clonal strain SJ (Jungnickel 1978). Nutrient medium according to Kuhl (as reported by Bornkamm 1965) was modified in the following way (Jungnickel and Augsten 1986): KH_2PO_4 (60 μM), $Ca(NO_3)_2$ (1 μM), KNO_3 (8 mM), $MgSO_4$ (1 mM), H_3BO_3 (5 μM), $MnCl_2$ (13 μM), Na_2MoO_4 (0.4 μM), FeEDTA (25 μM). For mixotrophic or heterotrophic cultivation 50 mM D-glucose were added. For vegetative growth phosphate concentration may be increased to 1.5 mM. However, in routine work 60 μM KH_2PO_4 were used in order to avoid precipitation, always observed at higher phosphate concentration in daylight and caused by photochemical destruction of FeEDTA (Armstrong *et al.* 1982). Only for stock cultivation the higher phosphate concentration was used in nutrient media supplemented with 0.65 % (m/v) agar (high gel strength, *Serva*, Heidelberg, Germany). All solutions were sterilized for 20 min at 120 °C at 0.22 MPa. The temperature was 25 °C. The treatments for cultivation were: *1*) autotrophic nutrition and continuous white light (cW), *2*) mixotrophic nutrition and cW, and *3*) heterotrophic nutrition and continuous dark. The strain SJ showed the following growth rates: *1*) 0.0156 ± 0.0009 h^{-1}, *2*) 0.0196 ± 0.0008 h^{-1}, *3*) 0.0054 ± 0.0006 h^{-1}.

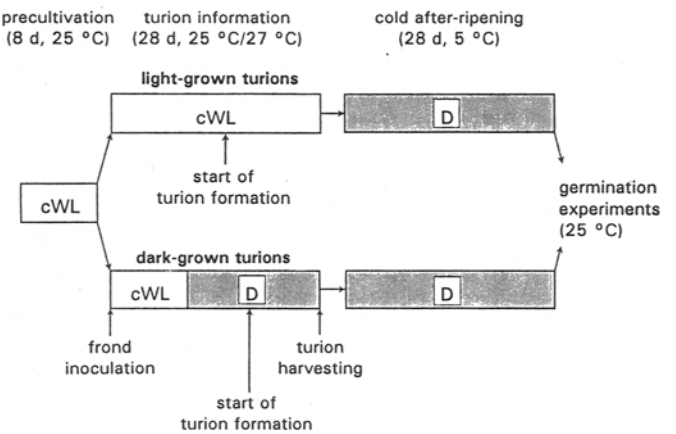

Fig. 1. Schematic presentation of the formation of light-grown (25 °C) and dark-grown (27 °C) turions of *Spirodela polyrhiza* under laboratory conditions.

Plant cultivation and formation of turions: Cultures were started with a single 3-frond colony taken from 8-d-old stock cultures (200-cm^3 Erlenmeyer flasks containing 120 cm^3 of nutrient solution plus glucose). All flasks were closed by air-permeable stoppers (cotton wool). 300-cm^3 Erlenmeyer flasks containing 180 cm^3 of nutrient medium plus glucose were further used. Vegetative fronds were cultivated at 25 ± 1 °C at cW (Table 1). Formation of turions was induced by phosphate-limitation caused by the vegetative growth. Turion formation started at day 16 after inoculation and turions were harvested after 28 d as mature turions detached from the parent plants. They sank to the bottom of the flasks. The senescent mother plants were

removed and the turions were washed twice with sterilized distilled water. Turions from two culture flasks were transferred to another 300-cm^3 Erlenmeyer flask containing 200 cm^3 of nutrient medium without glucose. The yield was about 600 turions per flask (about 0.6 g of fresh mass). These fresh harvested L-turions were dormant and were after-ripened for 28 d in D at 5 ± 1 °C before germination experiments (Fig. 1).

Production of D-turions started as before. However, about 10 d after inoculation flasks were transferred to D (27 ± 0.5 °C) before turion formation started. At this time the surface of the nutrient solution was covered by vegetative fronds (about 400 per flask) which showed the first sign of increased anthocyanin formation at the bottom side. The formation of D-turions started at day 21 and they were harvested in dim green light in the bench box at day 28. The yield was about 250 turions per flask (about 0.23 g of fresh mass). D-turions were harvested as described before and transferred from three culture flasks into one culture flask for cold after-ripening under the same conditions as L-turions.

Turions can be produced also under autotrophic conditions using the method of phosphate limitation (Appenroth *et al.* 1989a). However, this method is scarcely used because yield is low (about 30 % in comparison with L-turions) and the time of harvest should not be shorter than 50 d after inoculation.

Turion germination: After-ripened turions were filtered through a nylon mesh and rinsed with sterile water in a dim green light. The content of several flasks was mixed in order to avoid flask-specific variances in the germination. Turions were transferred either into plastic Petri dishes (diameter 50 mm) containing 8 cm^3 glucose-free nutrient medium or into 100-cm^3 Erlenmeyer flasks containing 50 cm^3 of nutrient medium, closed with a cotton wool stopper.

Turions were regarded as germinated if the new sprout was visible with the naked eye (operational criterium). Fig. 2 shows turions before and after germination induced by a R pulse. In continuous light development of roots is more pronounced.

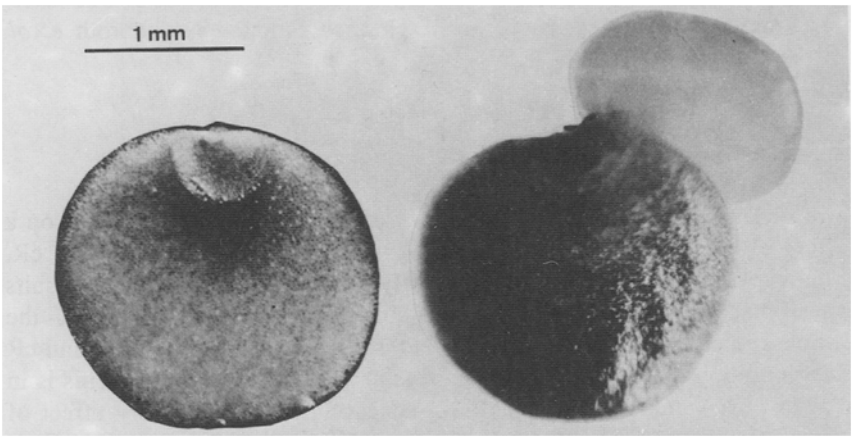

Fig. 2. Dark-grown turions of *Spirodela polyrhiza* before (*left*) and after germination (*right*), induced by a single red light pulse.

Under some conditions only a root initial started to grow or the pocket sheet on the upper surface shifted away without emergence of the new sprout. Such turions were normally regarded as non-germinated.

Nine germination vessels (Petri dishes or Erlenmeyer flasks) were used for each run and repeated in three independent experiments in order to average the results of at least three independent cultures.

Each data point represents at least 500 evaluated turions. Flasks with more than 75 turions were not included in statistical evaluation because germination percentage is decreased by the effect of overcrowding (Appenroth *et al.* 1990b).

Light sources and irradiation: Vegetative fronds were cultivated under cW. Red light and blue light for continuous irradiation (cR, cB) were used within an air-conditioned (25.0 ± 0.1 °C) growth chamber *I 35LL* (*Percival*, Boone, USA). All details including pulse treatments are given in Table 1 (*cf.* Mohr and Drumm-Herrel 1981). Dim green light was used for all manipulations except actinic irradiation. Fluence rates were measured with radiometer *IL 1700* (*International Light*, Newburyport, USA) in conjunction with a detector *SED 033/2767*. Emission spectra were recorded with a spectrofluorimeter *FICA 55 NKII* (*ARL*, Le Mesnil-Saint-Denis, France).

Statistics: Because samples of germinating turions can be considered as binomial distributed the common estimate of the standard deviation is $SD=100\sqrt{k/n^2}\ (1 - k/n)$, with n = total number of turions, k = number of germinated turions. The common statistical method for the comparison of two germination percentages $100\ k_1/n_1$, and $100\ k_2/n_2$ is the fourfold table test. In case of large n_1 and n_2 the corresponding χ-square test is recommended. The numbers of turions required to be at a certain level of significance depend strongly on the germination percentage. As a rule for the two-sided test, between about 300 turions (for low or high germination percentage) and 550 (for medium germination percentage) are required (Horn and Hothorn 1990).

To compare means (*e.g.* turions formed per flask) Student's *t*-test was used. Germination kinetics were fitted using the Mitcherlich function (Appenroth *et al.* 1989b).

Results and discussion

Formation of turions in blue and red light: Malek and Cossins (1979) reported on a stronger effect of cB on turion formation in *S polyrhiza* in comparison with cR. However, the experimental conditions were poorly described. Because these results might suggest that a specific blue light receptor controls turion formation, the influence of cB and cR was re-investigated. Yield of L-turions at the both cB and cR (mixotrophic conditions) was increased by increasing fluence rate (Fig. 3). This is in agreement with observations of Jacobs (1947) concerning the stimulatory effect of higher fluence rate and corresponds with results demonstrating that turion yield is increased with increasing daily light period (Appenroth *et al.* 1990a). However, no specific effect of cB was detected (Fig. 3). It implies that the spectral composition of

the light source used for turion formation is not important for turion yield and that only level of irradiance is important. This supports our previous experience: yield of mixotrophic turions was similar when different light sources (fluorescent tubes neutral white, warm white, day light, *etc.*) from different producers were used.

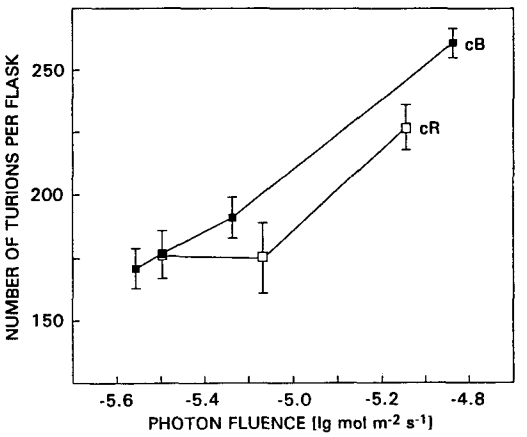

Fig. 3. Influence of continuous red (cR) and blue light (cB) on the yield of turions of *Spirodela polyrhiza* under mixotrophic conditions. Results are given as average from 9 flasks ± standard error of the means.

The influence of cB and cR on the formation of turions was also investigated under autotrophic conditions. Number of vegetative fronds, mature and immature turions (*i.e.* not yet detached from the mother plants) were determined per flask (Table 2). Whereas the number of vegetative fronds was identical, both, number of

Table 2. Influence of continuous red and blue light on the yield of vegetative fronds, and mature and immature turions of *Spirodela polyrhiza* under autotrophic conditions. Vegetative fronds were cultivated in 100-cm^3 Erlenmeyer flasks for 20 d in continuous white light (8 W m^{-2}). Flasks were subsequently transferred into continuous blue or red light (3.2 µmol m^{-2} s^{-1} each). 50 d after inoculation number of vegetative fronds, of mature turions (*i.e.*detached from mother plants), and of immature turions (*i.e.* still fixed in the pockets of mother plants) per flask were evaluated. Results are given as means from 9 flasks ± standard error of the mean.

	Vegetative fronds	Mature turions	Immature turions
Red light	274 ± 9	1.8 ± 0.9	5.7 ± 1.0
Blue light	274 ± 5	11.0 ± 1.0	10.0 ± 1.0

mature and immature turions was increased in cB in comparison with cR. The total number of turions formed was threefold higher in cB. Consequently, in contrast to mixotrophic formation of turions, under autotrophic nutrition action of a specific blue light receptor has to be assumed. The reason for the different behaviour under mixotrophic and autotrophic conditions is not known. For experimental purpose we

can suggest the use of white fluorescence tubes with a high portion of blue light (*e.g. TLD 86, Philips*) or additional blue fluorescence tubes (*e.g. TL 18, Philips*).

Germination of turions - the experimental system: Turions contain two distinct frond primordia (embryonic-like organs) within two pockets which are covered by a common pocket sheet (prophyllum) (Fig. 4). The right hand side primordium is more developed and causes germination by emerging from the bag. At temperature ≥ 13 °C (Guppy 1895) and after induction by light only one of the frond primordia starts with cell extension within the pre-germination period (Appenroth and Bergfeld 1993). This sprout pushes aside the prophyllum whereas the other one is simultaneously inhibited (Fig. 2). Only under certain circumstances, *e.g.*, after induction of germination by jasmonate, correlative inhibition is overcome and both sprouts emerge simultaneously (Appenroth *et al.* 1991). The second primordium emerges in continuous light.

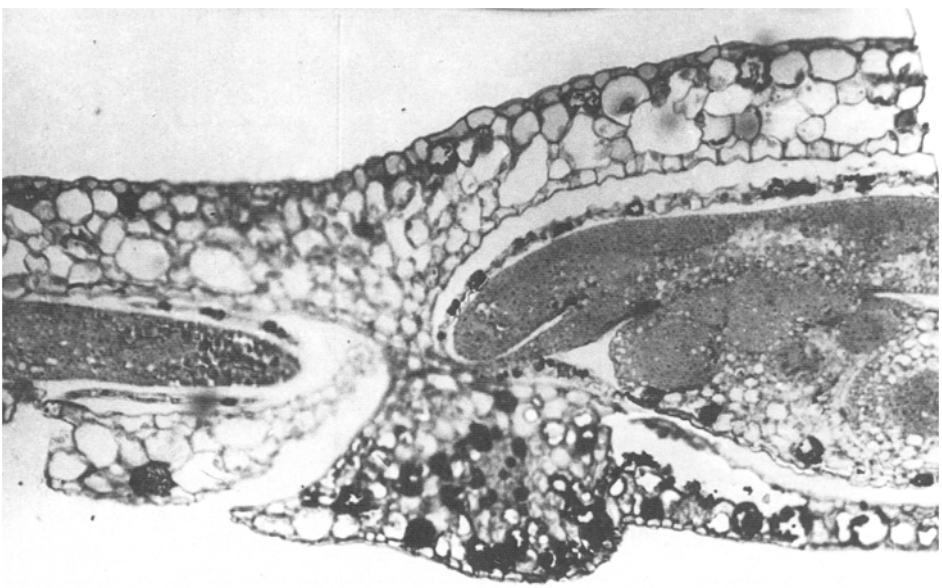

Fig. 4. Section of a turion of *Spirodela polyrhiza* through the pockets. The central part of the promoted embryo-like frond primordium on the *right side* and the correlative inhibited frond primordium are shown on the *left side*. Turions were transferred in continuous red light and fixed after 72 h at 25 °C in 2 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4 °C for 2 h. The tissue was embedded in araldite after dehydration in a series of solutions with increasing concentrations of acetone. Magnification: 120 ×.

Light-grown and dark-grown turions - comparison of germination kinetics: In a previous paper, germination kinetics of L-turions and D-turions after induction with a single R pulse were compared (Appenroth *et al.* 1989b). D-turions showed higher

maximal germination percentage and higher rate of germination than L-turions. Together with the lower germination in complete D, D-turions were regarded as more suitable objects for photophysiological investigations.

The germination kinetics of D- and L-turions in cR are very similar (Table 3). It can be concluded that L-turions require continuous irradiation to the rapid germination similarly as etiolated turions after R pulse. The molecular basis of this effect is not yet known but it may be related to the fact that D-turions never received any light before.

Table 3. Evaluation of cumulative germination of turions of *Spirodela polyrhiza* in continuous red light using the non-linear approximation function according to Mitscherlich (Appenroth *et al.* 1989b). Irradiation was started immediately after transfer from after-ripening conditions to germination conditions (z - lag in germination, k - rate of germination increase, M - asymptotic value of maximal germination)

	z [d]	k [d^{-1}]	M [%]
D-turions	2.1 ± 0.2	1.2 ± 0.2	86 ± 2
L-turions	1.9 ± 0.4	1.2 ± 0.3	88 ± 4

Germination of etiolated turions - the influence of light application and pre-treatment: In dependence on the question to be studied, induction of germination may be done by light pulses or in continuous light. Moreover, investigating different nitrate assimilating enzymes in turions (*e.g.* Appenroth *et al.* 1993, Teller and Appenroth 1994) we observed that immediately following cold after-ripening (defined as time zero) light induction of the enzymes is hardly possible. However, after inserting

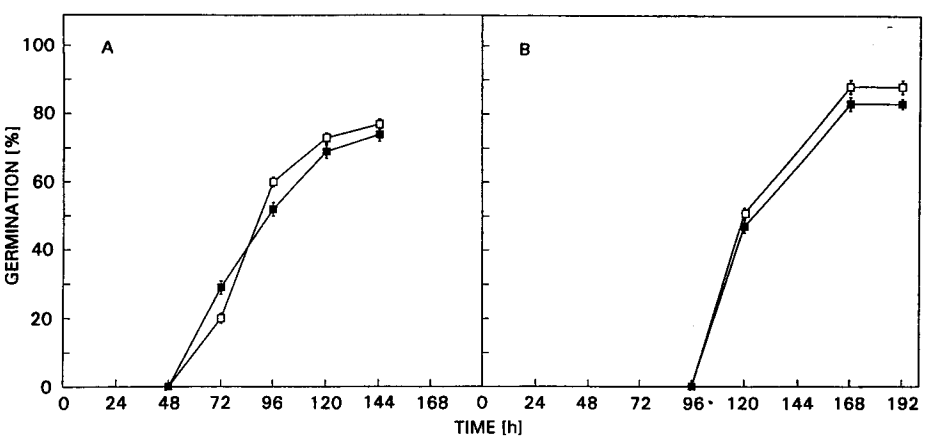

Fig. 5. Germination kinetics of dark-grown turions of *Spirodela polyrhiza* in Erlenmeyer flasks. Germination was induced either by a single red light pulse (*closed squares*) or in continuous red light (*open squares*). *A*: Light treatment immediately after transfer from after-ripening conditions (5 °C) to germination conditions (25 °C). *B*: Light treatment following a storage for 72 h in darkness at 25 °C.

a D period of 72 h at 25 °C the system became responsive to light and enzyme activities were increased. This pre-treatment may have also a certain influence on germination kinetics which would be important for the system of reference in enzymatic investigations. Up to now, germination properties of turions induced under these various conditions were not yet compared. Therefore, in the following experiments germination kinetics was investigated *1*) after induction by light pulses and in continuous light, *2*) either directly following after-ripening or after pre-treatment for 72 h in D at 25 °C and *3*) using Erlenmeyer flasks or Petri dishes as germination vessels.

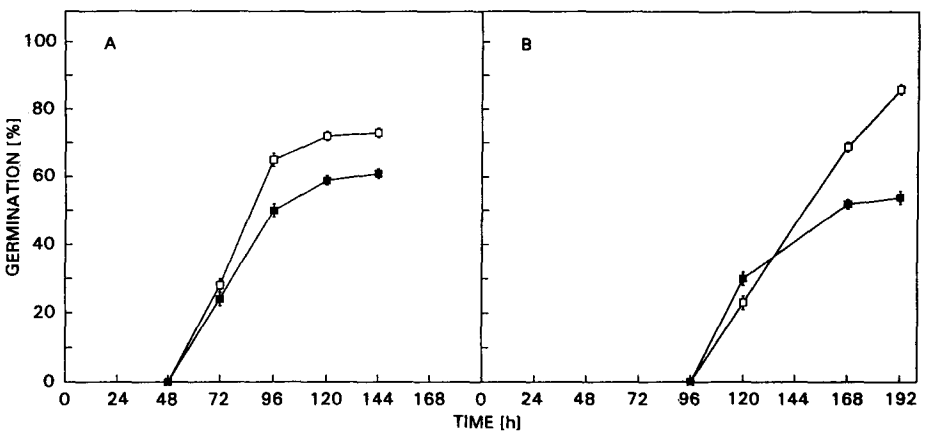

Fig. 6. Germination kinetics of dark-grown turions of *Spirodela polyrhiza* in Petri dishes. For further explanations see Fig. 5.

Figs. 5 and 6 confirm our previous results (Appenroth *et al* 1989b, Appenroth and Bergfeld 1993) that germination kinetics of D-turions were very similar after induction with light pulses or in continuous light - when Erlenmeyer flasks were used. In Petri dishes, however, pulse-induced germination was retarded and maximal response was decreased in comparison with continuous light. The difference is of minor importance in short time experiments because the starting point of germination was not changed. However, for experiments with extented germination time (more than 24 h) the use of Erlenmeyer flasks is strongly recommended. A very important change of germination kinetics was observed by pre-treatment for 72 h in D. The lag phase was shortened from about 48 h (Appenroth *et al.* 1989b) to about 24 h after start of the light treatment whereas the rate of increase of germination was changed only slightly. This corresponds with the appearance of responsiveness to light in the induction of several enzymes in turions and may be connected with speeding up metabolic events by adaptation to the germination temperature (25 instead of 5 °C during after-ripening). Thus, insertion of a pre-treatment at 25 °C in D may be recommended.

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