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Early quantitative method for measuring germination in non-green spores of *Dryopteris paleacea* using an epifluorescence-microscope technique

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A method is described to determine germination by blue-light excited red fluorescence in the positively photoblastic spores of *Dryopteris paleacea* Sw. This fluorescence is due to chlorophyll as evidenced from 1) a fluorescence-emission spectrum in vivo, where a bright fluorescence around 675 nm is obtained only in red light (R)-irradiated spores and 2) in vitro measurements with acetone extracts prepared from homogenized spores. Significant amounts of chlorophyll can be found only in R-treated spores; this chlorophyll exhibits an emission band around 668 nm, when irradiated with 430 nm light at 21°C.

Compared to other criteria for germination, such as swelling of the cell, coat splitting, greening, and rhizoid formation, which require longer periods after induction for their expression, chlorophyll fluorescence can be used to quantify germination after two days. This result is confirmed by fluence-response curves for R-induced spore germination; the same relationship between applied R and germination is obtained by the evaluation with the epifluorescence method 2 days after the light treatment as compared with the evaluation with bright-field microscopy 5 days after the inducing R.

Using this technique we show for the first time that Ca^{2+} contributes to the signal-transduction chain in phytochrome-mediated chlorophyll synthesis in spores of *Dryopteris paleacea*.

Key words – Calcium buffers, chlorophyll formation, *Dryopteris paleacea*, epifluorescence microscope, fluorescence-emission spectrum, greening, phytochrome, spore germination.

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Introduction

Fern spores serve as a single-celled model system to study the regulation of germination by various factors. Induction of germination can be mediated by red light (Furuya et al. 1982, Haupt 1985, Kendrick and Bossen 1987, Tomizawa et al. 1983, Wayne and Hepler 1984), Ca^{2+} (Föhr et al. 1987, Wayne and Hepler 1984, 1985), gibberellins (Fechner and Schraudolf 1986, Weinberg and Voeller 1969) and antheridiogens (Naef 1979). Inhibition of germination can be caused by blue light (Sugai and Furuya 1985, Sugai et al. 1984), ethylene (Edwards and Miller 1972) and various environmental pollutants that may interfere with signal transduction (Minamikawa et al. 1987, Wada et al. 1987; see also Raghavan 1980 and Furuya 1983 for review). Fern spores are ideal for the study of signal-transduction chains, since they are whole organisms yet, single cells, haploid, easily synchronized and readily available in large quantities.

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Fern spores can be divided into two groups, green and non-green, based on the presence or absence of significant amounts of chlorophyll in the quiescent spore. In the green spore species, germination can be assayed two days after light induction by using the acetocarmine-chloral hydrate method of Edwards and Miller (1972). In the non-green species, germination is usually determined by assaying swelling of the cell, splitting of the spore coat (perine and exine) and the presence of significant amounts of chlorophyll (greening) using bright-field microscopy (Mohr 1956). However, due to the difficulties of determining the presence of chlorophyll within the dark-pigmented spore coat and the evaluation of coat splitting under conditions of low contrast, the assay for germination usually is undertaken 5 to 6 days after induction in order to accurately score germination. By that time, the rhizoid has emerged and is easily identified, and often this is the criterion used for germination (Sugai et al. 1984). However, in studying signal-transduction chains in a developmental system it is advantageous to measure the response as soon after induction as possible in order to minimize the secondary or tertiary effects, such as migration of the nucleus, mitosis or cell division. Here we report an early, quantitative and simple assay, which enables measurement of germination in non-green spores as early as two days after the induction of germination with R.

Abbreviations – EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); R, red light; λ_m, transmission maximum of the interference filter, measured at the center of the halfband width; λ_{ex}, wavelength used for excitation; λ_{em}, wavelength used to measure emitted fluorescence.

Materials and methods

Plant material

Spores of *Dryopteris paleacea* Sw. (Dryopteridaceae; Tryon and Tryon 1982) were collected from plants growing in the Botanical Garden at the Univ. of Erlangen-Nürnberg (Erlangen, FRG) during the summer of 1985 and they were stored in a desiccator at 5°C in darkness.

Treatments

Spores were sown on an aqueous medium containing 7.4 m*M* $Ca(NO_3)_2$, 3.45 m*M* KNO_3, 1.01 m*M* $MgSO_4$, 10 m*M* EGTA and 20 m*M* PIPES at pH 6.0±0.1 (standard medium). The free Ca^{2+} concentration was 0.1 m*M*, the free Mg^{2+} concentration 1 m*M* (R. Wayne, 1985. Thesis, Univ. of Massachusetts, Amherst, MA, USA). Additionally, a medium was used with a free Ca^{2+} concentration $<10^{-8}$*M* and with a free Mg^{2+} concentration of 1 m*M*: 3.45 m*M* KNO_3, 1.03 m*M* $MgSO_4$, 10 m*M* EGTA, 20 m*M* PIPES at pH 6.0±0.1 (Ca^{2+} free medium). The media were prepared with milli-Q water (Millipore Corp., Bedford, MA, USA) with a resistance ≥ 18 MΩ.

Spores remained in darkness for 20 h at 22°C. Thereafter, they were R-irradiated. Red light was obtained by passing light from a 500 W projector bulb (DAY/DAK, General Electric, USA) through a heat absorbing filter and an Al666 interference filter (λ_m = 666 nm, halfband width = 23 nm, Schott, Mainz. The irradiance was 5.5 or 2.7 W m^{-2}, as measured with a Li-Cor light meter (LI 185; Lincoln, NE, USA) and a quantum sensor. Dark controls were run parallel to each irradiation experiment. Afterwards, spores were stored in darkness at 22°C until evaluation. All manipulations were carried out under dim green light.

Evaluation

Spores were examined with a Zeiss Univ. Microscope, equipped with either bright field or epifluorescence optics. In the epifluorescence mode, spores were illuminated with a 100 W mercury vapor lamp. The exciting light was passed through either a 1) BP-490 excitation filter combined with a dichroic mirror that allows excitation light of wavelengths less than 500 nm to pass and an LP520 barrier filter (blue excitation) or 2) a G365 excitation filter combined with a dichroic mirror that allows excitation light of wavelengths less than 395 nm to pass and an LP429 barrier filter (UV excitation). Germination was assayed with either 16×/0.4 or a 40×/0.75 NA Neofluar objective combined with an optovar set at 1.25× and 10× eye-pieces. Spores were counted in lots of 50 or 100 spores.

Photographs were taken with a Zeiss 35 Camera using Ektachrome 400 daylight film. Exposures lasted from 1 to 30 s, when the Zeiss automatic exposure meter was set at reciprocity = 3.

Chlorophyll determination in vivo

Chlorophyll fluorescence was measured using a Zeiss photomicroscope II equipped with a Zeiss microspectrophotometer and a Zeiss Zonax microprocessor. The optical path consisted of the following components: 40×/0.5 NA Neofluar objective; BP450–490 excitation filter; dichroic mirror <500 nm, LP520 barrier filter; optovar, 1.25×; eye-pieces 10×; aperature at the image plane, 100 μm. The voltage to the photomultiplier tube was set to 800 V and the gain to 100. The V. 821211 Lambda scan program was used and the errors due to the wavelength dependence of sensitivity of the photomultiplier tube and the chromatic aberration of the optic system were corrected with the V I.O Black Body Radiation correction program.

Chlorophyll determination in vitro

Chlorophyll was extracted from 80 mg spores (dry weight). Spores were prewashed in 20 ml of 10 m*M* EGTA at pH 6.0±0.1 for 20 h, thereafter they were sown on 20 ml culture medium. Two days after sat-

urating R, spores were separated from the culture medium by filtration through a Millipore filter (8 μm pore size; Millipore Corp., Bedford, MA, USA) and homogenization was performed in a glass-bead homogenizer (B. Braun, Melsungen) with 9 ml of 100% acetone (Merck, Art. 822251, Darmstadt). After centrifugation for 10 min at 1500 *g*, the chlorophyll contents in the supernatant was determined by measuring the absorbance in a 10 mm quartz cuvette at 663 and 645 nm with an Uvikon 860 spectrophotometer (Kondron, Analytik GmbH, München, FRG) according to Arnon (1949).

The in vitro fluorescence-emission spectrum of chlorophyll in 100% acetone was determined with a Perkin-Elmer 650-105 fluorescence spectrophotometer, measured in a 10 mm quartz cuvette. λ_{ex} was 430 nm; both slits controlling excitation as well as emission were set to 5 nm.

Statistics

In all figures regarding germination the 68% confidence interval is given as estimated from the equation

$$\pm\sigma = \sqrt{\frac{p(1-p)}{n}};$$

where p = germinated fraction and n = number of counted spores. Otherwise, data are presented as the mean ± SE.

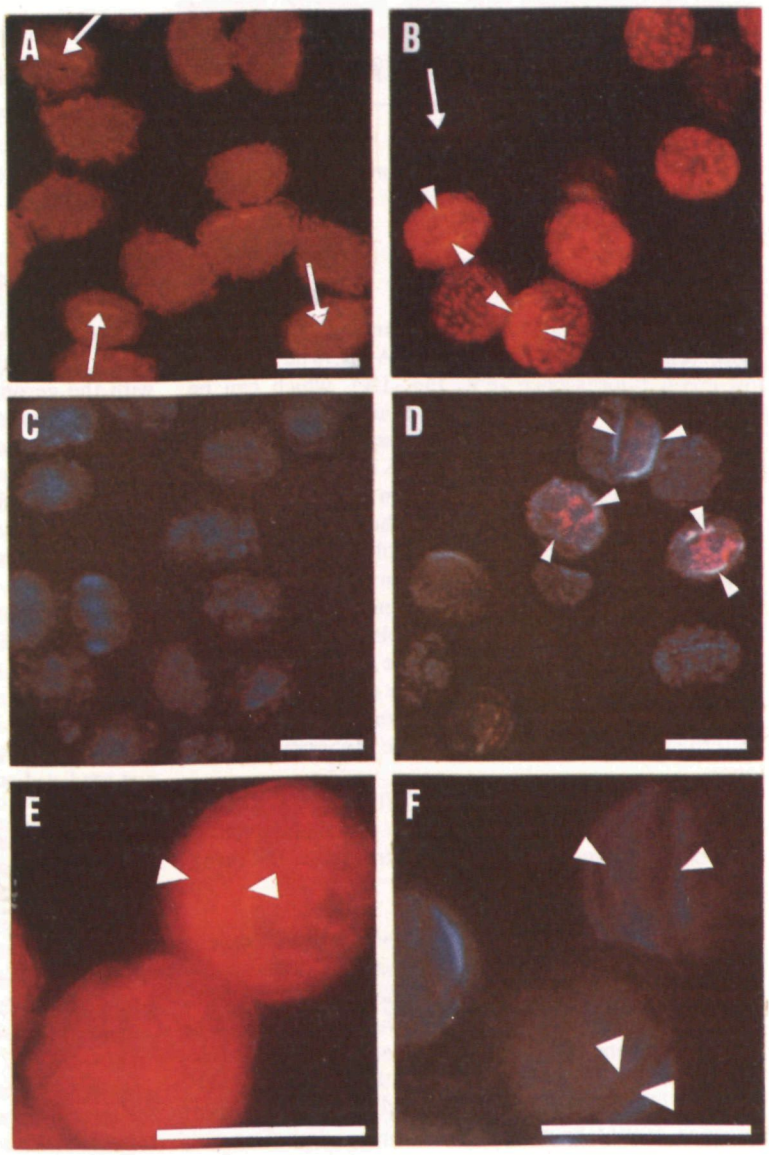


Fig. 1. Photomicrographs of non-irradiated (A, C) or R-irradiated (B, D to F) spores, taken 3 days after imbibition in standard medium. A and B (the same as E but with higher magnification) show fluorescence, induced by blue excitation. C and D (the same as F but with higher magnification) show fluorescence induced by UV excitation. R is saturating (60 s, 5.5 W m^{-2}). Triangles indicate the site of coat splitting; ↓, laesura; bars correspond to 50 μm. The visual impression of the weak fluorescence of non-germinated spores, observed through the microscope, is equal in A and B. In order to show this weak fluorescence in A in more detail the exposure time for taking the photomicrographs was prolonged. Photomicrographs of R-irradiated spores show selected areas containing a high amount of cells with coat splitting.

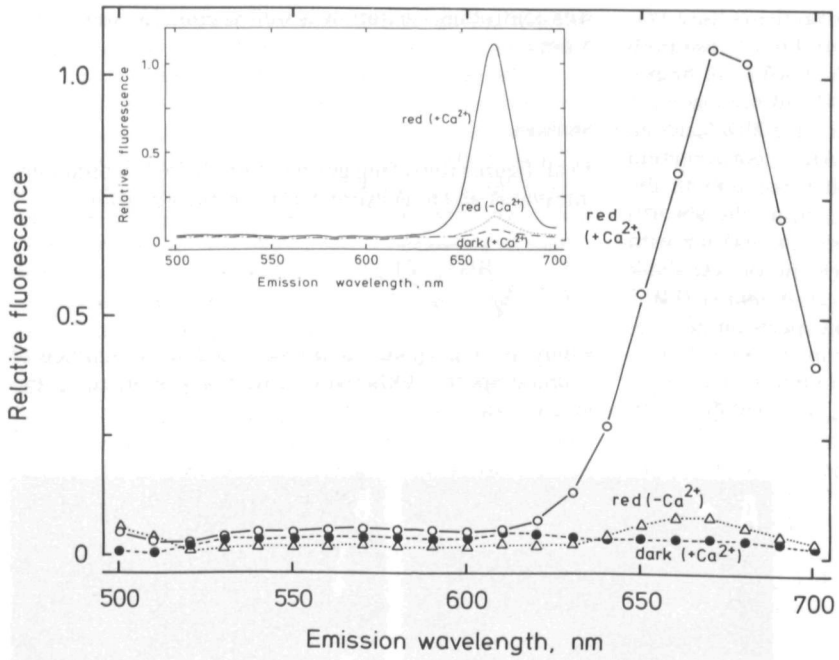


Fig. 2. In vivo fluorescence-emission spectrum of chlorophyll at 21°C measured in single spores 3 days after imbibition and after continuous darkness (●) or saturating R (○), 60 s, 5.5 W m^{-2}, 10^{-4} *M* Ca^{2+}; △, 60 s, 5.5 W m^{-2}, Ca^{2+} $<10^{-8}$ *M*). Excitation: blue light (n = 14). Inset: In vitro fluorescence-emission spectrum of chlorophyll in 100% acetone at 21°C. Eighty mg spores were sown on 20 ml of culture medium; 3 days after imbibition and after continuous darkness (10^{-4} *M* Ca^{+2}) or saturating R (300 s, 2.7 W m^{-2}, 10^{-4} *M* Ca^{2+} or Ca^{2+} $<10^{-8}$ *M*) chlorophyll was extracted with acetone. λ_{ex} = 430 nm. Relative fluorescence is given as photons nm^{-1} m^{-2} s^{-1}.

Results and discussion

Spores contain endogenous compounds that fluoresce blue, yellow-green and red (Fig. 1A–F). The dim yellow-green fluorescence (e.g. Fig. 1A), which can be quenched by the addition of KI and the blue fluorescence (e.g. Fig. 1C) is found in the spore coat; moreover, the yellow-green fluorescence may also be localized in the intine, as concluded from the bright yellow-green fluorescence shown around the laesura (Fig. 1A and B) and by the borders of the split spore coat (Fig. 1B). Both types of fluorescence seem to be independent of the light treatment. The blue fluorescence may be due to the presence of phloroglucinols (Tryon and Tryon 1982, Tryon et al. 1973); whereas the yellow-green fluorescence may be at least partly due to the presence of cellulose (Pettitt 1979). The autofluorescence color and pattern (Fig. 1E, F) may be of some taxonomic importance.

The red fluorescence two days after the inducing R (Fig. 1B and D–F) is localized in the chloroplasts and is due to chlorophyll, as evidenced from the following investigations.

1) The in vivo fluorescence-emission spectrum shows a peak around 675 nm (Fig. 2). Comparable results were reported for fluorescence measurements of chlorophyll at room temperature in bean leaves (Thorne 1971), maize leaves and in isolated, light treated etioplasts (H. L. Kraak 1986. Thesis, Agricultural Univ., Wageningen, The Netherlands). By contrast, this bright fluorescence cannot be observed in non-irradiated spores sown on standard medium or in R-irradiated spores sown on a Ca^{2+}-free medium. Both these conditions are known to inhibit germination (Wayne and Hepler 1984). Thus, this fluorescence seems to be correlated with the germination response. This latter observation is nicely confirmed in Tab. 1. Bright red fluorescence (relative fluorescence >0.1) can only be observed in R-treated spores. Under these conditions some spores with reduced fluorescence (relative fluorescence <0.1) can also be found. As a result, ca 85% show the bright fluorescence and this is in agreement with the germination response obtained for R-irradiated spores in the following experiments (see below, e.g. Fig. 3). By contrast, no bright fluorescence is obtained in darkness. For the expression of this R effect, Ca^{2+} ions are required in

Tab. 1. Relative red fluorescence (λ_{ex} = blue, λ_{em} = 670 nm) of non-irradiated and R-irradiated spores, sown on standard medium (+[Ca^{2+}]) or Ca^{2+} free medium (−[Ca^{2+}]). Measurement on a Zeiss photomicroscope 3 days after imbibition. For each treatment n = 14. [1] Calculated only with germinated spores, as determined by bright-field microscopy 6 days after imbibition; cf. also Fig. 4. [2] Calculated only with non-germinated spores.

Treatment	Relative red fluorescence	Germination (%)
Dark, $+Ca^{2+}$	0.03±0.002	0
Red, $+Ca^{2+}$	1.06±0.23 (1.22±0.23)[1]	85.7±9.4
Red, $-Ca^{2+}$	0.08±0.05 (0.04±0.004)[2]	7.1±6.9

Tab. 2. Contents of chlorophyll, extracted with 100% acetone from 80 mg spores (dry weight). Three days after imbibition on 20 ml culture medium and after continuous darkness (standard medium, $+[Ca^{2+}]$) or saturating R (300 s, 2.7 W m^{-2}, standard medium, $+[Ca^{2+}]$ or Ca^{2+} free medium, $-[Ca^{2+}]$) spores were separated from the culture medium and chlorophyll was extracted. Each treatment was repeated 3 times. For comparison the germination response, obtained for the various treatments, is given.

Treatment	Chlorophyll, μg (80 mg spores)$^{-1}$	Germination, %
Dark, $+Ca^{2+}$	0.83±0.25	0
Red, $+Ca^{2+}$	9.55±0.80	56.1±1.6
Red, $-Ca^{2+}$	1.55±0.19	7.7±0.9

the culture medium. Only 7% bright fluorescence is observed after saturating R in spores sown on a Ca^{2+}-free medium. Using this technique we show for the first time that Ca^{2+} contributes to the signal-transduction chain in phytochrome-mediated chlorophyll synthesis in *Dryopteris* spores; but it cannot be decided yet what step in the transduction chain is involved.

2) Significant amounts of chlorophyll can be extracted from R-irradiated spores as shown in Tab. 2; by contrast, considerably reduced quantities were found in non-irradiated spores or in R-irradiated spores sown on Ca^{2+}-free medium. Furthermore, the fluorescence-emission spectrum of chlorophyll, extracted from *Dryopteris* spores, is shown in Fig. 2 (inset). Again, significant fluorescence with a maximum around 668 nm is measured only in the extract obtained from R-irradiated spores sown on culture medium with a free Ca^{2+} concentration of 10^{-4} *M*. In general, coincidence is found for the fluorescence-emission spectrum in vivo and in vitro. This further confirms that the red fluorescence measured results from light emission from chlorophyll. Both the slight hypsochromic shift of the fluorescence-emission maximum and the more condensed form of the fluorescence-emission band, found under in vitro conditions, may be due to the altered hydration of chlorophyll, as well as to changes in the chlorophyll *a*/*b* ratio during extraction (Bruisma 1963).

The kinetics of the formation of the red fluorescence as compared to other characters used as indicators for the germination response, are given in Figs 3 and 4. In spores that have been synchronized, germination can be determined as early as 2 days from irradiation by using the chlorophyll-fluorescence method (Fig. 3a). At that time other indications of germination are still lacking. Coat splitting can be detected after another day's delay (Figs 3a, 4), and greening after at least 2 days delay. However, all 3 methods show a corresponding germination response of ca 80%. By contrast, a reduced value is indicated by rhizoid formation, and in darkness none of the germination criteria can be observed up to 5 days after irradiation (Figs 3a, 4). Red light causes a gradual spore swelling (Figs 3b, 4) during the first 5 days. After the fifth day a rapid increase is obtained, indicating the formation of a multicellular gametophyte (data not shown). By contrast, dark controls show a continuous decrease in cell size during the first 5 days (Fig. 3b). These observations characterize chlorophyll fluorescence as an early criterion to quantify germination within two days in non-green fern spores. This result is confirmed in Fig. 5, showing the fluence-response curve for the induction of spore germination with R: evaluation with the epifluorescence method 2 days after the light treatment as well as evaluation with bright-field microscopy 5 days after the light treatment show the same relationship between applied R and germination response. Moreover, since light requirement for spore germination can be replaced by gibberellins, e.g. in

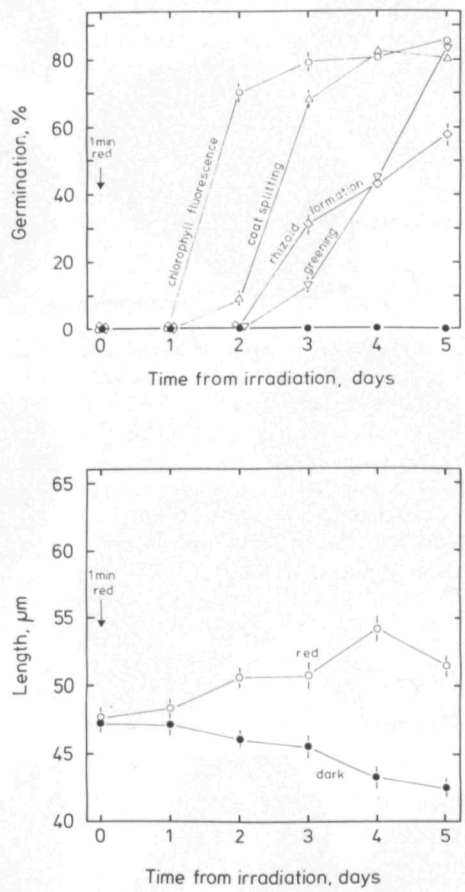
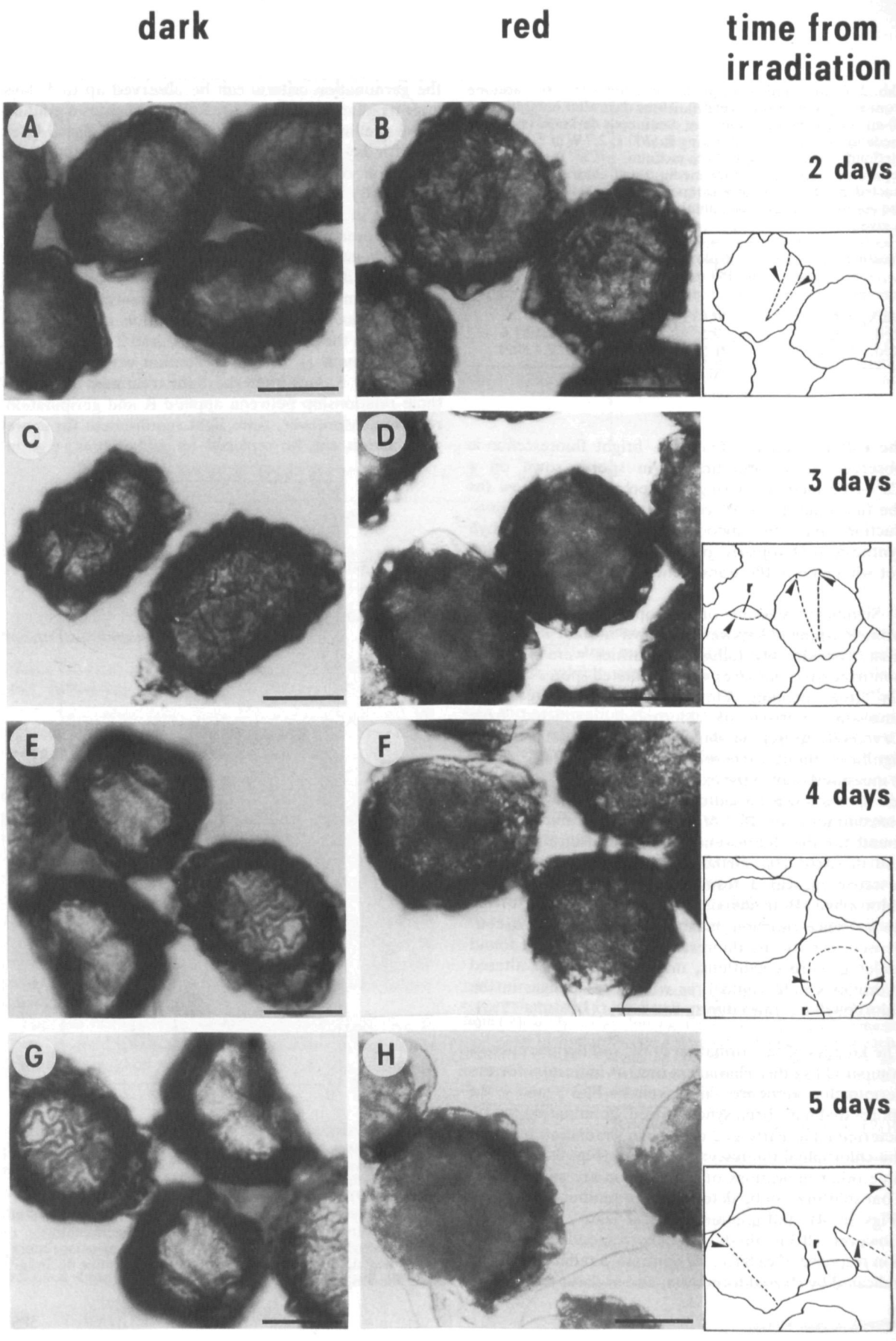


Fig. 3. Various criteria of germination scored daily after a saturating R-irradiation (60 s, 5.5 W m^{-2}, standard medium) (○). For comparison dark controls at different time intervals are given (●).
a) Chlorophyll fluorescence, coat splitting, rhizoid formation and visible greening were used as criteria for germination.
b) Spore length was used as a criterion for germination; spore length was determined as the distance from intine to intine along the longest axis.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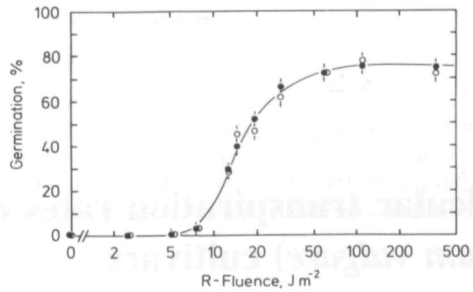

Fig. 5. Fluence-responce curve for the induction of spore germination with R (2.7 W m^{-2}, 1 s to 2 min) on standard medium. Germination was evaluated either with the fluorescence method 2 days after R (●) or with bright-field microscopy 5 days after R (○).

Anemia phyllitidis, (Schraudolf 1962, 1985) or in *Lygodium japonicum* (Manabe et al. 1987) the new method may also be used for chemical induction of germination by antheridiogens.

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Fig. 4. Photomicrographs of non-irradiated or R-irradiated spores taken at different intervals after light treatment. R was saturating (60 s, 5.5 W m^{-2}, standard medium). In the schematic drawings, added to the photomicrographs with R-irradiated spores, rhizoid formation (r) and the site of coat splitting (triangle) is indicated. Bar = 25 μm.