Spore Germination and Early Gametophyte Development of *Platycerium bifurcatum*

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Fern spores and gametophytes are excellent biological systems for the analysis of physiological and developmental problems (Raghavan, 1989). Gametophytes provide opportunities for studying various aspects of growth and differentiation (Hickok et. al., 1987). Furthermore, their existence is a boon to fern genetics (Chasan, 1992). An *in vitro* culture technique has been used to study different aspects of germination and early gametophyte development in many fern species (Nester & Coolbaugh, 1986; Von Aderkas & Raghavan, 1985; Whittier, 1981). Considerable information has also accumulated on the effect of different substances on spore germination and gametophyte growth (Melan & Whittier, 1990; Miller & Wagner 1987; Miller et al., 1983).

In *Platycerium,* these kinds of investigations have received little attention. Either the pattern of initial cell divisions during germination of spores was investigated (Nagmani & Raghavan, 1983) or older developmental stages of gametophytes were studied (Thentz & Moncousin, 1984; Camloh & Gogala, 1992). Therefore, in this investigation the process of spore germination and early gametophyte development of *Platycerium bifurcatum* was followed in detail. The effects of different culture conditions on various developmental stages of germination were examined. Stained whole-mount preparations were used for a more exact determination of cell divisions.

MATERIAL AND METHODS

Spores of *Platycerium bifurcatum* (Cav.) C. Chr. were kindly provided by Dr. B.J. Hoshizaki, University of California. They were harvested in her garden in Los Angeles in September 1991 and stored at 5°C in the dark until use. Spores were isolated from sporangial debris by filtering water suspensions of impure spores through a 60 μm nylon filter (Nybolt PA-60/33). They were collected on an 11μm nylon filter (Nybolt PA-11/4/C) and sterilized, unless indicated otherwise, first by a 5s exposure to 70% ethanol, followed by a 10 min. exposure to 10% (v/v) commercial bleach (4% NaOC1) with a drop of Tween 80 added, and thoroughly rinsed with sterile water. The water used for the sterilization procedure and media preparation was purified with a Milipore purification system (Milli Ro Plus and Milli-Q Plus unit).

Approximately 1000 spores were sown for each replicate on the surface of 3ml Miller medium (Miller & Miller, 1961) in which Fe citrate was replaced with NaFeEDTA. The pH was adjusted to 5.7-5.8 before autoclaving. The combinations of added Difco-Bacto agar and sucrose as well as the sterilization are presented in Table 1. For solidified media Petri dishes (diameter 3cm) were used. Liquid media were placed in tubes with 16mm diameter covered by aluminum caps. Cultures were maintained at 23±2°C, under a 16h photoperiod at 8.1–10.8Wm^{-2}, provided by 65-Watt fluorescent lamps. The spores were kept in these conditions from 3 to 10 days.

The germination percentage was determined 3 and 6d after spore sowing. The definition of spore germination was the emergence of a rhizoid through the spore coat. One hundred spores per replicate were scored. The length of the first rhizoid was measured 4,

Table 1. The % agar and sucrose used in different media. Spore sterilization is also indicated.

medium	A	B	C	D	E	F	G*
agar (%)	0.8	0.6	0.4	0	0	0	
sucrose (%)	3	3	3	0	3	0	0

*unsterilized spores

6 and 8d after sowing the spores and the gametophyte cell number was determined after 6, 8 and 10d of culture. For the determination of rhizoid length and number of cells, 25–35 samples were examined per replicate.

There were 2–3 replicates per treatment and all experiments were repeated twice. All parameters were scored on fresh material with a Carl Zeiss microscope. The Student t-test was used to calculate the levels of statistical significance (p) between the data obtained in different media. The mean values and 2SE are shown in tables and histograms.

For detailed observation of cell divisions, spores were sown on medium F in tubes with a 24mm diameter and plastic caps. In 1–2d intervals during the period from the second to the tenth day gametophytes were cleaned and stained with acetocarmine-chloral hydrate according to Edwards & Miller (1972). Three to four hundred gametophytes were examined each time. Photomicrographs were obtained by an Opton Axioskop.

Results

The % of spore germination 3 and 6d after sowing on different media are shown in Table 2. The germination percentages obtained after 3 and 6 days were similar. The best germination occurred when unsterilized spores were used (G) and the germination percentage was significantly higher ($p<0.05$) than in most of the other media. On the solid media (A–D), no significant differences on spore germination were observed in spite of

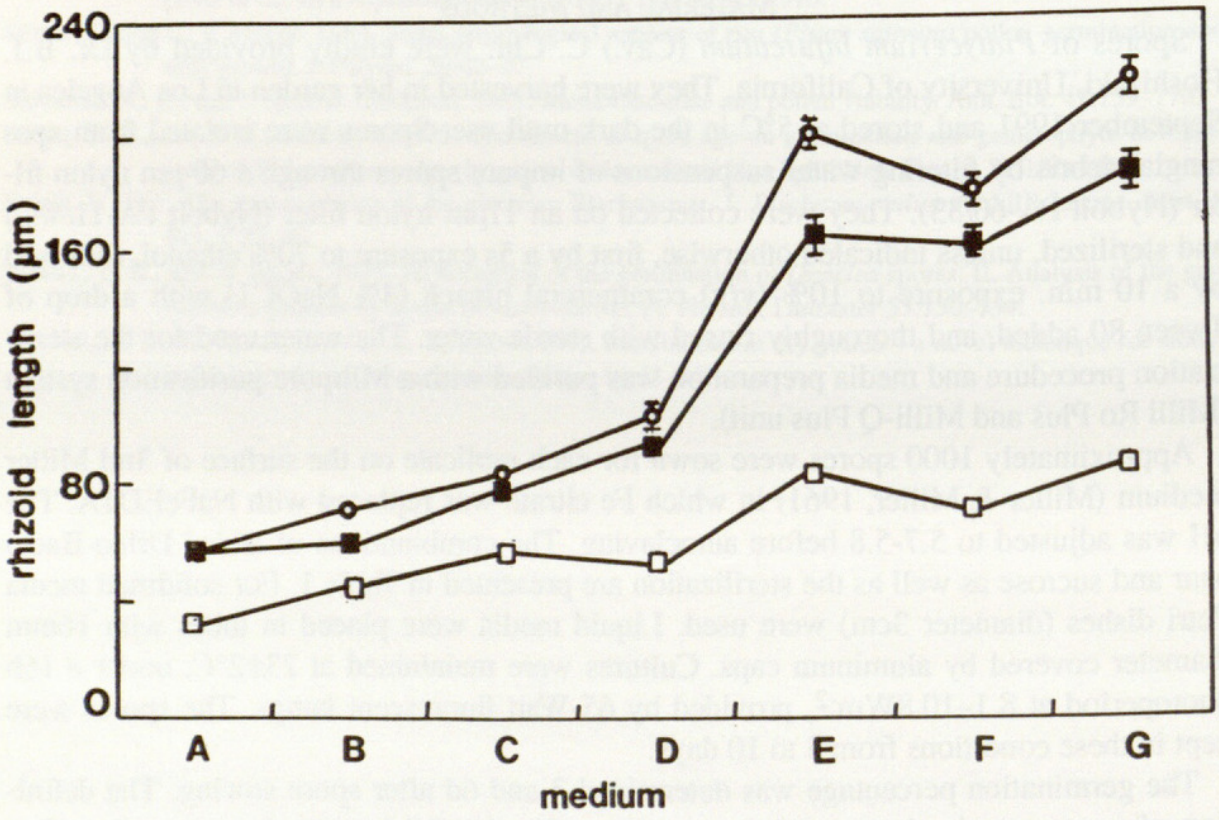


Fig. 1. Effect of various media on rhizoid length after 4 (open squares), 6 (solid squares) and 8 (open circles) days in culture (for medium labels see Table 1). Symbols without error bars had standard errors smaller than the symbols.

Table 2. The effect of various media and sterilization on spore germination. Percentage of spore germination determined 3 and 6d after sowing. Data represents mean values from 12–3 replicate samples ± SE.

medium[a]	% germination 3d	6d
A	56.0±2.2	66.0±3.8
B	54.4±0.8	73.0±2.2
C	52.4±5.3	64.1±0.3
D	53.5±5.5	67.6±6.0
E	47.3±1.6	59.8±3.8
F	50.3±3.6	68.8±1.4
G	68.2±3.6	87.2±0.6

[a]for medium labels see Table 1.

various agar and sucrose concentrations. On liquid media (E and F), the germination percentages were slightly lower (after 3d) or similar (after 6d) to the solid media.

Rhizoid elongation was greatly influenced by the medium (Fig. 1). The presence of agar in the medium greatly inhibited rhizoid elongation which can be seen not only from comparisons between agar-free and solid media but also from the increased rhizoid length at reduced agar concentrations. When 0.4% agar was added to the medium (C), the rhizoids were significantly longer ($p<0.001$) than in media with higher concentrations (A and B). The average rhizoid length of gametophytes grown for 6 or 8d in liquid media increased 2–3 fold as compared to solid media.

The absence of sucrose in the solid media (D) significantly ($p<0.001$) increased the rhizoid length, but not in liquid media. It is worthwhile to mention that the unsterilized

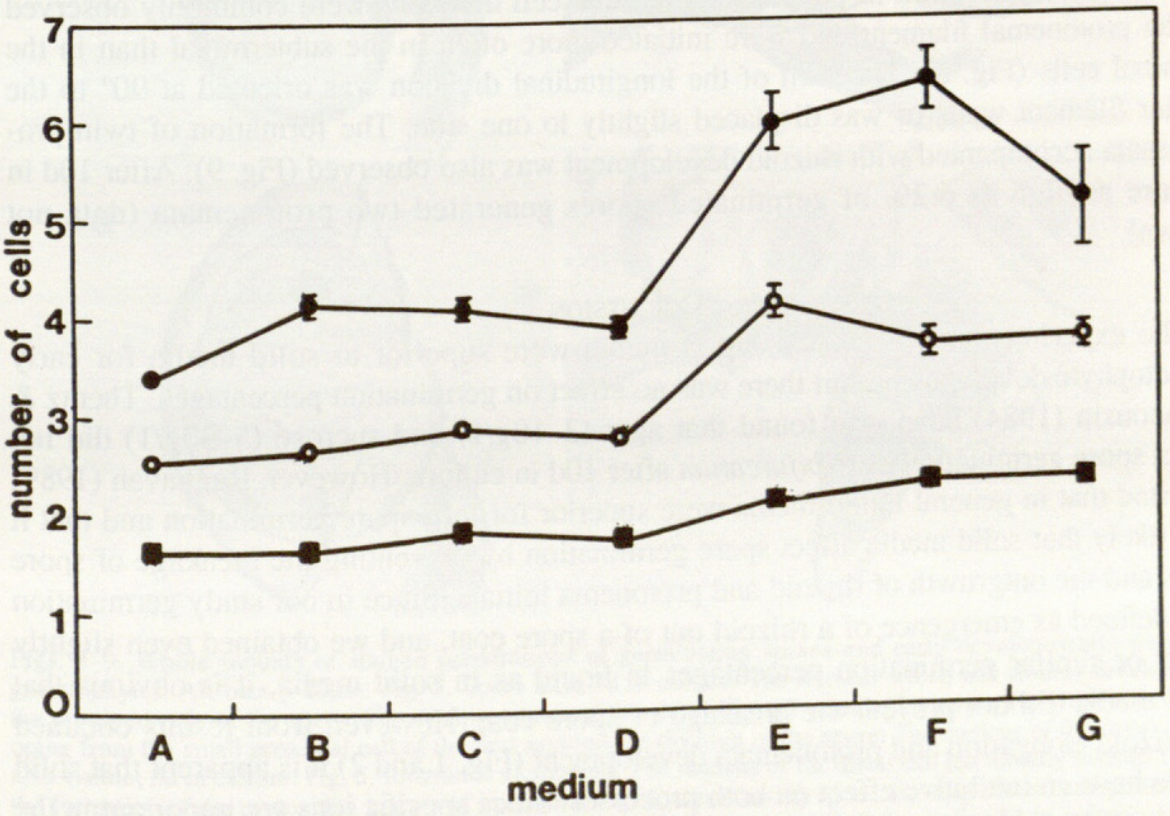


Fig. 2. Effect of various media on gametophyte cell number after 6 (solid squares), 8 (open circles) and 10 (solid circles) days in culture (for medium labels see Table 1). Symbols without error bars had standard errors smaller than the symbols.

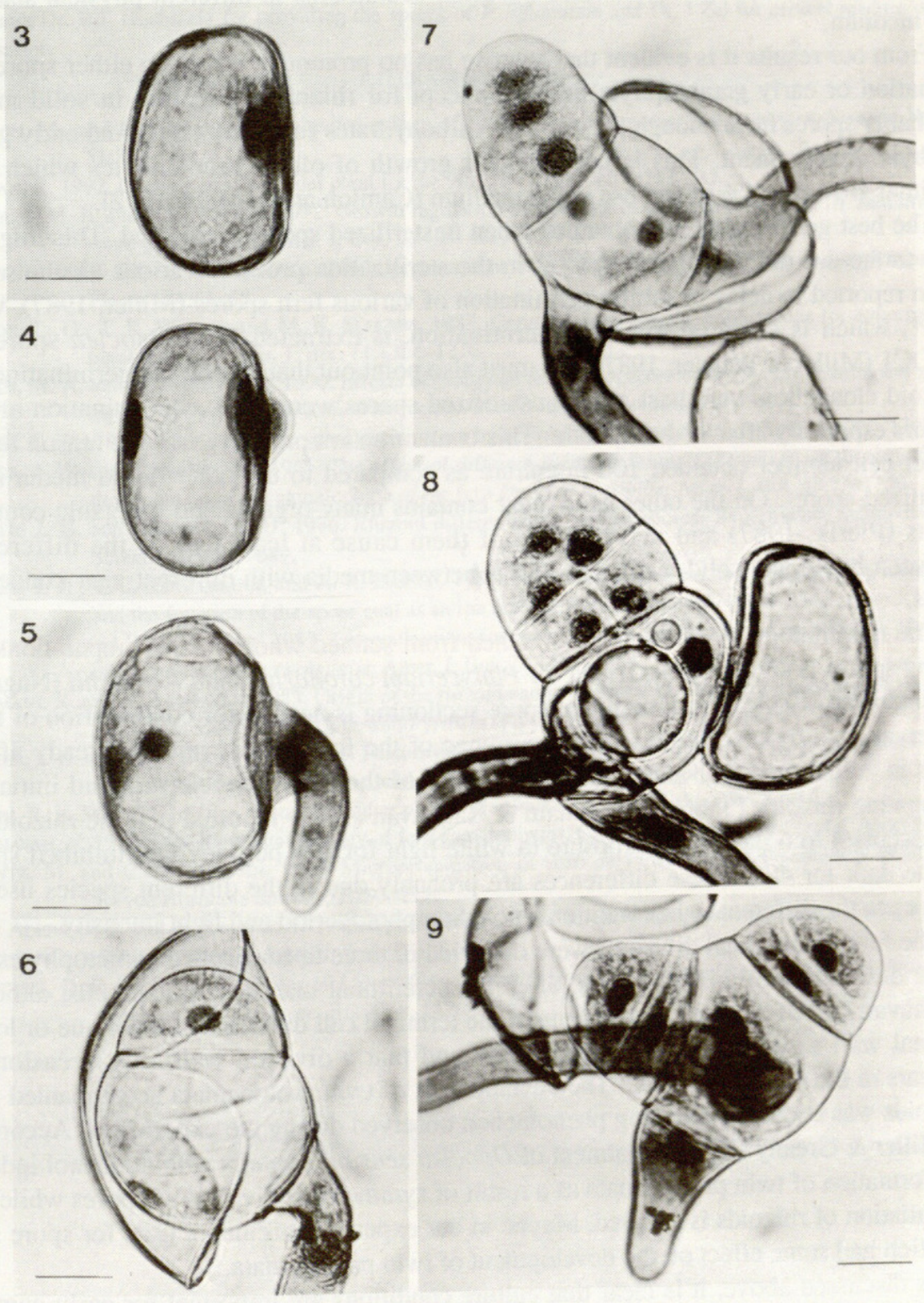
spores in liquid media had the longest rhizoids. No morphological rhizoid abnormalities were observed during these experiments.

The different media also affected the gametophyte cell number (Fig. 2). On liquid media gametophytes have a significantly ($p<0.001$ or $p<0.01$) higher number of cells than on solid media. The difference between liquid and solid media was visible after 6d in culture and increased considerably to the end of the experiment. Among the agar concentrations tested, a significantly lower number of cells ($p<0.001$) was obtained only after 10d of growth on the medium with 0.8% agar (A). The presence of sucrose had no significant effect on cell numbers on solid media (B and D), while in liquid media (E and F) the significantly higher cell numbers were obtained only after 8d in a sucrose enriched medium (E) ($p<0.05$). The cell number from unsterilized spores after 6 and 8d was comparable to sterilized spores, while after 10d it was lower.

The dry spores of *P. bifurcatum* are tan, slightly kidney-shaped with a smooth exine. After 2d in culture the nucleus was generally at the proximal pole of the spore and in some spores it had already divided (Fig. 3). After the division of the nucleus, the first asymmetric division of the spore cell occurs which yields the rhizoid initial. The rhizoid initial which grew through the exine at the proximal pole, represented the first microscopically visible sign of germination and was seen in a majority of spores after 3–4d in culture (Fig. 4). The rhizoid initial elongated and formed the primary rhizoid (Fig. 5). After approximately 6d in culture, the larger cell arising from the first asymmetric division divided asymmetrically by a wall parallel to the polar axis of the spore and gave rise to a protonemal cell (Fig. 6). This cell divided further until the protonemal filament was three to four-celled after about 8d in culture. At the same time a second rhizoid usually developed (Fig. 7). After 10d in culture, longitudinal cell divisions were commonly observed in the protonemal filament and were initiated more often in the subterminal than in the terminal cells (Fig. 8). The wall of the longitudinal division was oriented at 90° to the earlier filament walls or was displaced slightly to one side. The formation of twin protonemata accompanied with rhizoid development was also observed (Fig. 9). After 10d in culture as high as 6.2% of germinated spores generated two protonemata (data not shown).

Discussion

The experiments showed that liquid media were superior to solid media for early gametophyte development, but there was no effect on germination percentages. Thentz & Moncousin (1984) have also found that agar (3–10g/1) and sucrose (5–30g/1) did not affect spore germination of *P. bifurcatum* after 10d in culture. However, Raghavan (1989) reported that in general liquid media were superior for fern spore germination and that it was likely that solid media affect spore germination by preventing the breakage of spore coats and the outgrowth of rhizoid and protonema initials. Since in our study germination was defined as emergence of a rhizoid out of a spore coat, and we obtained even slightly lower or similar germination percentages in liquid as in solid media, it is obvious that solid media did not prevent the breakage of spore coat. However, from results obtained on rhizoid elongation and protonemata development (Fig. 1 and 2) it is apparent that solid media have an inhibitive effect on both processes. Since specific ions are important in the germination and development of fern spores (Miller & Wagner, 1987; Haupt, 1985) the reason for differences in rhizoid elongation and protonemata development obtained in



Figs. 3–9. Whole mounts of stained preparations of germinating spores and early developmental stages of gametophytes. All bars = 20μm. Fig. 3. Spore after 2d in culture. The nucleus which has already divided is at the proximal pole of the spore. Fig. 4. Appearance of the rhizoid initial outside the exine. The rhizoid is developing from the small proximal cell of the first asymmetric division of the spore. The nucleus of the distal cell is also visible; 3d in culture. Fig. 5. Rhizoid is elongating. The nucleus of the distal cell has already divided before the formation of the protonema initial; 4d in culture. Fig. 6. Germinating spore showing rhizoid and protonemal cell; 8d in culture. Fig. 7. Two rhizoids developing on a protonema with a few cells; 8d in culture. Fig. 8. Longitudinal division has already occurred and is clearly visible in the subterminal cell; 10d in culture. Fig. 9. Formation of twin protonemata accompanied by rhizoid development. Division in both cells to form filaments are visible; 10d in culture.

various media may lie in the altered diffusion of nutrients in different physical states of the medium.

From our results it is evident that sucrose has no promotive effect on either spore germination or early gametophyte growth, except for rhizoid elongation in solid media. Probably spores have enough endogenous carbohydrates for germination and early gametophyte development. This is not valid for growth of older gametophytes which gave optimal growth at 4% of sucrose in the medium (Camloh and Gogala, 1992).

The best germination was obtained when unsterilized spores were used. This might be due to the use of ethanol and NaOC1 in the sterilization process. Various alcohols have been reported to delay or inhibit germination of various fern spores (Miller, 1987), while Ca^{2+}, which is essential for spore germination, is extracted from *Onoclea* spores by NaOC1 (Miller & Wagner, 1987). We must also point out that even if the germination and rhizoid elongation were best when unsterilized spores were used, contamination always occurs especially after 10d of culture. This contamination probably was the reason for the lower cell number obtained 10d in culture as compared to the other liquid media using sterilized spores. On the other hand, agar contains many organic and inorganic contaminants (Pierik, 1987) and maybe some of them cause at least part of the differences between liquid and solid media as well as between media with different agar concentrations.

The morphology of germination obtained from stained whole-mount preparations and living spores is similar as described for *Platycerium coronarium* and *P. veitchii* (Nagmani & Raghavan, 1983) even though the spore sectioning is needed for confirmation of these observations. The exception is the occurrence of the first rhizoid initials already after 2 days in culture and that after 3 days about half of the spores develop rhizoid initials or elongating rhizoids (Table 2). Nagmani & Raghavan (1983) reported that the rhizoid initial occurs 4 to 6 days after exposure to white light for 12h but they first imbibed spores in the dark for 48h. These differences are probably due to the different species used as well as to the different light conditions including photoperiod and light intensity.

The first longitudinal divisions were observed in three to four-celled gametophytes and these divisions were initiated more often in subterminal than in terminal cells, although Raghavan (1989) reported that in general the terminal cell divides by an oblique or longitudinal wall to initiate planar morphology and that a division wall only occasionally appears in the subterminal cell. The development of twin protonemata accompanied with rhizoids was another interesting phenomenon observed during the experiment. According to Miller & Greany (1976), treatment of *Onoclea sensibilis* spores with methanol induces the formation of twin protonemata as a result of symmetrical division of spores while differentiation of rhizoids is delayed. Maybe in our experiments ethanol used for spore sterilization had some effect on the development of twin protonemata.

As discussed above, it is clear that culture conditions are important for early gametophyte growth of *P. bifurcatum.* In conclusion, spore sterilization is needed to study more than a few day old gametophytes; liquid media are superior for gametophyte growth when compared to solid, and sucrose is not required for early gametophyte growth. The optimal culture conditions as determined in this study represent an experimental system appropriate for further developmental and physiological studies of *P. bifurcatum* spore germination and gametophyte development.

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