American Fern Journal 77(3):95–99 (1987)

Germination of *Helminthostachys* Spores

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For nearly a century the spores of the Ophioglossaceae have been sown, but few investigators have succeeded in germinating them (Boullard, 1963). Campbell (1895, 1907) was the most successful in germinating spores of the Ophioglossaceae on soil. He reported the early stages of germination of spores of *Botrychium virginianum* (Campbell, 1895) and of three species of *Ophioglossum* (Campbell, 1907). An earlier report on spore germination in *B. ternatum* by du Buysson (1889) remains in question (Whittier, 1981) because the gametophytes that developed had essentially the same morphology as gametophytes of leptosporangiate ferns.

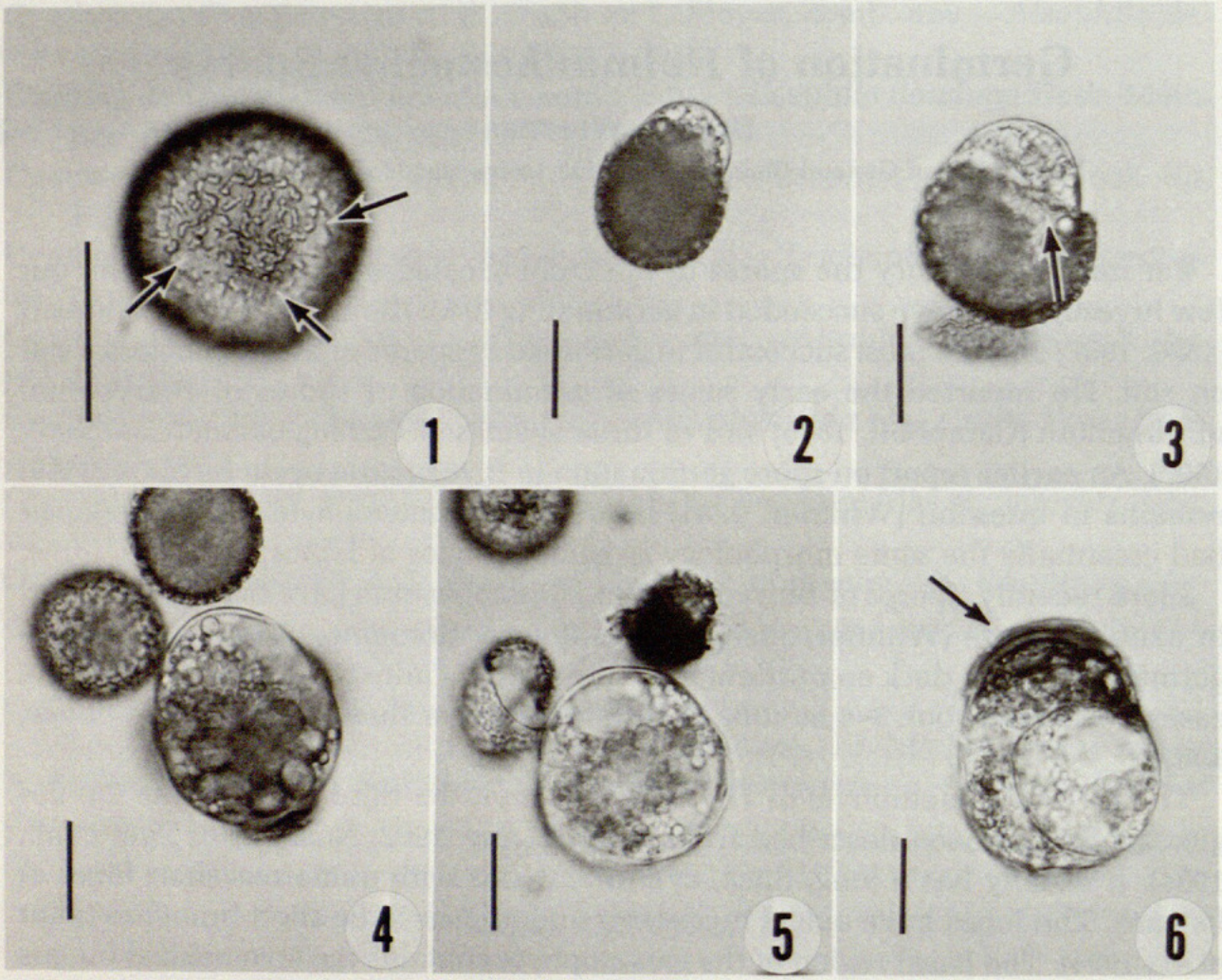
More recently, spores of *Botrychium* and *Ophioglossum* have been germinated in axenic culture (Whittier, 1972, 1981; Gifford & Brandon, 1978). These spores germinated in the dark on nutrient media containing minerals and sugar. In some cases, the spores took 3–4 months to germinate under these conditions (Whittier, 1981).

The mature gametophyte of *Helminthostachys*, the third genus of the Ophioglossaceae, has been described from nature (Lang, 1902; Nozu, 1961; Pant et al., 1984). It usually has a long, thick, cylindrical axis with numerous short lobes at its base. The lobes have apical meristems and appear to be short branches (Pant et al., 1984). The basal region of the gametophyte contains the mycorrhizal fungus and the cylindrical portion bears the gametangia. Spore germination and early stages of gametophyte development are unreported for *Helminthostachys*. Since germination and gametophyte development in *Botrychium* and *Ophioglossum* have been studied with the techniques of axenic culture, the aim of this study was to use these techniques to determine the conditions that promote germination and early gametophyte development in *Helminthostachys*.

MATERIALS AND METHODS

Fertile spikes of *Helminthostachys zeylanica* (L.) Hooker were obtained from plants grown in greenhouses at the New York Botanical Garden, Longwood Gardens, and the University of Massachusetts. The spores were sown within two weeks of their collection from the fertile spikes.

The techniques of Whittier (1973) were employed. Spores were sown on 15 ml of nutrient medium in culture tubes with a diameter of 20 mm. The tubes had screw caps which were tightened to reduce moisture loss. The nutrient medium was composed of a modified Moore's solution of mineral salts, minor elements, FeEDTA, and 0.6% agar. A liter of the modified mineral salt solution contained 100 mg NH_4Cl; 100 mg $MgSO_4 \cdot 7H_2O$; 40 mg $CaCl_2$; and 100 mg K_2HPO_4. The medium was supplemented with 0.2% glucose and had a pH of 5.3 after autoclaving. The spores were cultured at 24 ± 1°C in light at an intensity of 1400 lux from cool white fluorescent lamps or in darkness.



FIGS. 1–6. Germination and early gametophyte development of *Helminthostachys*. All bars = 25 μm. 1. Spore with cristate spore coat. Three arrows indicate ends and point of intersection of two arms of the triradiate ridge. 2. Rupturing of spore coat in early germination. 3. Two-celled gametophyte with spore coat attached. Arrow indicates cell wall separating proximal and distal cells. 4. Two-celled gametophyte with completely ruptured spore coat. 5. Young gametophyte free of spore coat. 6. Three-celled gametophyte with mucilage (arrow) on surface of proximal cell.

RESULTS

The hydrated spores of *Helminthostachys* are almost spherical and have an average diameter of 34 μm. The spore coat is cristate (Fig. 1) which makes observing the contents of the spores difficult. A major portion of the storage materials appears to be lipids since crushing the spores releases large amounts of oil which can be stained with Sudan IV. The triradiate ridge is difficult to observe (Fig. 1) because it is indistinct and small and all surfaces of the spore have the cristate ornamentation. The arms of the triradiate ridge are short with an average length of 10 μm.

After 8 months, spores on the nutrient medium in the dark began to germinate. Spores that were kept on the nutrient medium in the light for over a year failed to germinate.

Rupturing of the triradiate ridge initiates germination. The spore cracks open and the enlarging cell protrudes. This original protrusion has a small diameter

initially (Fig. 2) because of the small-sized triradiate ridge. The ornamented spore coat hinders observation of the first cell division in the enlarging cell. However, as the protrusion enlarges, a cell wall can be seen at the base of the protrusion (Fig. 3). The first division of the enlarging cell is perpendicular to the polar axis of the spore. This division produces a proximal cell (near the triradiate ridge), the protruding cell, and a distal cell (away from the triradiate ridge), which initially remains inside the spore coat.

The enlarging two-celled gametophyte, especially the distal cell, ruptures the spore coat more completely (Figs. 3, 4). Even after the tears in the spore coat extend to its distal surface, the spore coat can remain attached to the distal cell or its derivatives (Fig. 4). With additional enlargement of the young gametophyte the spore coat is sloughed off (Fig. 5).

The second cell division of the young gametophyte occurs in the distal cell and is parallel to the polar axis of the spore (Fig. 6). A small globular gametophyte is produced by additional cell divisions occurring in cells derived from the distal cell. The proximal cell, at least in the early stages of gametophyte development, remains undivided. The surface wall of the proximal cell is thicker than other cell walls in the young gametophyte (Fig. 6). In addition to the thicker wall, there is an accumulation of mucilage on the surface of the proximal cell (Fig. 6, arrow).

Discussion and Conclusions

The dark requirement for the germination of spores of *Helminthostachys* is the same as for spores of *Botrychium* and *Ophioglossum* in axenic culture. However, the time necessary for germination of *Helminthostachys* spores, 8 months, is longer than for other spores of the Ophioglossaceae that have been germinated in axenic culture (Whittier, 1981).

Helminthostachys spores have smaller triradiate ridges than comparable-sized spores of *Botrychium* (e.g., *B. dissectum* forma *obliquum*). The arms of the triradiate ridge are 10 μm long for *Helminthostachys* and 17 μm long for *B. dissectum*. The small triradiate ridge is probably responsible for the narrow diameter of the original protrusion of the enlarging cell during earlier germination. In *Helminthostachys* the protrusion is only about half the diameter of the spore. The protrusion in *Botrychium*, which has the larger triradiate ridge, has essentially the same diameter as the spore.

The pattern of early divisions in the young gametophytes of *Helminthostachys* is essentially the same as has been found for *Ophioglossum* and *Botrychium* (Campbell, 1907; Whittier, 1981). The first division is perpendicular to the polar axis of the spore producing proximal and distal cells. The second division occurs in the distal cell and is parallel to the polar axis of the spore. In *Botrychium* and *Ophioglossum*, the subsequent divisions in the cells derived from the distal cell produce the gametophyte (Whittier, 1981).

The proximal cell in the early stages of gametophyte development, as with *Botrychium* and *Ophioglossum* (Whittier, 1981), remains undivided. The thickened surface wall of the proximal cell and the secretion of mucilage through areas of this wall are characteristic for the Ophioglossaceae. Mucilage production

by the proximal cell has been reported for *Botrychium* (Melan, 1985) and observed in *Ophioglossum* (Whittier, unpublished).

The gametophytes of the Ophioglossaceae are slow growing (Boullard, 1963) and often live for more than one year (Jeffrey, 1897; Lang, 1902; Bruchmann, 1904; Campbell, 1911). The subterranean habitat appears to be a more protected site for these long-lived gametophytes than the soil surface. More water should be available for growth below the soil surface. Bruchmann (1906), St. John (1949), and Foster (1964) have reported finding living gametophytes in the soil during or after drought conditions. The subterranean habitat appears to provide more stable environmental conditions throughout the year which allows many of these gametophytes to be perennial.

The subterranean habitat is also important to the gametophytes of the Ophioglossaceae because they are dependent on an association with fungi in the soil for organic nutrients. In addition, the mycorrhizal fungus is also probably responsible for increasing the absorptive surface for uptake of mineral nutrients. The dependency on this mycorrhizal association occurs in early gametophyte development. The spores do not appear to contain enough reserves to support extended gametophyte growth because these young gametophytes undergo only a few cell divisions if not infected by fungi in soil (Campbell, 1907) or if no carbon energy source is available in the nutrient medium in axenic culture (Whittier, 1973). The initiation of gametophyte development in the soil would appear to increase the possibility that germinating spores and young gametophytes will be in close proximity to mycorrhizal fungi in the soil when organic nutrients are first required for development.

Evidence from this and other studies (Whittier, 1973, 1981) carried out with axenic techniques suggests that the spores of the Ophioglossaceae germinate only in the dark. Even short exposures of low light intensities are sufficient to prevent germination in *Botrychium* (Whittier, 1973). Presumably the spores sift or percolate down into the soil before germination can occur. The prevention of germination by light would be an effective mechanism to insure that germination will occur only after the spores are buried in the soil. The time delay, weeks or months, for germination in the dark probably also is important in assuring that the spores percolate to appropriate depths before germination. Since the gametophytes are adapted for the subterranean habitat, it is important for early gametophyte development to occur there.

ACKNOWLEDGMENTS

I thank John T. Mickel (New York Botanical Garden), Donald G. Huddleston (Longwood Gardens), Edward L. Davis (University of Massachusetts), and Ronald H. Beckwith (University of Massachusetts) for supplying the spores used in this study. This study was supported in part by the Vanderbilt University Research Council.

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