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# Delayed Growth in Mycoheterotrophic Gametophytes of Seedless Vascular Plants

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ABSTRACT.—Growth of the mycoheterotrophic gametophytes of *Sceptridium dissectum* and *Ophioglossum crotalophoroides* stalls at a few cells after germination on a nutrient medium lacking sugar. Resumption of growth when the cultures are supplied with glucose reveals that they are still alive. The stalled gametophytes of *S. dissectum* and *O. crotalophoroides* on media without sugar contain lipid droplets from the spores and a few starch grains that form during germination. The starch and lipid do not support the further growth of the stalled gametophytes because the size of the gametophytes does not change over time. These storage products slowly disappear from the stalled gametophytes. It appears that the storage materials are used to keep the stalled gametophytes alive. About 9% of the stalled gametophytes of *O. crotalophoroides* remain alive for ten months and about 5% of those of *S. dissectum* remain alive for 34 months. Delayed gametophyte growth is also exhibited by young gametophytes of *Botrypus virginianus*, *Lycopodium obscurum*, and *Psilotum nudum*. Delayed growth of mycoheterotrophic gametophytes under natural conditions would increase the time available for colonization by appropriate mycorrhizal fungi.

KEY WORDS.—Ophioglossales, Lycopodiales, Psilotales, mycorrhiza, sugar-free medium

Spores from plants with mycoheterotrophic gametophytes germinate in the dark (Whittier, 1973, 1998; Whittier and Braggins, 1994) whether on a nutrient medium in culture or embedded in soil (Whittier, 1984). The spores require the absence of light or more specifically the absence of red light (Whittier, 2006, 2008) before germination occurs. The spores of *Ophioglossum crotalophoroides* Walt. and *Sceptridium dissectum* (Spreng.) Lyon are trilete and contain significant amounts of lipid. In *S. dissectum*, lipid makes up 32% of the weight of the dry spores (Melan, 1985). Starch is not present in the dry spores. However, once the spores are wet and have swollen starch appears before the triradiate ridge has cracked open. *Sceptridium* spores germinate on nutrient media with or without sugar. However, on a medium without sugar, gametophytes form only a few cells and bulge only slightly from the spore coats. The gametophytes reach maturity only on a nutrient medium with sugar (Whittier, 1972, 2003). The small gametophytes that form on a nutrient medium without soluble carbohydrates do not appear to die when their growth stalls. These gametophytes contain lipid but it does not seem to be used for continued gametophyte growth on a medium lacking sugar (Whittier, 1984).

The mycorrhizal symbionts of *Botrychium* were characterized by Winther and Friedman (2007) at three stages in the life cycle of *Botrychium*. Two stages include the subterranean gametophytes and the young subterranean sporophytes that are completely dependent on the symbionts for fixed carbon. The

last stage includes the symbionts of the photosynthetic sporophytes that are assumed to have a mutualistic relationship with the host sporophytes. Because the gametophytes are totally dependent on the mycorrhizal fungi, they must be colonized early in their development. There is evidence that small few-celled gametophytes of *Ophioglossum* are colonized by mycorrhizal fungi before subsequent gametophyte growth occurs. This raises the question of how long these non-growing, few-celled mycoheterotrophic gametophytes (stalled gametophytes) can remain alive. The objective of this study is to examine whether these stalled mycoheterotrophic gametophytes can remain alive for a long time and whether they can resume growth if soluble carbohydrates are made available. Finally it would be of interest to determine whether delayed gametophyte growth occurs in any of the other groups of seedless vascular plants with mycoheterotrophic gametophytes.

## Materials and Methods

Species investigated for the study were: *Ophioglossum crotalophoroides* Walt., *Sceptridium dissectum* (Spreng.) Lyon, *Botrypus virginianus* (L.) Michx., *Lycopodium obscurum* L., and *Psilotum nudum* (L.) Pal. Beauv. The nomenclature for *Botrychium* used in this report follows Barker and Hauk (2003), that is *Sceptridium dissectum* (=*Botrychium dissectum*) and *Botrypus virginianus* (=*Botrychium virginianum*). Spores were collected in Tennessee except for those of *O. crotalophoroides* from Alabama and those of *P. nudum* from the greenhouse plants at Vanderbilt University, Nashville. Gametophytes of these five species require mycorrhizal symbionts to provide the organic carbon for their growth in nature. These symbionts have been identified using DNA data as *Glomus* spp. (Glomeromycota) for *Botrychium* and *Lycopodium* (Winther and Friedman, 2007, 2008).

The nutrient medium contained 100 mg $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2$, 100 mg $K_2HPO_4$, and 100 mg $NH_4Cl$ per liter. In addition, 0.5 ml of a minor element solution (Whittier and Steeves, 1960) and 4 ml of a FeEDTA solution (Sheat *et al.*, 1959) were added. The control medium contained 0.2% glucose and the experimental medium lacked glucose. Both media were adjusted to pH 6.0 before autoclaving and they were solidified with 1.1% agar.

The spores were surface sterilized with 1.1% sodium hypochlorite (Whittier, 1964), suspended in sterile water and sown on 12 ml of nutrient medium in culture tubes (20 × 125 mm) with screw caps that were tightened after inoculation. The cultures were maintained at 22±1° in the dark. After the spores were sown, the culture tubes were covered with aluminum foil before being placed in the dark. At irregular intervals after germination had reached its maximum level (unpub. data) additions of glucose were made to the experimental cultures. The additions were made at 4, 6, and 8 months for *O. crotalophoroides* and at 7, 14, and 32 months for *S. dissectum*. In all cases the cultures were examined two months later for gametophyte growth. The additions were made to the foil covered tubes under dim green light (Pratt, 1973) to reduce any possible effects that short exposures of dim light would

have on the stalled gametophytes. Sterile water (0.7 ml) or a sterile 0.5% glucose solution (0.7 ml) or nothing was added to the surface of glucose-absent cultures. The 0.5% glucose solution rather than a 0.25% glucose solution was used for sugar addition to the glucose-absent culture to reduce any dilution problems. The stalled gametophytes were exposed to the dim green light for about 30 s while the addition was being made. The caps were replaced and the tubes were returned to the dark for two months. No additions were made to the tubes with the control medium already containing 0.2% glucose.

After two months, the contents of the tubes with or without the additions were removed to ascertain the percentage of germination, gametophyte cell numbers, gametophyte lengths, and storage contents of the gametophyte cells. Gametophyte length was measured from the base of the spore coat, or if absent, from the base of the gametophyte to the apex of the gametophyte. Stained gametophytes were treated with acetocarmine for nuclei, $I_2KI$ for starch, or Sudan IV for lipid (Jensen, 1962). The sample sizes were 500 for the germination percentages, 200 for the gametophyte cell numbers, and 30 for the gametophyte length. The standard deviations were included with the averages for the cell numbers and gametophyte lengths. Gametophytes were considered to be growing if they had not turned brown and were larger than the stalled gametophytes.

Spores of *Botrypus virginianus, L. obscurum*, and *P. nudum* were germinated in the dark on a nutrient medium lacking glucose. Spores of *B. virginianus* and *L. obscurum* were examined at 14 months and those of *P. nudum* were examined at 9 months for gametophyte growth.

## Results

The germination process was the same for *O. crotalophoroides* and *S. dissectum* although the timing was different. Earliest germination occurred within two weeks for the spores of *O. crotalophoroides* and about six weeks for those of *S. dissectum* (unpub. data).

*Ophioglossum crotalophoroides.*—Germination (98%) was achieved by six months in this experiment whether the spores were sown on media with or without glucose. Because almost all the spores germinated initially subsequent germination was insubstantial. The spores of *O. crotalophoroides* germinated on the nutrient media with or without glucose (Table 1). Growth of gametophytes on the medium without glucose stalled (Fig. 1A) shortly after the spores germinated. The average cell number for the stalled gametophytes was $5.3 \pm 0.6$ with 7% having four cells, with 70% having five cells, 17% having six cells, and 6% having seven cells. The average cell number for the stalled gametophytes was the same throughout the experiment. Gametophyte growth on the medium with glucose for the whole experiment did not stall (Fig. 1B) but increased in size and cell number (Table 1).

Stalled gametophytes in cultures initially without sugar that had glucose added for the last two months before being examined produced more than 30 cells and grew to approximately double the size of the stalled gametophytes

TABLE 1. Delayed gametophyte growth in *Ophioglossum crotalophoroides*.

| | Nutrient medium without glucose[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Nothing added last 2 mo | | | Water added last 2 mo | | | Glucose added last 2 mo | | |
| Time from sowing spores | 6 mo | 8 mo | 10 mo | 6 mo | 8 mo | 10 mo | 6 mo | 8 mo | 10 mo |
| Spore germination | 98.7 | | | 98.2 | | | 98.5 | | |
| Gametophyte length (μm) | 77.9±5.9 | 77.2±5.8 | 81.2±6.4 | 78.8± 7.4 | 81.8± 6.8 | 82.7± 5.6 | 186.9±26.3 | 182.0±25.4 | 176.3±30.9 |
| Growing gametophytes (%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 99.5 | 45.6 | 9.3 |
| Stalled gametophytes (%) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 0.0 | 51.2 | 86.3 |
| with starch (%) | 15.6 | 0.2 | 0.0 | 12.8 | 0.1 | 0.0 | -[2] | -[2] | -[2] |
| with lipid (%) | 71.4 | 15.7 | 1.5 | 75.8 | 17.2 | 1.1 | -[2] | -[2] | -[2] |

[1] No stalled gametophytes occurred when spores were sown on a medium with 0.2% glucose. All the gametophytes grew and they attained an average size of 942.7 ± 307 μm at 10 months. [2] No data taken. The small number of gametophytes that could not be classified as stalled or growing was omitted from the results. mo = months.

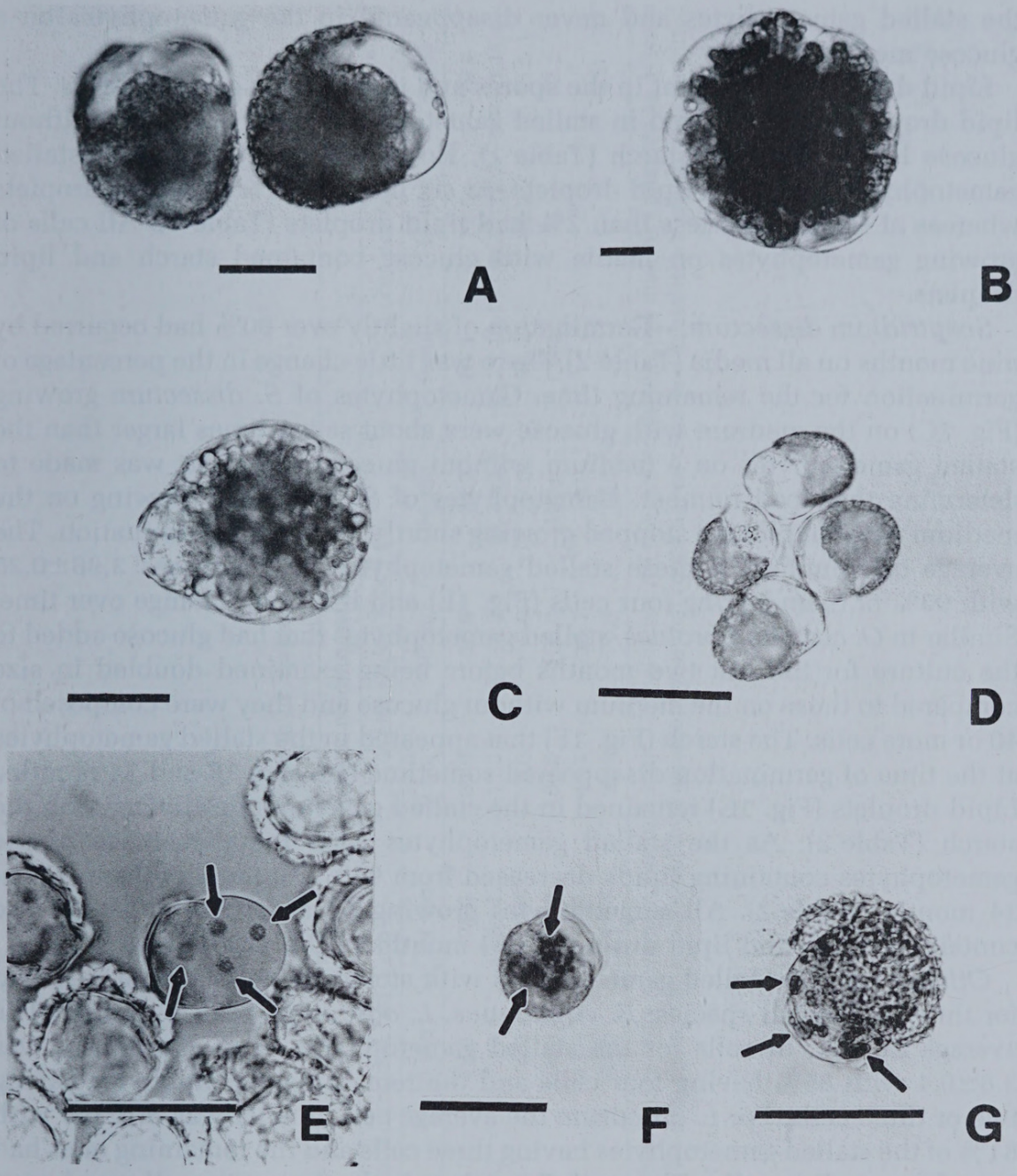


FIG. 1. Young gametophytes of *Ophioglossum* and *Sceptridium*. A–B, *O. crotalophoroides*. A) Stalled gametophytes. B) Growing gametophyte. C–G, *S. dissectum*. C) Growing gametophyte. D) Stalled gametophytes. E) Stalled gametophyte with four stained nuclei (arrows). F) Starch grains (arrows) in stalled gametophyte. G) Lipid droplets (arrows) in stalled gametophyte. Scale bars = 50 μm.

not supplied with glucose. Likewise these gametophytes on the medium with glucose for ten months were over ten times longer than the stalled gametophytes on a medium without glucose (Table 1).

On either medium, starch initially appeared as small grains next to the nuclei at germination. This starch essentially disappeared by eight months in

the stalled gametophytes and never disappeared in the gametophytes on a glucose medium.

Lipid droplets are present in the spores and in all young gametophytes. The lipid droplets were retained in stalled gametophytes on the medium without glucose longer than the starch (Table 1). However, with time, fewer stalled gametophytes contained lipid droplets. At six months 76% had lipid droplets whereas at ten months less than 2% had lipid droplets (Table 1). All cells of growing gametophytes on media with glucose contained starch and lipid droplets.

*Sceptridium dissectum*.—Germination of slightly over 80% had occurred by nine months on all media (Table 2).There was little change in the percentage of germination for the remaining time. Gametophytes of *S. dissectum* growing (Fig. 1C) on the medium with glucose were about seven times larger than the stalled gametophytes on a medium without glucose. No effort was made to determine their cell number. Gametophytes of *S. dissectum* growing on the medium without glucose stopped growing shortly after spore germination. The average cell number of these stalled gametophytes (Fig. 1D) was 3.98±0.25 with 95% of them having four cells (Fig. 1E) and it did not change over time. Similar to *O. crotalophoroides,* stalled gametophytes that had glucose added to the culture for the last two months before being examined doubled in size compared to those on the medium without glucose and they were composed of 40 or more cells. The starch (Fig. 1F) that appeared in the stalled gametophytes at the time of germination disappeared sometime between 16 and 34 months. Lipid droplets (Fig. 1G) remained in the stalled gametophytes longer than the starch (Table 2). As the stalled gametophytes aged the percentage of the gametophytes containing lipids decreased from 93% at nine months to 7% at 34 months (Table 2). All gametophytes growing on a medium with glucose contained starch and lipid during the 34 months of the experiment.

*Other species*.—Stalled gametophytes with storage materials were observed for three additional species: *B. virginianus*, *L. obscurum*, and *P. nudum*. The average number of cells for the stalled gametophytes of *B. virginianus* was 3.8±0.4 with 85% having four cells and the remaining gametophytes having two or three cells. For *L. obscurum* the average cell number was 3.1±0.5 with 81% of the stalled gametophytes having three cells and the remaining ones had two, four, or five cells. Those of *P. nudum* had an average cell number of 1.9±0.2 with 94% having two cells and 6% having one cell.

## Discussion

Gametophyte growth of *O. crotalophoroides* and *S. dissectum* stalls after a few cells on a medium lacking soluble carbohydrates. Gametophytes of *B. virginianus*, *L. obscurum*, and *P. nudum* also stop growing under the same conditions. Stalling of gametophyte growth appears to be a general phenomenon for mycoheterotrophic gametophytes of seedless vascular plants. These small, non-growing gametophytes are not dead but living in a stalled state with growth resuming when soluble carbohydrates are added.

TABLE 2. Delayed gametophyte growth in *Sceptridium dissectum*.

| | Nutrient medium without glucose[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Nothing added last 2 mo | | | Water added last 2 mo | | | Glucose added last 2 mo | | |
| Time from sowing spores | 9 mo | 16 mo | 34 mo | 9 mo | 16 mo | 34 mo | 9 mo | 16 mo | 34 mo |
| Spore germination | 79.3 | 79.1 | 79.5 | 83.9 | 78.8 | 81.7 | 84.1 | 78.1 | 79.8 |
| Gametophyte length (μm) | 42.9±3.7 | 42.7±3.8 | 43.3±2.7 | 43.1±3.8 | 42.1±3.5 | 41.3±2.4 | 96.7±16.1 | 94.9±18.7 | 88.2±11.4 |
| Growing gametophytes (%) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 98.1 | 92.2 | 15.5 |
| Stalled gametophytes (%) | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 0.0 | 5.1 | 82.2 |
| with starch (%) | 43.5 | 12.8 | 0.0 | 48.1 | 15.1 | 0.0 | -[2] | -[2] | -[2] |
| with lipid (%) | 93.1 | 58.9 | 11.4 | 92.6 | 70.3 | 7.0 | -[2] | -[2] | -[2] |

[1] No stalled gametophytes occurred when spores were sown on a medium with 0.2% glucose. All the gametophytes grew and they attained an average size of 1104 ± 408 μm at 34 months. [2] No data taken. The small number of gametophytes that could not be classified as stalled or growing was omitted from the results. mo = months.

In earlier studies on *S. dissectum* (Whittier, 1984) it was shown that the absence of soluble carbohydrates stopped the growth of young and mature gametophytes. Both young and mature gametophytes were unable to use external insoluble carbohydrates (starch and cellulose) for growth (Whittier, 1984). The use of sterile soil instead of a nutrient medium without sugar had the same effect on the young gametophytes. This would be expected because it has been concluded by Smith (1966) and Stevenson (1994) that there are basically no soluble carbohydrates available for plants in soil. Gametophyte growth stalls in the absence of a soluble carbohydrate in culture or without nutrients supplied by a mycorrhizal fungus in nature.

The young gametophytes seem unable to use their storage materials for extended gametophyte growth. The stalled gametophytes of *O. crotalophoroides* and *S. dissectum* contain ample storage materials in the form of starch and lipid droplets. These storage materials would be expected to support the gametophyte growth but they do not, as revealed by the unchanged size of the stalled gametophytes after ten months for *O. crotalophoroides* and 34 months with *S. dissectum*. However, the storage materials appear to be used to keep the cells of the stalled gametophytes alive.

The delayed growth of these fern gametophytes is similar to a condition found in the early development of orchids (Arditti, 1979; Smith and Read, 1997). Orchid embryos under asymbiotic conditions, like these fern gametophytes, need sugar for their continued early growth. For orchids, continued growth beyond the early protocorm stage needs soluble carbohydrates. Orchid embryos in their early protocorm stage only utilize their lipid storage materials very slowly (Arditti, 1979). They cannot use externally supplied large molecules like starch and cellulose. These seedlings, which cannot use their storage materials for growth, come to a 'resting stage' and remain alive by slowly utilizing their storage products. In the simplest case this 'resting stage' continues until soluble sugars are available for growth. The sugar necessary for growth is provided by mycorrhizal fungi in soil and by an appropriate nutrient medium in culture. The 'resting stage' (Arditti, 1979) or 'waiting time' as noted by Rasmussen (1995) and reported by Smith and Read (1997) of orchid embryos is similar to the 'stalled' state of mycoheterotrophic fern gametophytes.

Although the storage materials in the stalled gametophytes (starch and lipids) do not support further gametophyte growth, they do disappear from these gametophytes over time (Tables 1, 2). Starch is lost faster than the lipid (Tables 1, 2). Many gametophytes retain the storage products, especially the lipid droplets, for considerable lengths of time. A few of the stalled gametophytes of *O. crotalophoroides* had lipid at ten months; the comparable time for *S. dissectum* was 34 months (Tables 1, 2). Stalled gametophytes without storage products increased in number over time and they usually turned brown. These gametophytes were considered to be dead because they did not resume growth if supplied with sugar.

Fewer stalled gametophytes with storage products were present as time in culture increased and fewer gametophytes could be stimulated to grow. By the

time all the stalled gametophytes had run out of storage products, none would have the potential to resume growth whether it involved the addition of soluble carbohydrates to the cultures or colonization by a mycorrhizal fungus under natural conditions. Species with stalled gametophytes that used their storage products slowly would have an advantage over other species with gametophytes that used their storage products more rapidly. A longer time period increases the probability of colonization by mycorrhizae and subsequent growth.

Campbell (1907) reported that young gametophytes of *Ophioglossum* did not continue to grow until they were colonized by a mycorrhizal fungus. Bruchmann (1910) observed a similar situation with young *Lycopodium* gametophytes. This is consistent with what has been found for *Botrychium* and *Ophioglossum* gametophytes in culture. The stalled gametophytes can remain alive by using their storage materials for extended periods of time, but additional growth only occurs after soluble carbohydrates are provided.

Photoinhibition of spore germination insures that spores of species with mycoheterotrophic gametophytes will only germinate underground (Whittier, 2006, 2008). The subterranean germination of these spores would improve the possibility of sufficient moisture for gametophyte growth and development. These spores in the soil would be a temporary component of a spore bank (Sharpe and Mehltreter, 2010) because they germinate in the dark. Once germination and gametophyte growth occurs, they would be considered as belonging to a gametophyte bank (Farrar *et al.*, 2008) or to the belowground structure bank recognized for *Botrychium* (Johnson-Groh *et al.*, 2002).

The subterranean germination insures that the gametophytes develop underground and it enhances the possibility that young gametophytes will be in the proximity of mycorrhizal fungi. Delayed gametophyte growth has the potential to give the gametophytes more time for colonization. The use of the storage materials for increased cell growth likely has a shorter time frame than keeping the small but stalled gametophytes alive for a long time. The presence of small, but living gametophytes in the soil would improve the possibility for colonization to occur and mature gametophytes to develop.

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