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Gametophytes of *Diphasiastrum ×habereri*

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Diphasiastrum ×habereri (House) Holub is recognized as a hybrid of *D. digitatum* (Dill. ex A. Br.) and *D. tristachyum* (Pursh) Holub, because it is morphologically intermediate between the two parents (Wilce, 1965; W. Wagner, 1969; F. Wagner, 1980; Hersey and Britton, 1981; Wagner et al., 1985). Normally, pteridophyte hybrids have meiotic problems and abortive spores (Wagner et al., 1985) but *D. ×habereri* is different. Its meiosis is normal and its spores are not aborted (F. Wagner, 1980; Hersey and Britton, 1981).

The complete pairing of the chromosomes during meiosis suggests that *D. ×habereri* is fertile (Wagner, 1980; Hersey and Britton, 1981). It also has been noted that the frequency of these plants is greater than expected for diploid interspecific hybrids (Wagner et al., 1985) and that it can occur in areas with only one or neither of the parental species (F. Wagner, 1992). These observations support the possibility that *D. ×habereri is* a fertile hybrid.

If *D. ×habereri* is fertile, then its spores must germinate to form sexually mature gametophytes. No gametophytes of this hybrid have been found in nature, although this is not unexpected for *Lycopodium* sensu lato (Bruce and Beitel, 1979). Gametophytes of the Lycopodiaceae have been grown successfully in axenic culture, and the present study was initiated in an attempt to grow these unknown gametophytes. Problems with spore germination and gametophyte development would certainly raise questions as to whether *D. ×habereri* is fertile.

MATERIALS AND METHODS

Spores of *Diphasiastrum ×habereri* were obtained from strobili collected in the Parry Sound District of Ontario, Canada. A cytological examination of material from this site had been done previously (Hersey and Britton, 1981). A voucher specimen (*Britton 11507*) is on deposit at the Vanderbilt University Herbarium (VDB).

The spores were surface sterilized with 20% Clorox by the techniques of Whittier (1973), and sown on 15 ml of nutrient medium in culture tubes having a diameter of 20 mm and screw caps that were tightened to reduce moisture loss. Fresh spores and those stored at room temperature for two months gave essentially the same results. The cultures containing the spores were maintained in darkness or under a 12 hr photoperiod (50 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) from Gro-Lux fluorescent lamps.

The nutrient medium contained a modified Moore's solution (Moore, 1903). The spores almost never germinated on nutrient media with full strength Moore's solution. However, spore germination increased if a lower strength Moore's medium was employed. The highest percentages of germination were obtained if a liter of the basic nutrient medium contained 25 mg $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, 25 mg K_2HPO_4, and 50 mg NH_4Cl, plus FeEDTA and minor elements. In addition to the minerals, the nutrient medium was completed with 0.25% glucose and 0.9% agar. After autoclaving, the medium was at pH 5.1±0.2.

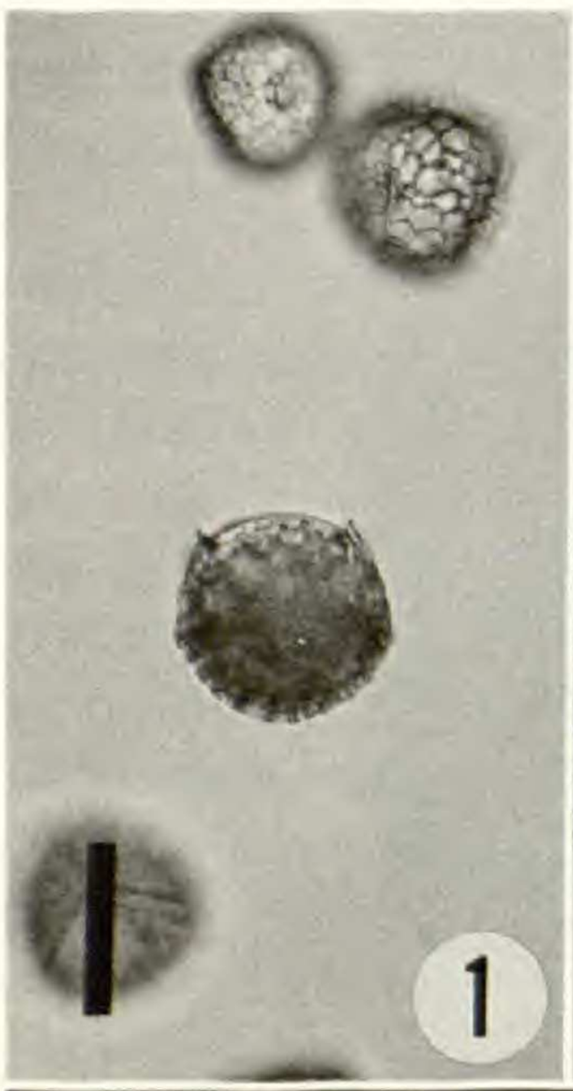
Gametophytes developing on the basic nutrient medium in the original cultures grew slowly. In an effort to accelerate gametophyte growth, the young gametophytes were transferred to new cultures that had double the mineral concentration and 0.5 or 1.0% glucose. The higher sugar concentrations enhanced gametophyte growth, and macroscopic gametophytes were periodically collected from the cultures. These gametophytes were fixed and stored in CRAF (Johansen, 1940) until sufficient numbers of gametophytes were available for study. The white color of the large macroscopic gametophytes changed during fixation and storage in CRAF to bluish-green. Hand sections of the gametophytes were stained with Toluidine blue O.

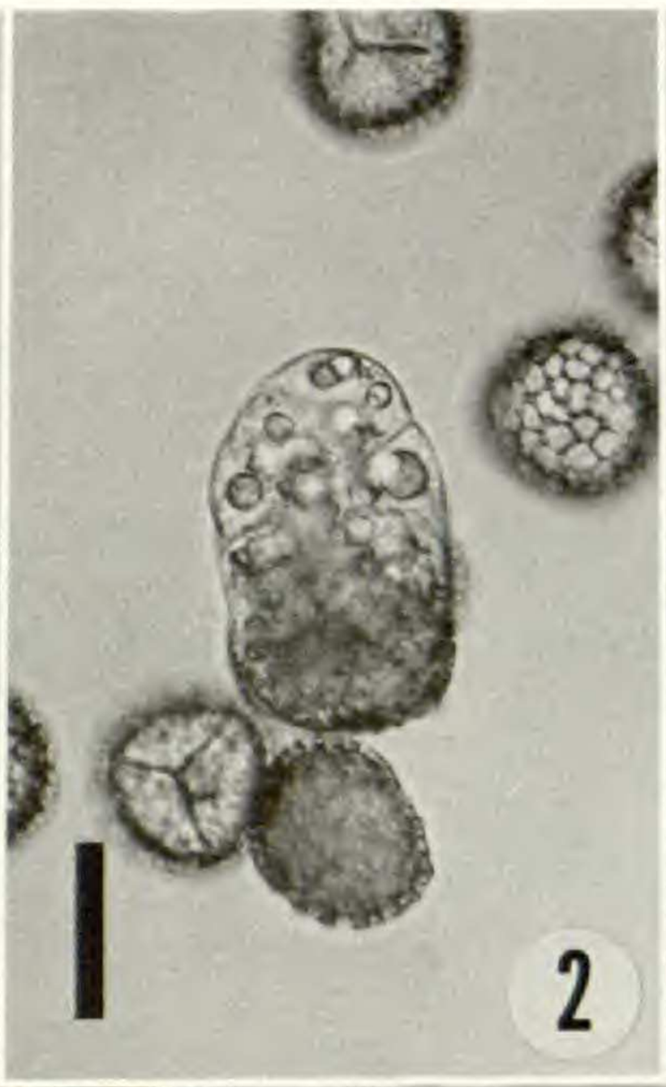
Results

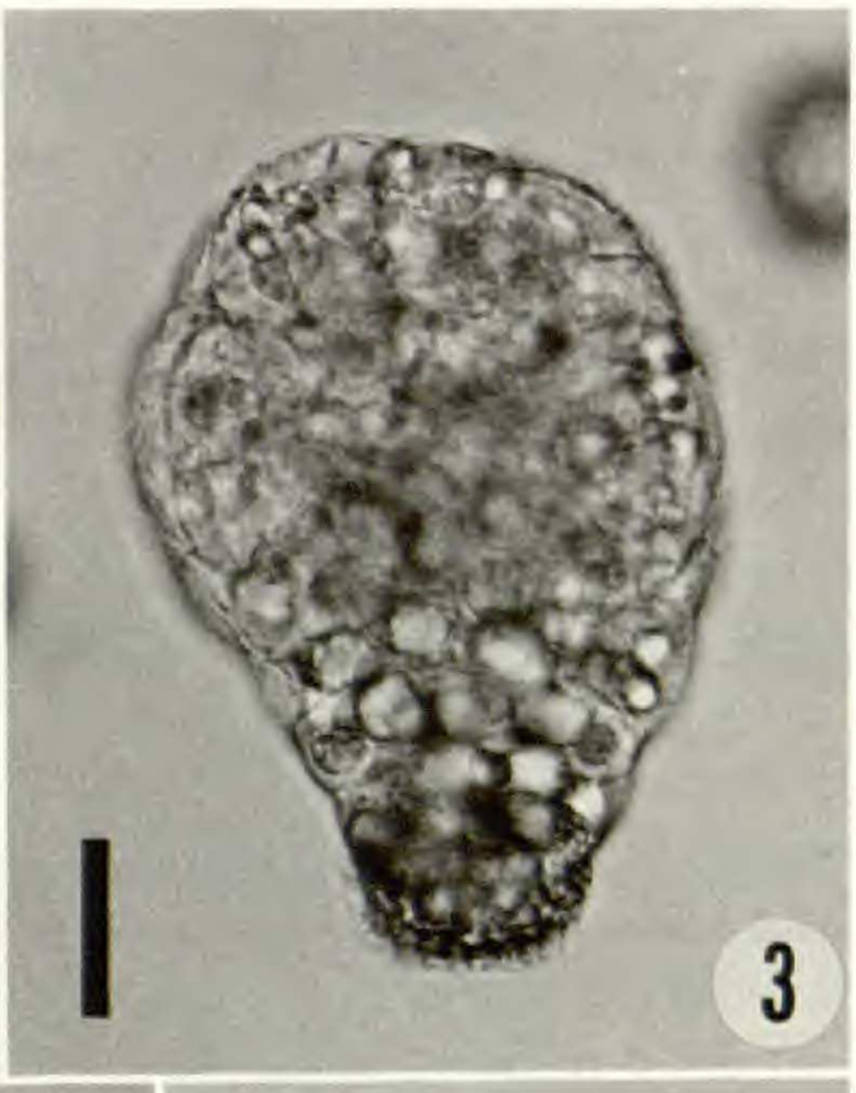
Spores of *D.* ×*habereri* germinated only in cultures maintained in the dark. No germination had occurred by three months, but 0.5% of the spores had begun to germinate at six months (Fig. 1). By nine months more spores (1.6%) had germinated and small multicellular gametophytes were present (Figs. 2, 3). The last observations on germination were made at one year when 2.5% of the spores had germinated.

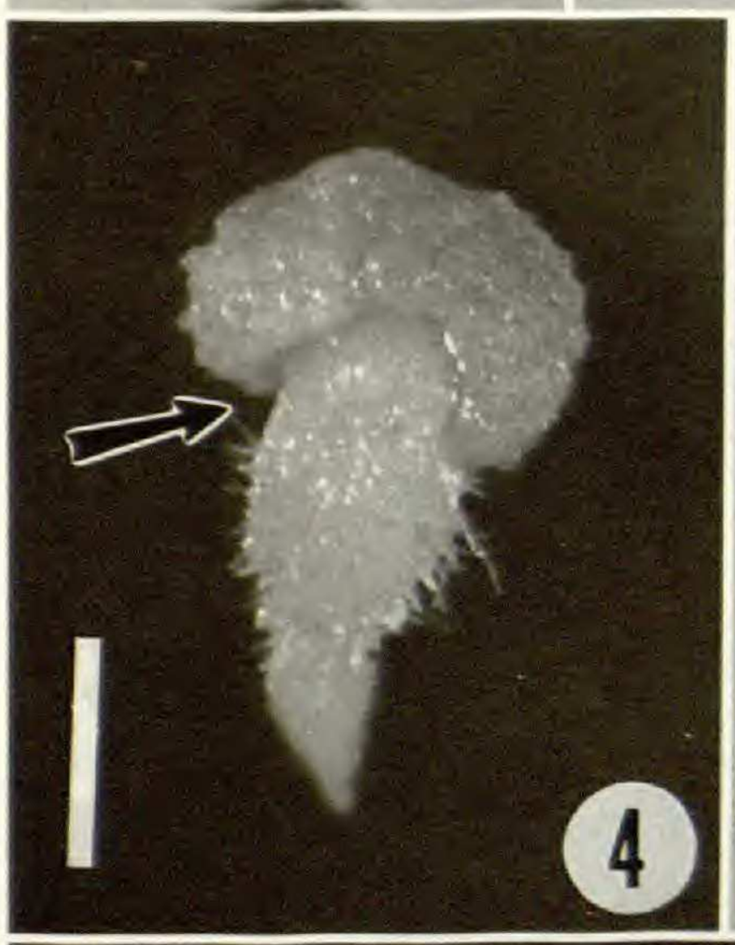
At one year there was some variation in the size of the gametophytes, but most were small globular gametophytes. All the gametophytes had accumulations of starch in their cells and appeared healthy. There was no abortion of gametophytes in any of the cultures. Mature gametophytes were obtained from gametophytes transferred to new cultures with higher mineral and glucose concentrations after another year or more of growth.

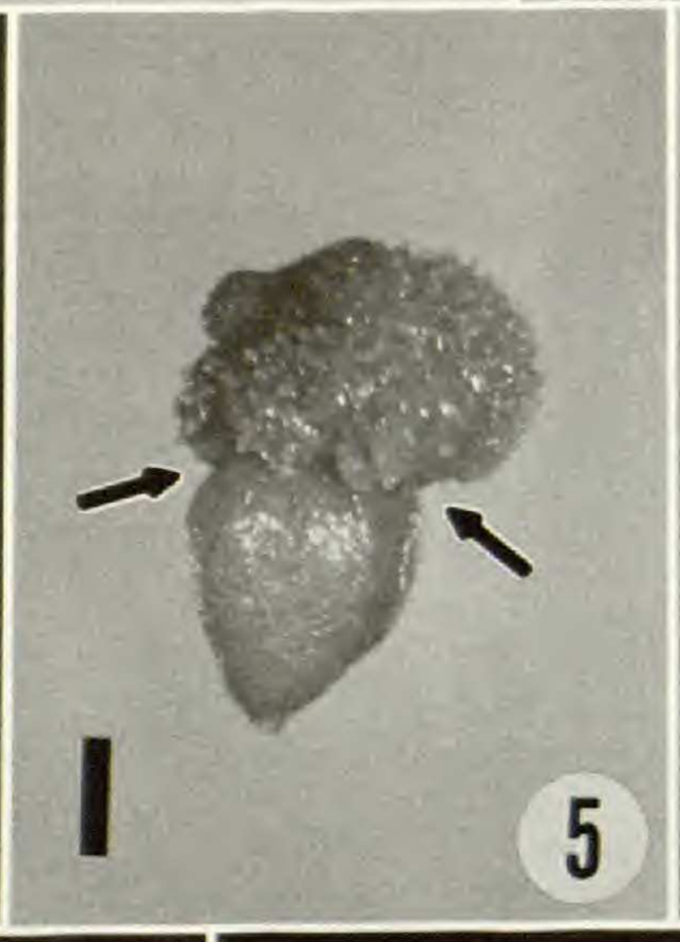
The mature, white gametophytes were carrot-shaped (Figs. 4, 5, 6) and the largest one grown was 8 mm long. The gametangial cap was separated from the conical base by a constriction at the ring meristem. Hand sections demonstrated several aspects of the internal structure. Below the ring meristem in the conical region some cell elongation was apparent in what would be the inner mycorrhizal zone of gametophytes from nature. Some gametangial caps only contained large sunken antheridia (Figs. 4, 9) which on the average were 310 μm long and 145 μm wide. On other gametophytes both antheridia and archegonia had developed (Figs. 5, 6, 7). The archegonial necks were long (Figs. 7, 10) and contained 10 or more neck canal cells. The average length from base of egg to tip of neck was 295 μm and the neck usually extended 150 μm above the gametophyte surface.

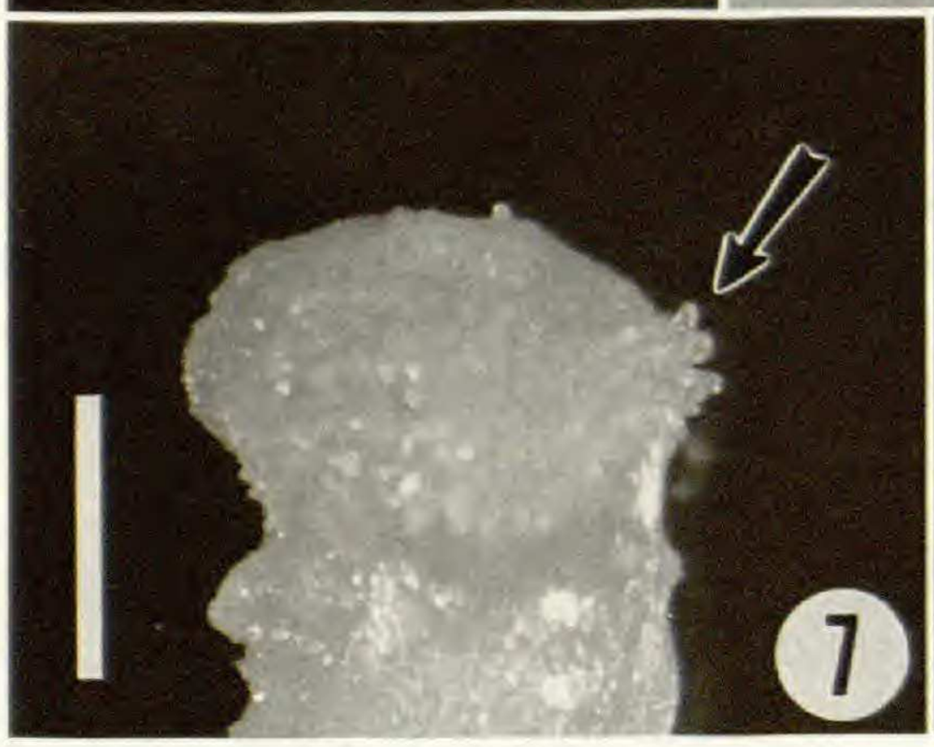


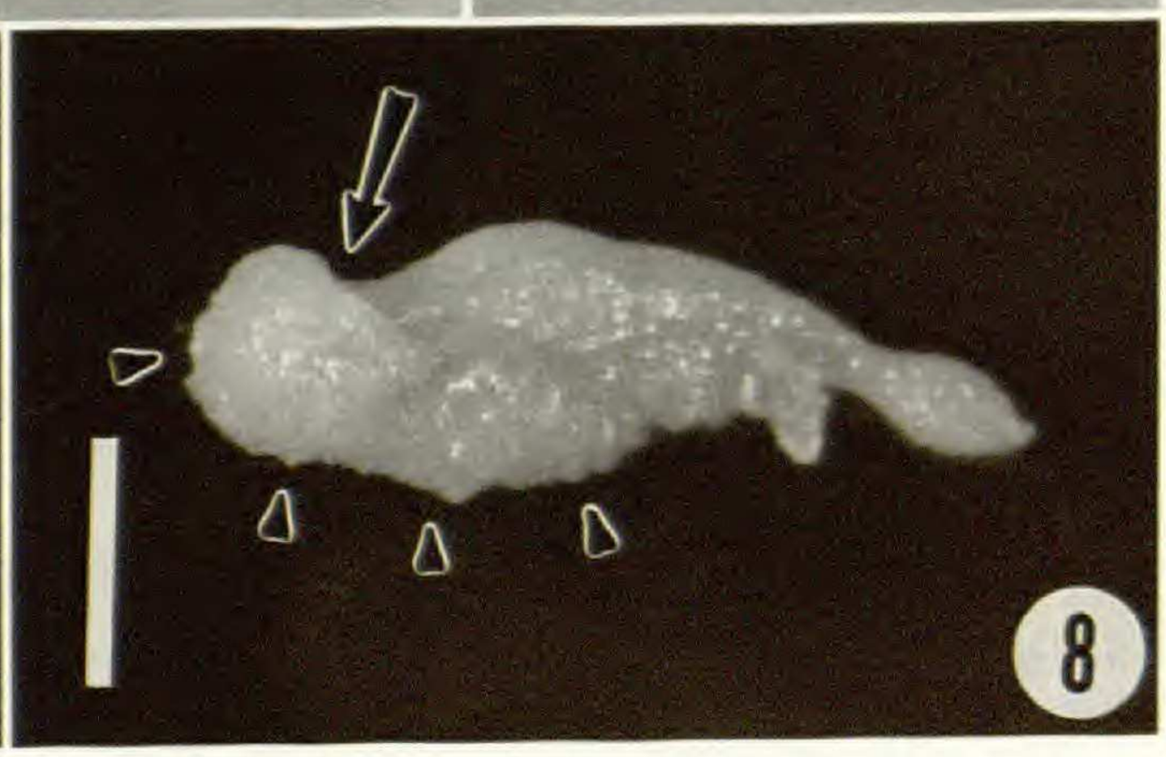




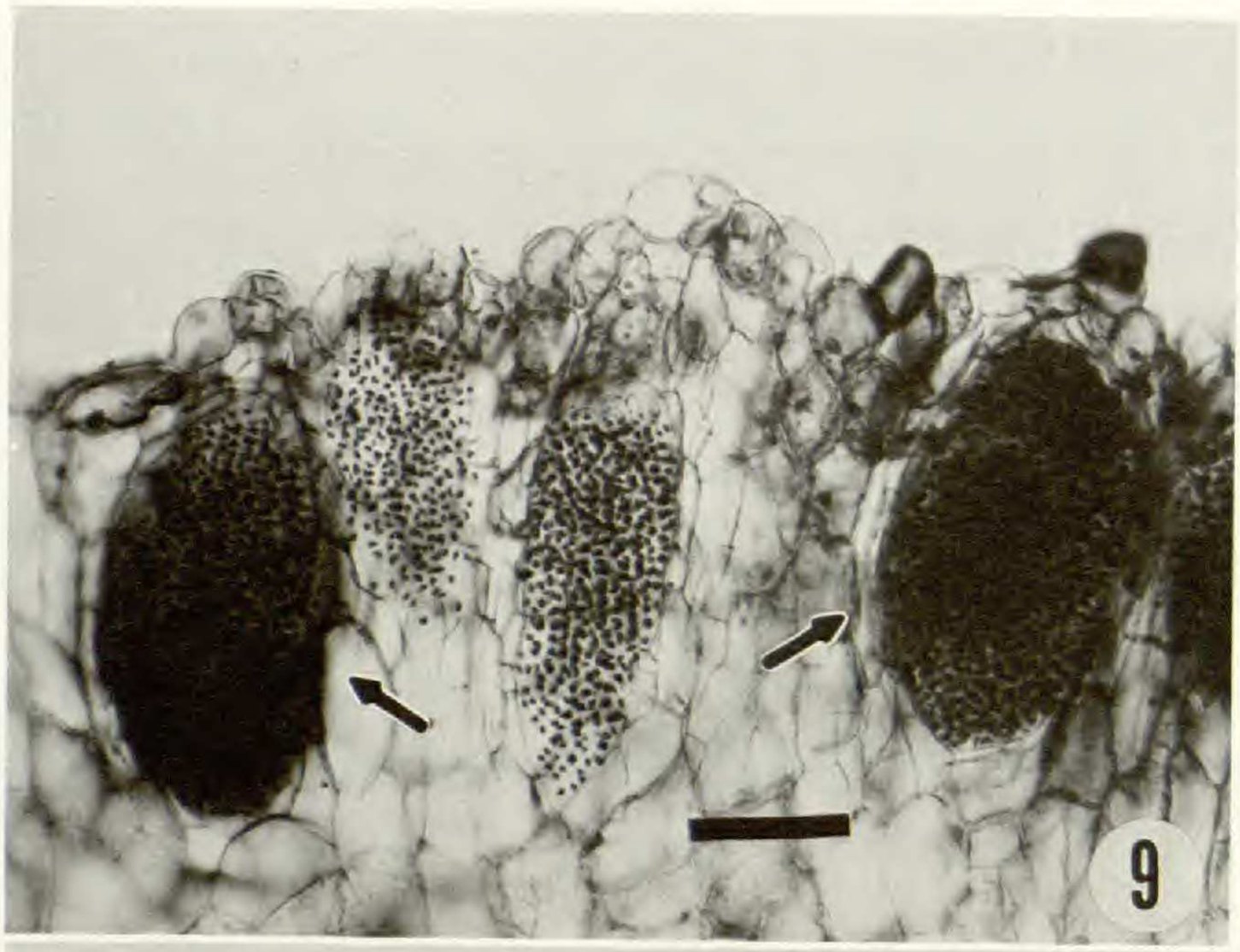


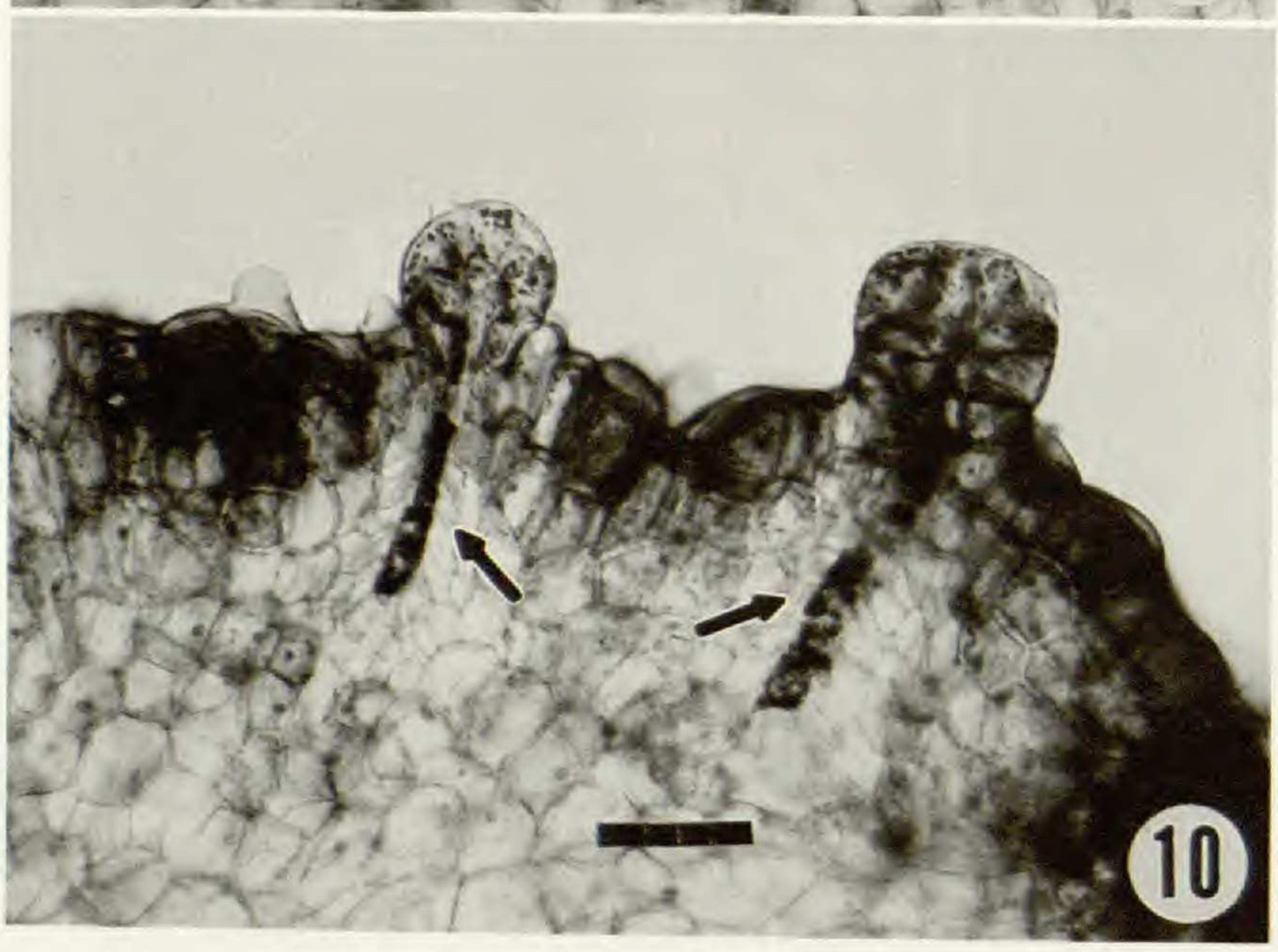






FIGS. 1–8. Gametophytes of *Diphasiastrum* ×*habereri*. FIG. 1. Germinating spore. FIGS. 2–3. Young multicellular gametophytes with spore coats attached. FIG. 4. Macroscopic gametophyte with conical base and gametangial cap having sunken antheridia. Arrows indicate site of ring meristem. FIG. 5. Mature gametophyte with conical base and gametangial cap having both gametangia. Arrows indicate ring meristem. FIG. 6. Mature gametophyte with enlarged gametangial cap that obscures ring meristem. FIG. 7. Gametangial cap showing long archegonial necks (arrow). FIG. 8. Gametophyte with incomplete ring meristem (arrow) and gametangial region down side of conical base (arrowheads). Bars = 30 μm for Figs. 1–3 and 1 mm for Figs. 4–8.





FIGS. 9–10. Hand sections of gametophytes of *Diphasiastrum* ×*habereri*. FIG. 9. Section of gametangial cap with antheridia. The more median a section through an antheridium the darker its contents (arrows). FIG. 10. Section of gametangial cap with two archegonia. The dark stained file of cells include neck canal cells and basal egg (arrows). Bars = 100 μm.

A few gametophytes with incomplete ring meristems developed in these cultures (Fig. 8). These gametophytes were bilaterally symmetrical along their longitudinal axes. The gametangial region started dorsal to the partial ring meristem and extended over the top of the gametophyte and down one side of the conical basal region.

DISCUSSION

The spores of *D.* ×*habereri* germinate in axenic culture. The percent germination (2.5%) is low but it is higher than previously obtained with spores

from three nonhybrid species of *Lycopodium* sensu lato having mycorrhizal gametophytes (Whittier, 1977, 1981; Whittier and Webster, 1986). The spores of *D.* ×*habereri* are no more difficult to germinate than those of the three nonhybrid species, which included *D. digitatum,* one of the parental species. The results from all four taxa suggest general problems in trying to germinate *Lycopodium* sensu lato spores in axenic culture and no special difficulties with those of *D.* ×*habereri.* Although improvements have been made in the techniques used to germinate the spores of those species of *Lycopodium* sensu lato with mycorrhizal gametophytes, the optimal conditions for germination in axenic culture have yet to be attained.

The small multicellular gametophytes of *D.* ×*habereri* are similar to young gametophytes of other species of the Lycopodiaceae with mycorrhizal gametophytes (Whittier, 1977, 1981). These gametophytes lack chloroplasts and have large amounts of starch in their cells. Older gametophytes with incomplete ring meristems were similar to those reported for *D. digitatum* from nature and culture (Bruce, 1979; Whittier, 1981).

Mature gametophytes of *D.* ×*habereri* are carrot-shaped with the gametangial cap above the ring meristem. They are very similar to those of *D. digitatum* from either nature or culture (Bruce, 1979; Whittier, 1981). The *D.* ×*habereri* gametophytes are somewhat smaller than those reported previously for *D. digitatum,* however no effort was made in these experiments to grow the largest gametophytes possible. No comparisons can be made with gametophytes of *D. tristachyum,* the other parental species, because they have yet to be found in nature (Bruce and Beitel, 1979) or grown in culture. However, it must be noted that Wilce (1965) has raised the possibility that some previously described gametophytes of *D. complanatum* may belong to other species including *D. tristachyum.* Since there is no way of substantiating Wilce's suppositions, the gametophytes of *D. tristachyum* are considered as being undescribed.

The internal structure of these gametophytes is also similar to those of *D. digitatum.* The antheridia are completely sunken and about the same size as those on gametophytes of *D. digitatum* from culture. A long archegonial neck is characteristic for *Diphasiastrum* (Bruce, 1976). The number of tiers of neck cells above the gametophyte surface is five or six for both taxa. The average length of the neck for *D.* ×*habereri* is about 10% longer than the neck of *D. digitatum.* The depth of the egg in the gametophyte is 10% less for *D.* ×*habereri* than for *D. digitatum.* Finally, the number of neck canal cells is similar for both taxa.

Because the hybrid should have an intermediate morphology and the gametophytes of *D.* ×*habereri* and *D. digitatum* are almost identical, the gametophytes of *D. tristachyum* should have essentially the same morphology. A carrot-shaped gametophyte for *D. tristachyum* would fit for this genus. It has been noted previously how similar the gametophytes of *D. digitatum* and *D. complanatum* are to each other (Bruce, 1979). The more similar these gametophytes are to each other the more difficult it would make the positive identification of those of *D.* ×*habereri* from nature unless a sporophyte was attached.

There are no abnormalities in the gametangial structures of *D.* ×*habereri*. They appear functional, although fertilization has not occurred in culture. Attempts to bring about fertilization by flooding the gametophytes with water have so far been unsuccessful. However, it should be noted that fertilization has not occurred in cultured gametophytes from nonhybrid species having mycorrhizal gametophytes. Efforts are underway to determine what conditions in axenic culture promote fertilization in species of *Lycopodium* sensu lato with mycorrhizal gametophytes.

Fertilization and subsequent sporophyte development would prove that *D.* ×*habereri* is fertile, and explain why there are more plants than expected for diploid interspecific hybrids present in areas lacking one or both parents. Even though fertilization has not been accomplished, the development of mature gametophytes from spores demonstrates that another portion of the life cycle of this hybrid is normal. Evidence from this study provides additional support for *D.* ×*habereri* being a fertile hybrid.

ACKNOWLEDGMENTS

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