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# Observations of the Development of Ascocarps in *Phaeocryptopus gaeumanni* and on the Possible Existence of an Anamorphic State

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## Introduction

*Phaeocryptopus gaeumanni* (Rohde) Petrak is a ubiquitous parasite on the needles of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco). Although it was first described from a Douglas fir plantation in Switzerland, it is in fact widespread throughout the moister portions of southwestern Canada and northwestern USA where Douglas fir is the dominant forest tree (Boyce, 1940; Michaels & Chastagner, 1984). The fungus typically produces rows of minute ascocarps on the lower surfaces of the needles, one ascocarp developing above each stoma. Each ascocarp is connected to the intramatricular mycelium within the needle by a group of thick-walled hyphal cells which project through the stoma and anchor the ascocarp to the surface of the needle (Müller & v. Arx, 1962).

While examining cleared Douglas fir needles for the presence of endophytic fungi, we noticed clusters of cells projecting through the stomata which appeared to be phialides. Closer observation revealed that such cells were often associated with young ascocarps of *Phaeocryptopus gaeumanni*. The present investigation with correlated light and electron microscopy was undertaken to discover whether these cells are indeed conidiophores which also produce ascocarp initials.

## Materials and Methods

Second and third-year needles bearing young and developing ascocarps of *P. gaeumanni* were collected in December, April, and June, 1984. Needle segments were fixed in 2% glutaraldehyde and 1.5% paraformaldehyde in .05 M cacodylate buffer, pH 6.8, in an ice bath. Segments were aspirated in the fixative for 2 hr., then washed 5 times in fresh buffer on ice and aspirated once again.

For scanning electron microscopy (SEM) segments were dehydrated to 100% ethanol, critical point dried from 100% acetone, sputtercoated, and examined in an AMR 1000 scanning electron microscope at 20 kV.

For transmission electron microscopy (TEM) segments were postfixed in 1% OsO4 in the above buffer for 12 h at 4°, washed 3 times in fresh buffer, dehydrated in an ethanol series with frequent aspiration, passed through 2 changes of anhydrous acetone, and embedded in an epon-araldite mixture. Sections 5–10 μm thick were cut

with glass knives and examined under the light microscope to select sections containing putative phialides. These sections were then affixed to flat-ended epoxy blocks, retrimmed, and thin-sectioned with a diamond knife. Thin sections were collected on formvar-coated grids, stained with 5% uranyl acetate followed by Reynolds' lead citrate (REYNOLDS, 1963), carbon stabilized, and examined under a Phillips 300 transmission electron microscope at 60 kV.

Whole needles were cleared for light microscopy in 1 N KOH at 60° C for several days with several fresh changes of KOH, washed several times with distilled water, and stained in .05% trypan blue in lactophenol at 60° for 10 min., then destained in lactophenol. Needles were then dehydrated to 100% ethanol followed by 2 changes of xylene to remove resistant resins, then mounted in Permount. Needles were rendered nearly transparent by this procedure while retaining good staining and differentiation of fungal cells.

## Results

Light micrographs of cleared, stained Douglas fir needles reveal small clusters of elongated cells projecting through stomata (Figs. 1A, 1B), The apices of such cells stain strongly with trypan blue, suggesting thickened walls typical of certain kinds of phialides. Roughly spherical cells are often seen attached to the apices of the putative phialides (Fig. 1B). Thick-sections of osmium-fixed material embedded in plastic show that these clusters of spherical cells completely fill the front cavities of the stomata and are indistinguishable from ascocarp initials of *Phaeocryptopus gaeumanni* (Fig. 1C).

Transmission electron micrographs through putative phialides (Figs. 1D & 2) show the following additional details: 1) the apex of the cell is nearly occluded by an electron-transparent, multilamellate, ring-shaped wall thickening; 2) the basal portion of the cell is completely filled with gray sphaerical bodies which are probably lipid; 3) the cross wall between the putative phialide and the cell above consists of a peripheral heavily melanized portion and an inner very thin-walled portion only 0.5 μm in diameter. The fungal cells shown in Figs. 1C and 1D came from needles collected in June, while the cell in Fig. 2 came from a needle collected in December. Although the cells in both figures appear similar in most respects, that from the December-collected material contains less lipid and has begun to vacuolate, a possible sign of incipient senescence.

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Fig. 1: Phialides of *Phaeocryptopus gaeumanni*. – A. Light micrographs of a group of phialides protruding through the stoma of a cleared needle, × 900. – B. Group of phialides from cleared needle showing branching habit, periclinal thickenings and attached cells, possibly conidia (arrows), × 1750. – C. 10 μm thick section through developing ascocarp. The cell indicated by curved arrow is shown in Fig. 1D. S = Subsidiary cell; G = Guard cell. Peripheral hyphae are indicated by arrowheads. – D. Transmission electron micrograph of cell in Fig. 1C showing periclinal wall thickening with successive deposition layers, × 14,800.

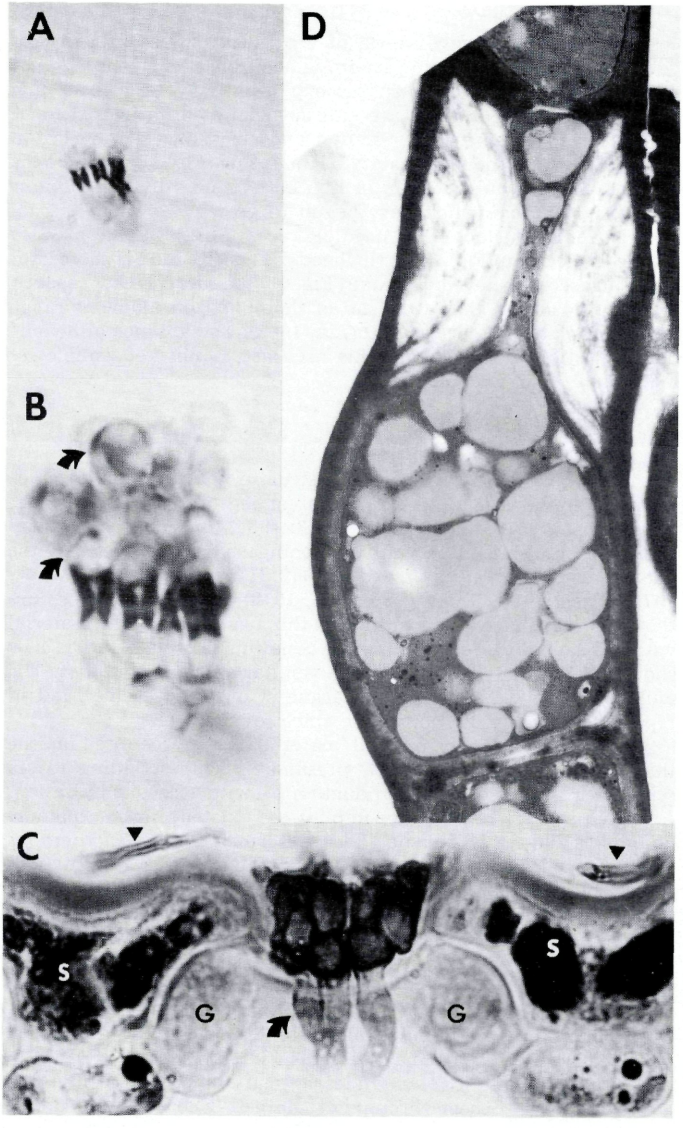

Fig. 3 shows surface views of sequential stages in ascocarp development seen under the scanning electron microscope. Fig. 3A represents the earliest stage of ascocarp initiation we have been able to detect. The fungal cells are still sufficiently thin-walled to be susceptible to collapse under vacuum, and the young ascocarp initial has not yet filled the front cavity of the stoma. Fig. 3B corresponds to the stage shown in section in Fig. 1C. Noteworthy are the four superficial hyphae which have begun to grow away from the ascocarp over the surface of the needle. Traces of these hyphae are also visible in section in Fig. 1C (arrowheads). Figs. 3C and 3D show later stages in ascocarp development. In Fig. 3D the ascocarp has reached a size of 50 μm, at the lower end of the 50–80 μm diameter range typical of mature ascocarps (MÜLLER, 1962). The absence of a well-defined ostiole suggests that this ascocarp is not yet completely mature.

## Discussion

Many investigations on the fine structure of conidium development in phialides have by now been carried out (See for reviews: SUBRAMANIAN, 1979; MINTER & al., 1983; 1984). In a number of cases periclinal multilamellate wall-thickenings have been seen at the apex of the phialide (e. g. CAMPBELL, 1972; HAMMILL, 1974; MIMS & al., 1976; JONES, 1976; HARDER & CHONG, 1978). These thickenings are thought to result from deposition of a thin new layer of wall material within the phialide apex each time a conidium is produced. The cells shown in Figs. 1D and 2 look very similar to such phialides. The multilamellate wall-thickenings argue strongly for some kind of repetitive spore production at the apices of such cells.

At least six layers of wall material cen be counted in the thickenings visible in Fig. 1D, presumably corresponding to six separate instances of spore production prior to ascocarp initiation.

The presence of phialides implies the existence of an anamorphic state. No anamorph has been described for *Phaeocryptopus gaeumanni*. However, inconspicuous small clusters of phialides whose collarettes barely project beyond the stomata could easily be overlooked. Studies on the infection of Douglas fir needles by *Ph. gaeumanni* (CHASTAGNER & BYTHER, 1983) suggest that in young trees the infection of needles by ascospores occurs during a relatively brief six-week period from mid-May through the end of June and that the initiation of new infections is limited to this period. Electron micrographs of phialide-like cells are shown from material collected both in June (Fig. 1D) and in mid-December (Fig. 2). In both cases the putative phialides appear to be quiescent, with abundant lipid present throughout the cell. The structure of the

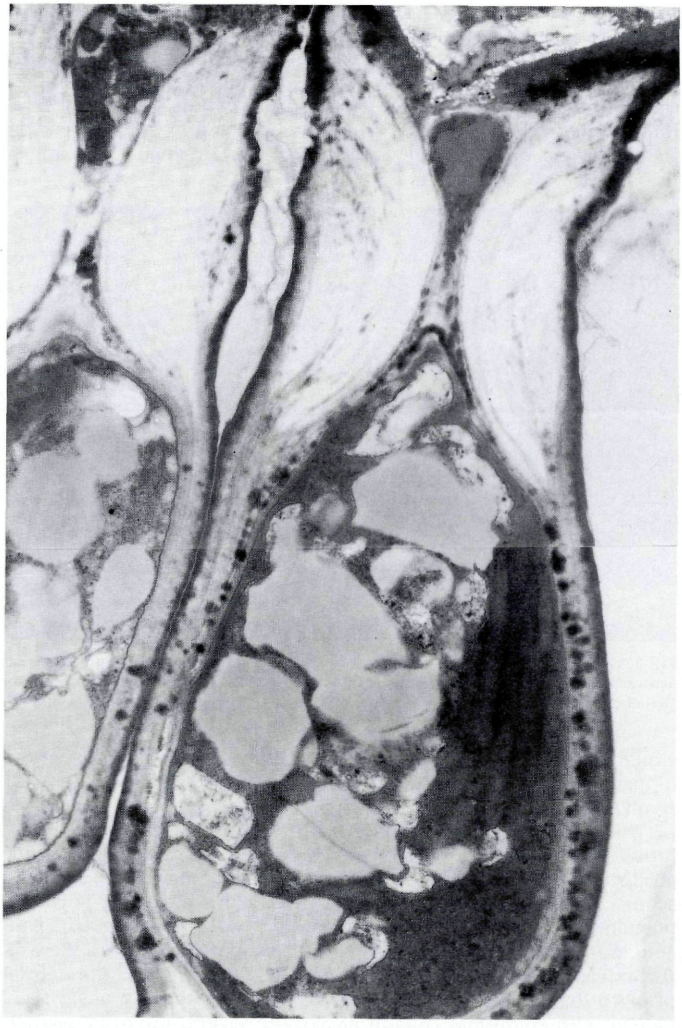

Fig. 2: Transmission electron micrograph of *Phaeocryptopus gaeumanni* phialide, × 15,400.

septum between the phialide and the cell above suggests a condition of arrested conidiogenesis, with centripetal secondary wall deposition and melanization as ascocarp development proceeds. Clearly neither of the cells shown in Fig. 1D or Fig. 2 were involved in conidiogenesis at the time of collection and fixation. We suggest that if an anamorphic state occurs, it should be found in October, just as the fall rains begin in the Pacific Douglas fir zone. This might

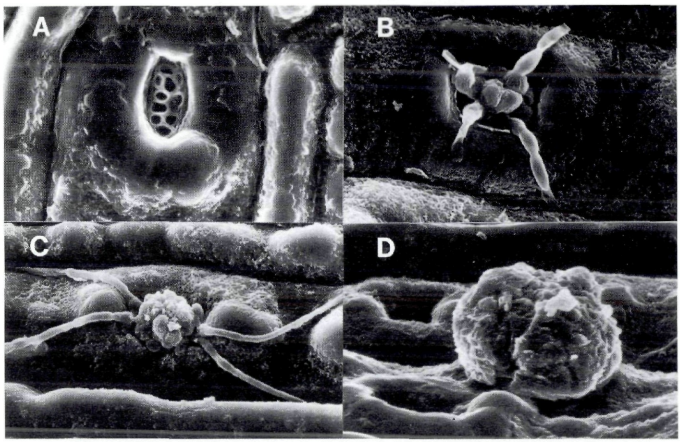


Fig. 3: Scanning electron micrographs showing successive developmental stages of ascocarps in *Phaeocryptopus gaeumanni*. – A. × 1400. – B. Stage corresponding to that shown in Fig. 1C. Note four peripheral hyphae, × 1200. – C. × 1000. – D. × 800.

explain the October infections of Douglas fir needles by *Phaeocryptopus* found by FATUGA (unpublished, but cited in CHASTAGNER & BYTHER, 1983).

Superficial hyphae associated with the ascocarps of *Phaeocryptopus* have not been reported previously, but have been seen by us consistently in material viewed under the scanning electron microscope. Such hyphae may serve to initiate infections in adjacent portion of the needle or may form assimilative haustoria in adjacent epidermal cells. Both of these possibilities seem unlikely in view of the extensive intracellular mycelium already present in the needle prior to the production of ascocarps. Further, we have never seen haustoria in epidermal cells in any of the several hundred needles examined under the light microscope or in any of the needle sections examined under the transmission electron microscope. Alternative-

ly, such superficial hyphae may serve to absorb critical nutrients leached from the canopy during the rainy season.

Precipitation in the moist Douglas fir forests of western States and Canada may exceed 250 cm/yr, and leached nutrients such as nitrogen may represent a significant fraction of the total cycled within the system.

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