

## SEM Studies on Tracheids of Lycopodiaceae; Observations on Adaptations in *Phylloglossum*

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**ABSTRACT.**—Scanning electron microscope (SEM) studies of stem and strobilus longisections of *Huperzia*, *Lycopodium*, and *Phylloglossum* were undertaken to explore ultrastructure of pit membranes in tracheids. The membranes do not characteristically have pores and may often lack evidence of cellulosic fibrils. Some pit membranes in *Lycopodium* did show cellulosic fibrils. Porose membranes were seen in some tracheids, an appearance probably related to scraping away of layers in pit membranes by the sectioning process, or in other cases, artifact formation. Metaxylem tracheids have wide borders on pits. Truly “reticulate” metaxylem tracheids are few. Protoxylem tracheids have helices with borders in *Huperzia* and *Lycopodium*, but in *Phylloglossum* protoxylem annuli and helices are non-bordered. *Phylloglossum*, which appears nested in *Huperzia*, lacks metaxylem and has numerous other adaptations to the distinctive ephemeral vernal bogs of Australia and New Zealand, similar to those in Droseraceae and Orchidaceae.

**KEY WORDS.**—adaptation to fire, *Huperzia*, *Lycopodium*, *Phylloglossum*, pit membranes, tracheid ultrastructure, vernal bogs, xylem

Tracheids of *Lycopodium* were studied by Bierhorst (1960, 1971) and by Wilder (1970) by means of light microscopy. Although the total number of species studied was small, the differences among species were not great, so a broad-based survey of tracheids in the family has not been undertaken by those authors or by us. Cook and Friedman (1998) and Friedman and Cook (2000) offered some fascinating new data on ultrastructure of *Huperzia* (formerly a section of *Lycopodium*) tracheids. They demonstrated that the secondary wall of *Huperzia* tracheids is composed of a “template layer” on which is superimposed a “resistant layer.” The template layer is lignin-poor, whereas the resistant layer is rich in lignin.

Cook and Friedman (1998) and Friedman and Cook (2000) as well as Kenrick and Crane (1991, 1997) and Edwards (1993) have sought to integrate information on tracheid ultrastructure of early vascular plants with facts on the ultrastructure of tracheary elements in extant groups of vascular plants.

Lycopodiaceae, along with *Equisetum*, play an important role in these considerations because they may retain some characteristics of tracheary element ultrastructure from early times (e.g., Devonian).

Our goals in the present study complement the abovementioned studies by offering scanning electron microscope (SEM) studies of primary walls in three of the four genera currently recognized in Lycopodiaceae. We investigated primary wall microstructure with respect to protoxylem versus metaxylem. Also, we have included the first studies on ultrastructure of *Phylloglossum* tracheids. We have focused on the possible existence of thin areas or porosities in tracheid primary walls, and whether or not cellulosic fibrils are visible with SEM in primary walls of tracheids. Such structures have been evident in tracheids and/ or vessel elements of ferns (Carlquist and Schneider, 2007), *Equisetum* (Carlquist and Schneider, 2011a), cycads (Schneider *et al.*, 2007), and various families of monocotyledons (Carlquist and Schneider, 2010a, 2010b; Carlquist, 2012). Presence of pores and abundance of cellulosic fibrils vary considerably among these groups. For example, the end wall of a grass vessel contains a single large pit membrane that dissolves as the vessel matures; we were unable to demonstrate cellulosic fibrils in those pit membranes (Carlquist and Schneider, 2011b). By contrast, the cellulosic fibrils in perforations of *Canna* vessel elements are striking and persist in the mature vessel elements (Carlquist and Schneider, 2010b). The presence of a reticulum of cellulosic fibrils or of pores in an intact pit membrane appears to mediate the balance between conductive safety (restricting air bubbles to a single tracheary element) and conductive efficiency (porousness permitting passage of greater volumes of water per unit time than non-porous pit membranes). Where the tracheids of Lycopodiaceae fall in this gamut is the point of interest in our investigation.

Study of three genera of Lycopodiaceae permits a preliminary examination of diversity of tracheid ultrastructure for the family. One of these genera, *Phylloglossum*, is of especial interest because of the distinctive habit, which in turn is related to a special ecological niche. *Phylloglossum* plants consist of a so-called tuber (coexisting with the production of a single new tuber), a root, several leaves, and a strobilus. There is no stem in any accepted sense, merely a junction among these organs. Each spring, as moisture permits, the tuber produces leaves, a root, and a strobilus, and a geotropic new tuber. The plant oversummers and survives fire by means of the tuber. The vascular system of *Phylloglossum* consists of a plexus of tracheids interconnecting root, leaves, and strobilus. No vascular tissue enters the tuber, and thus vascular tissue is not intercontinuous from one year's plant body to the next year's. The distinctive habitat occupied by *Phylloglossum* can be characterized as ephemeral bogs, shallow depressions of acid sand, underlain by a hardpan, that accumulate rainwater during the winter months but evaporate as spring progresses into summer. The interrelationships between this distinctive habitat and the unique morphology of *Phylloglossum* form an obvious reason for special attention to *Phylloglossum* xylem. The habits of remaining Lycopodiaceae, which consist of horizontal and/or upright or pendant stems,

is relatively uniform, but no less interesting in terms of mechanical and physiological aspects.

#### MATERIALS AND METHODS

Sources of material are as follows. *Huperzia lucidula* (Michx.) Trevis: supplied by Carolina Biological Supply Company. *Lycopodium annotinum* L., *L. dichotomum* L., and *L. complanatum* L.: collected by E. L. Schneider at the moose-viewing platform along the Gunflint Trail, Minnesota, on September 12, 2011. *Phylloglossum drummondii* Kunze: specimen used for paraffin sectioning: collected between 120 and 121 mile post on highway between Brookton and Mt. Barker, Western Australia, on flat with grasses and sedges and annual *Stylidium* species; 9 October 1974, by Sherwin Carlquist (RSA). *Phylloglossum drummondii* used for SEM work: collected at Forrestdale Lake Nature Reserve, Western Australia, in seasonally waterlogged sandy clay flat (palusplain) with *Drosera* spp., *Utricularia multifida* and *Philydrella pygmaea*, 6 September 2007 by C. Tauss 1640 (PERTH). Recognition of genera in this paper follows that of Wikström and Kenrick (1997). The system for the family by Wagner and Beitel (1992) preceded molecular investigations and some genera recognized by them have not been followed in subsequent treatments.

All collections were preserved in 50% aqueous ethanol. The 1974 collection of *Phylloglossum* was embedded in paraffin according to the usual techniques; sections were stained with a safranin-fast green combination. Collections of all other Lycopodiaceae were sectioned by hand with a single-edged razor blade. The sections were subjected to three changes of distilled water, dried (with pressure applied) on a warming table, mounted on aluminum stubs, sputter-coated with gold, and examined with a Hitachi S2600N SEM. These methods have been described in more detail by Carlquist and Schneider (2007).

#### RESULTS

*Huperzia lucidula* (Fig. 1A–D). SEM micrographs of *H. lucidula* in our preparations show a range of conditions. The metaxylem pit membranes in Fig. 1A show numerous holes of various sizes, but no convincing evidence of cellulosic fibrils. The holes may represent thin areas in the pit membrane, revealed only when portions of the pit membrane are shaved away by the sectioning process. No holes are evident in the pit membrane shown in Fig. 1B, which has not been affected by the sectioning process. Likewise, the metaxylem tracheids in Fig. 1C show no evidence of pores in the pit membranes (tears represent obvious artifacts).

In protoxylem of *H. lucidula* (Fig. 1D), primary walls (equivalent to pit membranes in metaxylem) without pores were observed. This appearance accords with the illustration for this species by Friedman and Cook (2000). Also in agreement with their illustration, we found a relatively abrupt shift from protoxylem to metaxylem wall patterns, with few reticulate tracheids.

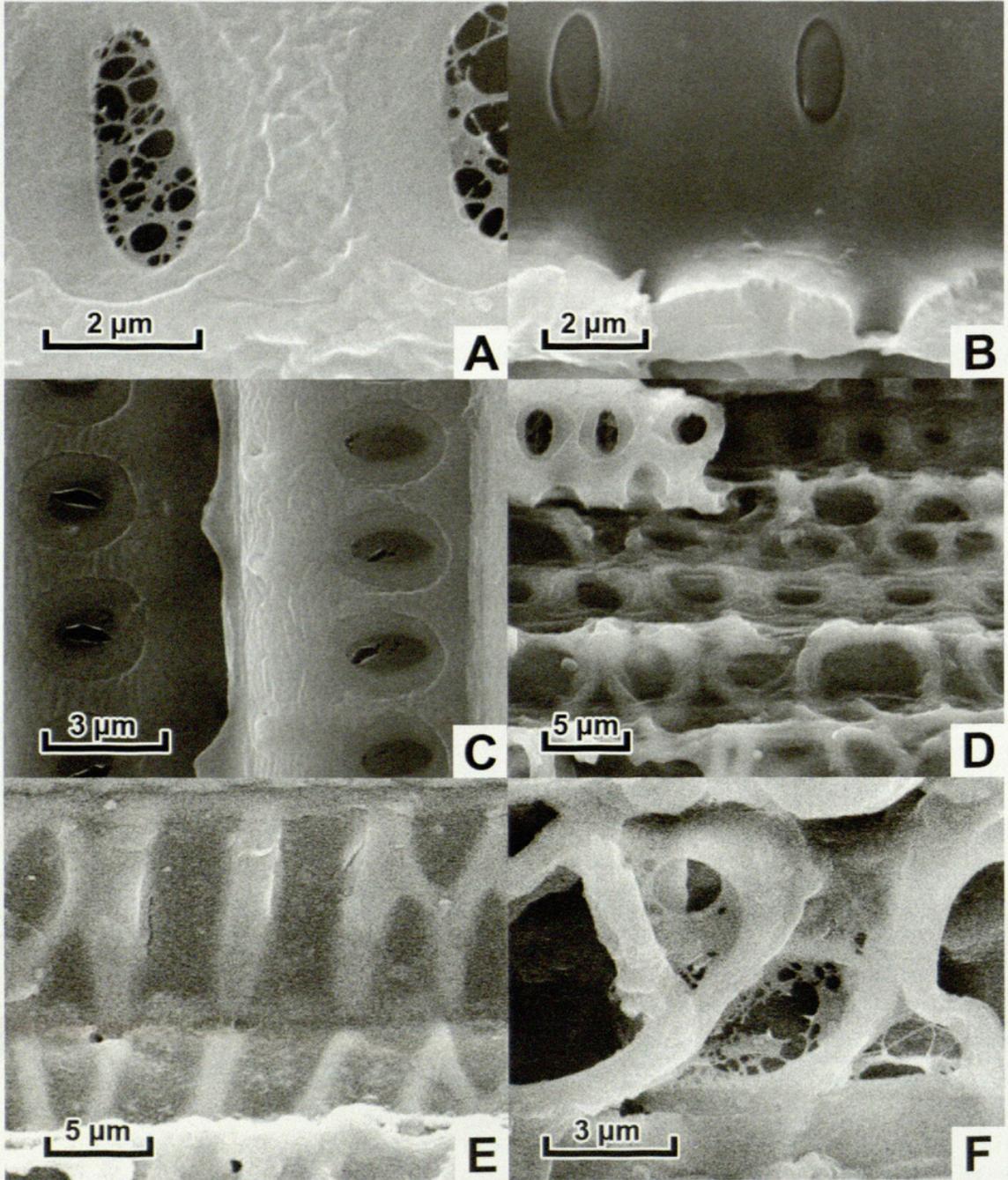


FIG. 1. SEM micrographs of tracheids from longisectons of stems of Lycopodiaceae. A–D. *Huperzia lucidula*. A. Metaxylem tracheid outer surface, showing porous appearance of the pit membrane. B. Metaxylem tracheid in sectional view, showing pit membranes in face view (above) and in sectional view (lower right); no porosities evident. C. Outer surfaces of two metaxylem tracheids; no pores evidence in pit membranes (which do show some tears). D. Protoxylem (below) and metaxylem (above). E–F. *Lycopodium complanatum*. E. Protoxylem; some interconnections between the secondary wall helices occur in the tracheid above; no pores evident in primary wall. F. Porous primary wall in protoxylem, probably related to the sectioning process.

*Lycopodium complanatum* (Fig. 1E–F).

Protoxylem tracheids of *L. complanatum* in Fig. 1E show thin non-porose pit membranes on helical (below) and transitional (above) tracheids. Pores may be found on primary wall areas scraped away by sectioning (Fig. 1F), although the primary walls are otherwise non-porose.

*L. annotinum* (Fig. 2A–D).

In metaxylem, pit membranes of *L. annotinum* tracheids are typically non-porose (Fig. 2A). Tracheid pits that presumably have not been affected by sectioning (because they are viewed from the tracheid inside and seem intact), some pit membranes have a few small holes (Fig. 2B–C). These porosities may be irregularly distributed (Fig. 2B), and may represent some degree of artifact formation. In pits that have experienced scraping from the knife, a fibrillar structure is evident (Fig. 2D).

*L. dichotomum* (Fig. 2E–H).

Scattered small pores were observed in an apparently intact metaxylem tracheid (Fig. 2E). Protoxylem tracheids show pores to the degree that portions of the primary wall are shaved away by the sectioning process (Fig. 2F–G). The pit membrane portion shown in Fig. 2H is suggestive of presence of a fibrillar reticulate background. In the other instances, there is no indication of a fibrillar background because the porosities are circular rather than angular.

*Phylloglossum drummondii* (Fig. 3A–F).

The xylem studied in this species is derived from study of the vascular plexus, which interconnects strobilus, leaves, and root (see Introduction for a description of the plant body). *Phylloglossum* has no metaxylem. The bands of secondary wall in protoxylem tracheids are annular in a few places, but mostly helical (Fig. 3A). There are very few interconnections (indicating an approach to reticulate wall pattern) between the helices. The secondary wall annuli and helices are not bordered (Fig. 3B).

Primary walls of *Phylloglossum* tracheids are thin and homogeneous (Fig. 3C, E). Some areas show faint dark spots as recorded by the SEM (Fig. 3D). This rendering suggests that they are depressions. Areas of primary wall that have experienced some shaving from the sectioning process show pores (Fig. 3F) that correspond to the depression seen in Fig. 3D.

## DISCUSSION AND CONCLUSIONS

*Ultrastructure of the primary walls of tracheids.*—Pits in metaxylem tracheids are oval in shape and prominently bordered in all Lycopodiaceae, as shown in the illustrations of Bierhorst (1960, 1971), Wilder (1970), Cook and Friedman (1998), and Friedman and Cook (2000). The appearances of pit

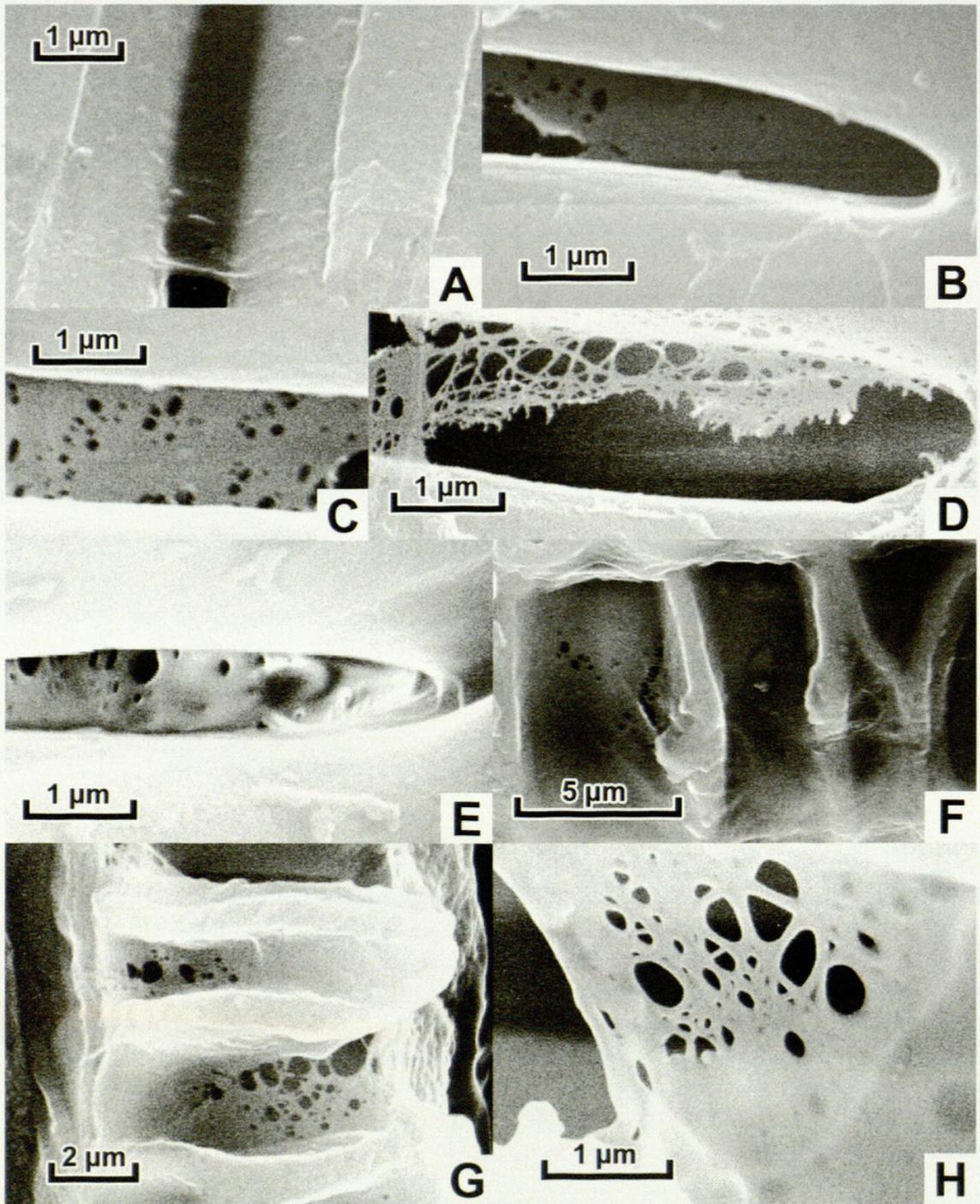


FIG. 2. SEM micrographs of tracheids from longisections of *Lycopodium*. A–D. *L. annotinum*. A. Outer surface of metaxylem tracheid, showing non-porous pit membrane extending across the pit border. B, C. Views of metaxylem pit membranes that have not been affected by sectioning, seen from inside the tracheid. B. A few small pores adjacent to a torn area (left.). C. Porosities scattered within pit membrane. D. Metaxylem pit membrane that has been partially scraped away by the sectioning process, revealing a reticulum with strands. E–H. *L. dichotomum*. E. Pit membrane seen from inside a metaxylem tracheid, showing some holes of various sizes. F–H. Portions of protoxylem tracheids seen from their outer surfaces. F. Primary wall non-porose, except at left; secondary wall thickenings are bordered. G. Porose appearance in primary wall of helical tracheid. H. A portion of a primary wall from a helical tracheid, showing a reticulate appearance suggestive of a fibrillar background.

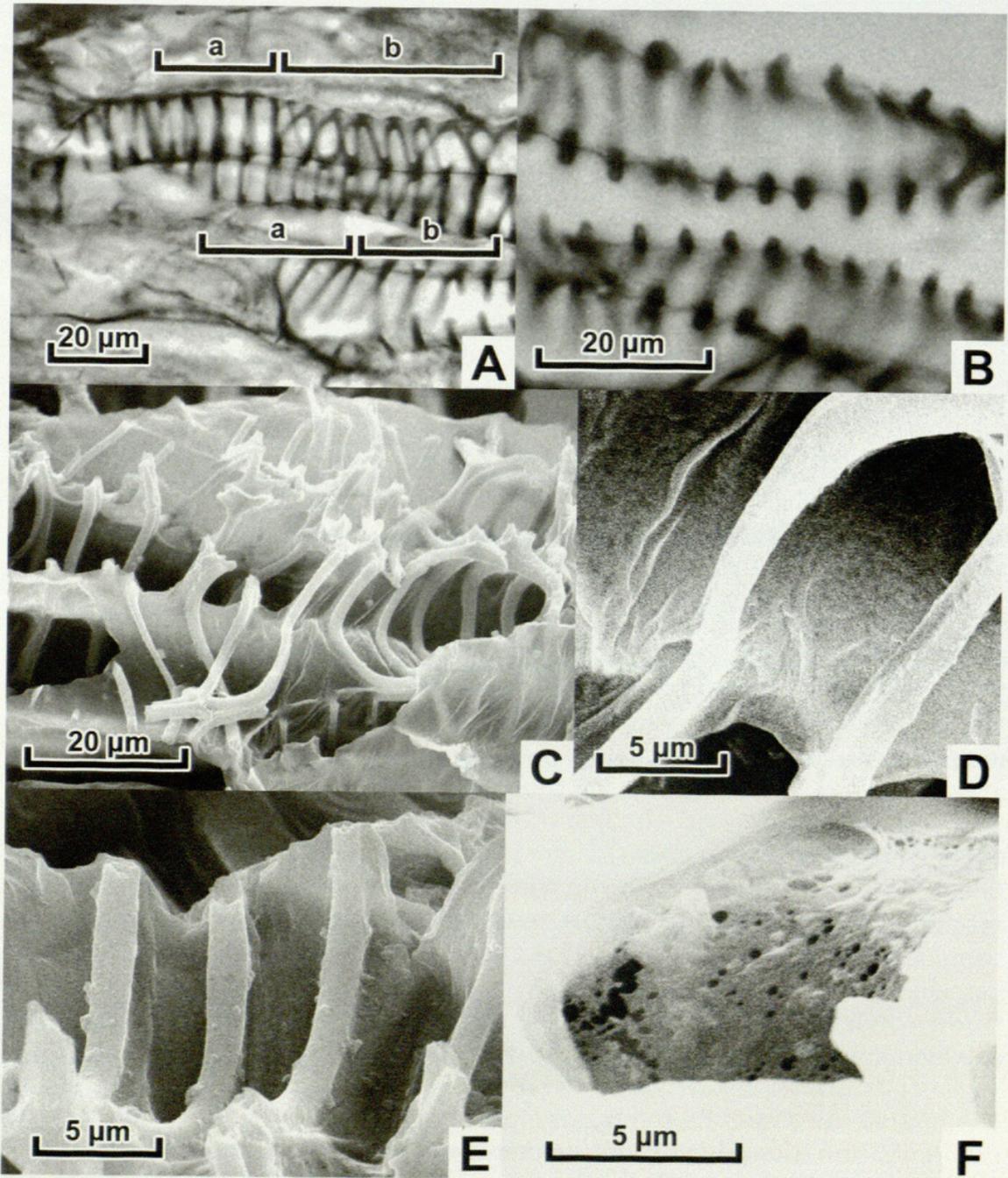


FIG. 3. Longisections of tracheids from the vascular plexus in plants of *Phylloglossum drummondii*. A, B. Light micrographs. A. Portions of tracheids to show annular (a) and helical (b) secondary wall bands. B. Optical sections of secondary wall bands (center), showing non-bordered condition. C-F. SEM micrographs. C. Portions of several adjacent tracheids, showing inner surfaces; primary wall portions non-porous. D. Portion of primary wall and two secondary wall helices from inner surface of tracheid; faint spots on the primary wall suggest presence of depressions. E. Inner surface of tracheid; secondary wall helices are borderless; primary wall is homogeneous in appearance. F. Shaved away portion of primary wall; minute pores are illustrated.

membranes that have experienced some degree of shaving away of wall material during the sectioning process suggest the presence of cellulosic fibrils, but this is not evident in the intact pit membranes we viewed. The circular shape and distribution of pores we observed in pit membranes that have not been affected by sectioning suggests the possibility that such holes may be artifacts caused by drying. However, porose appearances that are related to shaving away of wall material could indicate genuine thin areas in primary walls.

The abovementioned appearances are also found in primary walls of protoxylem tracheids. Homogeneous non-porose wall surfaces predominate in our material. This finding is in accord with the illustration of Friedman and Cook (2000) for *Huperzia lucidula*, which is based upon an SEM image of rotary microtome sections.

The paucity of cellulosic fibrils in primary walls of lycopodiaceous tracheids contrasts with appearances found in ferns (Carlquist and Schneider, 2007), cycads (Schneider *et al.*, 2007), and monocots (Carlquist and Schneider, 2010a, 2011b). In the monocots, fibrillar appearances are common in end walls of tracheary elements of families with more numerous plesiomorphic features (Carlquist, 2012). Cellulosic fibrils are, by contrast, few in the pit membranes of the end walls, prior to lysis, of vessel elements of grasses (Carlquist and Schneider, 2011b), which have simple perforation plates. More observations on a diversity of pit membranes, preferably with transmission electron microscopy (TEM) is desirable. The electron microscope data of Friedman and Cook (2000) were focused on the secondary walls of *Huperzia* tracheids.

In monocots (Carlquist, 2012) and in some genera of ferns, (e.g., *Blechnum*, Carlquist and Schneider, 2007), there is differentiation between end walls and lateral walls of tracheary elements with respect to size of pits (or perforations), pit membrane porousness, and presence of evident cellulosic webs. This may be related to incipient tendencies toward acquisition of some characteristics of vessels. Such differentiation was not evident to any appreciable extent in our studies on cycad tracheids (Schneider *et al.*, 2007). There is no differentiation between end walls and lateral walls in tracheids of Lycopodiaceae. This suggests that fascicles of tracheids, as in vascular cryptogam steles, serve as conductive units conjunctively, whereas individual vessels or tracheids with differentiation of end walls are the conductive units in monocots.

*Unique adaptations of Phylloglossum.*—Wikström and Kenrick (1997) found that *Huperzia* is paraphyletic, because *Phylloglossum* is nested within it (*Lycopodium* + *Lycopodiella* forms the other clade of the family). If we regard *Phylloglossum* as an extreme adaptation of the *Huperzia* clade, a number of adaptations become evident. The meristem that gives rise to each new geotropic tuber is exogenous, formed from surface tissue where the leaves and strobili join the old tuber. As the tuber elongates geotropically, a negatively geotropic meristem, which will give rise to the next year's leaves and stems, forms within the new tuber at its upper end (see Bierhorst, p. 25). This is a unique structure within vascular plants. At this stage when the parent plant has produced leaves and a strobilus, the new tuber has no vascular tissue. The

vascular plexus that interconnects root with juncture between leaves and strobilus does not even take the form of any discernable stem or stelar configuration. As the current year's plant body dries with the onset of the warm and dry season, the only surviving portion is the maturing new tuber, which contains no vascular tissue. Vascular tissue is initiated from the negatively geotropic meristem of the new tuber, apparently in response to leaf and strobilus initiation.

The vascular plexus, as we have seen, consists wholly of protoxylem. This fact is compatible with changing turgor in the plant, facilitated by the annular and helical patterns of the secondary wall within the tracheids. This correlates with changes in water availability in the wet vernal flats—ephemeral bogs of a sort—in which *Phylloglossum* grows.

Friedman and Cook (2000) illustrated borders on secondary wall in protoxylem tracheids of *Huperzia*. This corresponds with our observations on *Huperzia* and *Lycopodium* tracheids, although the sections of Friedman and Cook (2000) are clear in this respect because they observed rotary microtome sections with SEM. *Phylloglossum*, by contrast, lacks borders on the secondary wall annuli and helices of tracheids. The borderless condition confers more flexibility, because the investment in cellulosic wall material is less.

There are several interesting implications of the anatomy of *Phylloglossum* and its xylem. One can regard the plant body of *Phylloglossum* as paedomorphic, as Wikström and Kenrick (1997) do, in that it produces so few leaves and only one root, and yet produces a strobilus with this minimal vegetative apparatus. The tuber does not represent a juvenilization of a stem, but a new kind of appendage. This innovation within Lycopodiaceae is truly remarkable, because it runs counter to the intuitive idea that an ancient group of vascular plants is less likely to produce a vegetative structure *sui generis*. The gemmae of *Huperzia* spp. are rather easily categorized, in contrast, as products of shoot dimorphism.

The xylem of *Phylloglossum* may certainly be regarded as paedomorphic, because it consists of protoxylem only, whereas all other Lycopodiaceae have metaxylem as well as protoxylem. The wall strength of metaxylem is sufficient to promote a self-supporting stem structure. Although sclerenchyma develops in the cortex of some *Lycopodium* species, it develops later than metaxylem (original data). The central issue at hand is whether or not one invokes the term "paedomorphic," a functional correlation exists between the mechanical strength provided by cellulose deposition and the mechanical strength (or lack of it) in tracheids of *Phylloglossum* as compared to those of the remaining Lycopodiaceae. This relative lack of tracheids with reticulate wall thickenings in tracheids of Lycopodiaceae other than *Phylloglossum* suggests that elongation (congruent with annular and helical wall thickenings of protoxylem) abruptly yields to self-support (a characteristic of pitted tracheary elements with appreciable wall thickness).

Interestingly, *Phylloglossum* shares its habitat with other vascular plants that produce tuber-like structures with various modes of origin, notably

Droseraceae, certain Stylidiaceae, and *Utricularia menziesii* R. Br. (Utriculariaceae). Studies on the comparative physiology and timing of photosynthate storage and retrieval of these plant assemblages together with xylem characteristics would be of interest. One should point out, as do Wikström and Kenrick (1997) with respect to *Phylloglossum*, that tuber formation is also a strategy of fire avoidance. Data on soil temperature gradients with respect to the species with tuber-like perennation structures in these areas would be of special interest, because most of these structures are relatively close to the ground surface in the “vernal bog” habitats. More deeply buried tuber-like structures are formed in various Orchidaceae native to sand areas of Australia (especially Western Australia), a fact which correlates with the fact that those Orchidaceae tend to co-exist with more shrubs, which would provide greater heat when burned.

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