

G-fibers and Florin ring-like structures in *Dioon* (Zamiaceae)

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Abstract

Cuticle information and epidermal characters have great potential in systematic studies. However, micromorphology and anatomical studies on cycads are relatively scarce in comparison to similar studies on other gymnosperms. In this study leaflet anatomy and cuticles in the genus *Dioon* have been investigated with bright field microscopy, epifluorescence, and scanning electron microscopy. Structures hitherto not completely studied for the genus are reported such as lignified hypodermis, girder sclerenchyma, especially G-fibers, and Florin ring-like structures associated with the stomatal apparatus.

Key words: anatomy, cuticles, cycads, Cycadales, leaflets.

Fibras-G y estructuras de anillo tipo Florin en *Dioon* (Zamiaceae)

Resumen

La información de cutículas y caracteres epidérmicas tienen gran potencial para estudios sistemáticos. Sin embargo, la micromorfología y estudios anatómicos en las cícadas son relativamente escasos en comparación de estudios similares con otras gimnospermas. En este estudio se investigó la anatomía de foliolos y cutículas del género *Dioon* con técnicas de microscopía de campo claro, epifluorescencia y de microscopía electrónica de barrido y se reportan estructuras no completamente estudiadas antes para el género. Se observó hipodermis lignificada, presencia de esclerénquima de trabe y particularmente fibras gelatinosas y estructuras semejantes a anillos de Florin asociados con los aparatos estomáticos.

Palabras clave: anatomía de foliolos, cícadas, cutícula, Cycadales.

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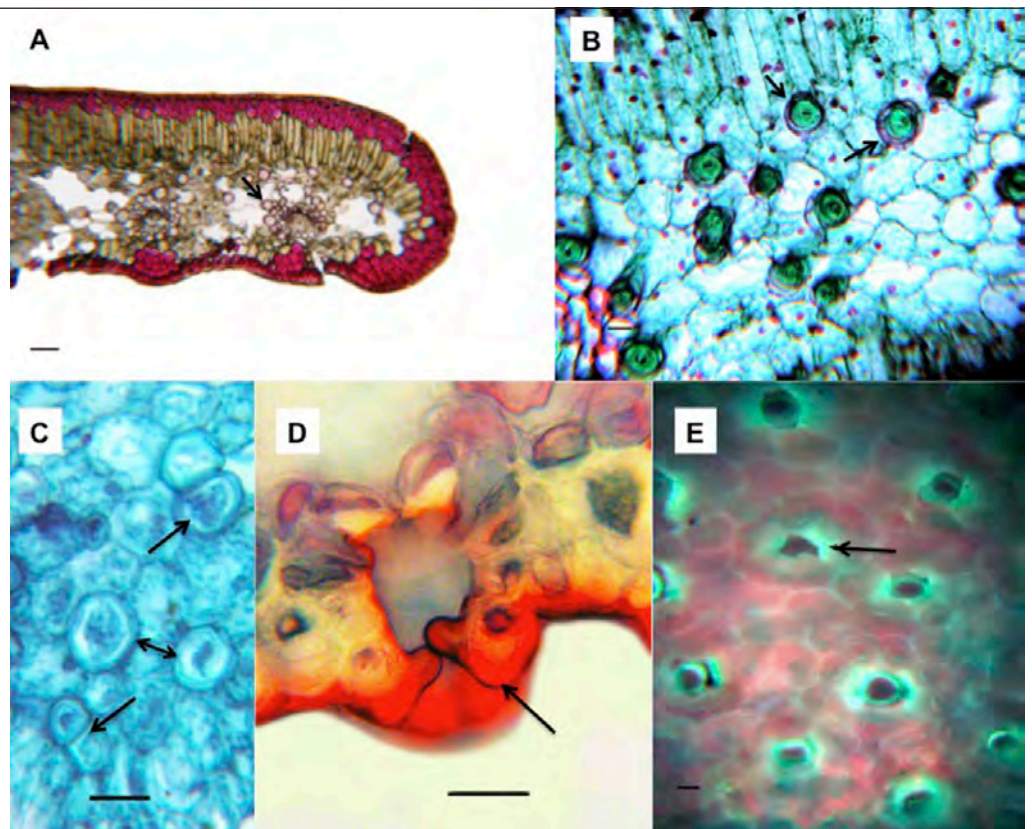
The importance of cuticular information and systematic value of epidermal characters has been emphasized by Stace (1965) who did extensive work on of about 250 species of angiosperms. However micromorphology and anatomical studies on living cycads have been scarce and patchy in comparison to the work done on other gymnosperms i.e. *Araucaria* de Jussieu (Stockey and Taylor, 1978), Podocarpaceae (Stockey, 1982; Stockey *et al.*, 1998, 1992; Mill and Schilling, 2009) among others since the classical works of Florin cited by Yoshie and Sakai (1958). Early work on cycad leaflets by Lamb (1923) and Papadopoulos (1928) described anatomy of selected species of nine genera and that of a hybrid cycad respectively. Work by Pant and Nautiyal (1963) described cuticle and epidermis of 59 species and Tang *et al.* (2004) described the leaflet anatomy of ten species from six genera. Mickle *et al.* (2011) studied the cuticular micromorphology with the scanning electron microscope (SEM) of five species of *Cycas* L. and drew taxonomic conclusions regarding the status of *C. normanbyana* F. Muell.

The present study is part of a leaflet anatomical study of the Neotropical cycad genus *Dioon* Lindl., and we report cuticular and anatomical features hitherto unexplored throughout the genus.

Transverse (TS) sections were taken from the mid portion of leaflets that were in turn taken from the mid section of healthy mature leaves from the 14 known species of *Dioon* held in the Mexican National Cycad Collection held at the Clavijero Botanic Garden, Xalapa. For observation with clear field microscopy sectioning was done using standard microtechnique (Johansen, 1940; Purvis *et al.*, 1966) on fresh or fixed material histochemical staining done with phloroglucinol HCl for lignin and Sudan III and IV for suberin and cutin. Permanent slides were prepared by double staining with safranin and fast green FCF and mounted in Histoclad[®]. For SEM observations of outer cuticular surfaces, cuticular peels were prepared using a modified technique with Jeffery's solution according to Stace (1965) to obtain clean surfaces as well as untreated material in order to preserve wax deposits, both dried in a desiccator and sputter coated with gold-palladium at 1.5 kv at 5 mA for eight minutes with a Jeol Fine Coat JFC 1100 sputter coater. Observations were made with a Jeol JSM-5600LV scanning electron microscope.

Transverse sections (TS) of the *Dioon* leaflets under clear field microscopy revealed thick adaxial cuticles ranging over nine μm in thickness depending on species, lignified epidermis

Figure 1. *Dioon* leaflet transverse sections. **A)** Leaflet stained with phloroglucinol-HCl to show lignified hypodermis and girder sclerenchyma (red), note hyaline G-fibers with lignified wall (arrow). Bar: 100 μm . **B)** Double stained with safranin and fast green-FCF, note G-fibers cellulose secondary wall (S3) green, lignified outer wall (S1 and S2) red (arrow). Bar: 20 μm . **C)** Young leaflet showing hyaline fibers with lesser thickening of secondary wall (arrows). Bar: 40 μm . **D)** Stomatal apparatus of *D. purpusii* stained with Sudan III and IV showing thickened periclinal wall of raised encircling cell, thick cuticle (arrow) and cuticular papillae protruding into the stomatal chamber. Bar: 20 μm . **E)** Abaxial epidermal surface of *D. edule* under epifluorescence microscopy showing thickened cuticle surrounding pores forming Florin rings (arrow). Bar: 20 μm .



and hypodermis with girder sclerenchyma varying in layers according to species (Figure 1A). Thick walled non-lignified hyaline fibers with virtually no lumen are interspersed between the hypodermis and the single row of palisade mesophyll as well as in the spongy mesophyll (intervascular) and surrounding the vascular bundles (perivascular) were observed in mature leaflets of all species. These have been referred to as bast fibers (Lamb, 1923); and bast cells (Papadopoulos, 1928). These are now known as tension wood fibers or gelatinous fibers (G-fibers) and have been reported for cycads by Tomlinson *et al.* (2014) associated in the secondary phloem in roots that undergo contraction, in *Gnetum*; and in angiosperms by Fisher and Blanco (2014). G-fibers are not restricted to wood or angiosperms and may have originated during an early period of spermatophyte evolution (Tomlinson *et al.*, 2014).

In mature leaflets of *Dioon* these fibers appear gelatinous-like, the first layers of the secondary wall (S1 and S2 layers) react for lignin with phloroglucinol-HCl forming a lignified sheath (Figure 1A). The rest of the secondary wall (S3 layer) does not and stains with fast green. The thick cellulose S3 layer often breaks away during sectioning that is a characteristic of G-fibers (V. Angyalossy pers. com.) being rich in cellulose and stains green when double stained with safranin and fast green (Figure 1 B). The hyaline fibers of young leaflets (three to four weeks after emergence) do not show these characteristics differing by having a much less thick cellulose secondary wall with a large lumen (Figure 1C).

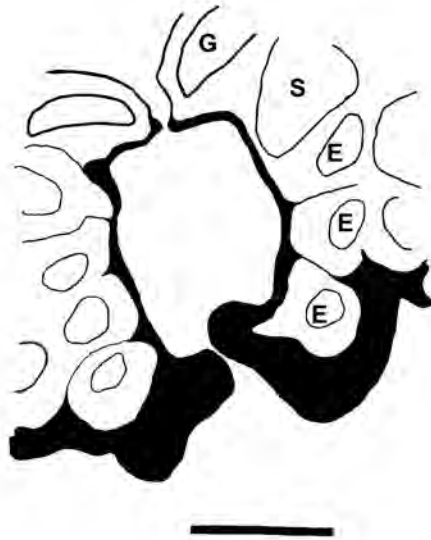
G-fibers or gelatinous fibers are commonly found in tension wood that forms in response to gravitational stresses. They may be clustered together forming compact tension wood or as single fibers or in small groups forming diffuse tension wood (Mauseth, 1988). G-fibers are poor in lignin but rich in cellulose (Coté *et al.*, 1969; Mauseth, 1988) and have a complex chemical structure (Bowling and Vaughn, 2008).

In healthy, mature and functioning leaflets of *Dioon* they appear to be single fibers dispersed within the spongy mesophyll and closely clustered around the vascular bundles in varying numbers. Most cycad leaves and leaflets tend to be very coriaceous, heavy but flexible and long lived, especially in *Dioon* where the leaflets are strongly reinforced by a lignified hypodermis and girder sclerenchyma that adds weight to the leaf. These cycads grow in exposed environments such as rocky outcrops on steep hills or on canyon walls that are exposed to wind and direct sunlight. Thus they are exposed to mechanical stress and water loss through transpiration. In *D. edule* leaf flushes occur every other year (Chamberlain, 1919; Vovides, 1990) and the life span of any given leaf in the field may be up to three years or more (pers. obs.). It is likely that once the long lived leaves of *Dioon* have reached maturity, usually less than three to six months after emergence, aspects such as flexibility, resistance to mechanical stress and the water absorbing ability of the G-fibers would thus offer some advantage to the plants occupying these harsh environments. Future research should follow the development and quantification of these fibres from early leaf emergence through to senescence.

All *Dioon* spp. are hypostomatic and the adult stomatal apparatus consists of three layers of encircling cells above the subsidiary cells forming an epistomatal chamber that varies in depth according to species. In TS through the stomatal apparatus a prominent thickening of the periclinal wall and cuticle of the outer or distal encircling cells occurs, forming a raised ring around the epistomatal pore. This can be seen when stained with Sudan III and IV (Figure 1D) and is reminiscent of Florin rings, for want of a better term. Florin rings are associated with the subsidiary cells observed in both living and fossil gymnosperms such as *Pinus strobus* by Deckert *et al.* (2001), *Papuacedrus* (Wilf *et al.*, 2009) and in *Falcatifolium* (Podocarpaceae) by Stockey *et al.* (1992). In *Dioon* these structures can also be seen under epifluorescence microscopy of the abaxial cuticular surface using 400-440 nm exciter filters (Figure 1E) comparable to the Florin rings reported by Wilf *et al.* (2009) on fossil *Papuacedrus* and Paull and Hill (2010) on fossil Cupressaceae. Dilcher (1969) describes Florin rings as “conspicuous thickened rings of cutin overlying the accessory cells and surrounding the stomata.”

In the stomata of *Dioon* the situation differs in that there are three layers of encircling cells above the subsidiary (accessory) cells, that together form a deep stomatal chamber. The outermost (distal) encircling cells have a thickened upper periclinal wall as well as a thickened cuticle and can be seen in the transverse section of the Sudan stained cuticle in figure 1D (arrow) also under fluorescence microscopy in figure 1E (arrow) and illustration (Figure 2). Another

Figure 2. Illustration showing section through stomatal apparatus of *Dioon purpusii* showing thickened cuticle (black), G = guard cell, S = subsidiary cell, E = encircling cells. Bar: 20 μm .

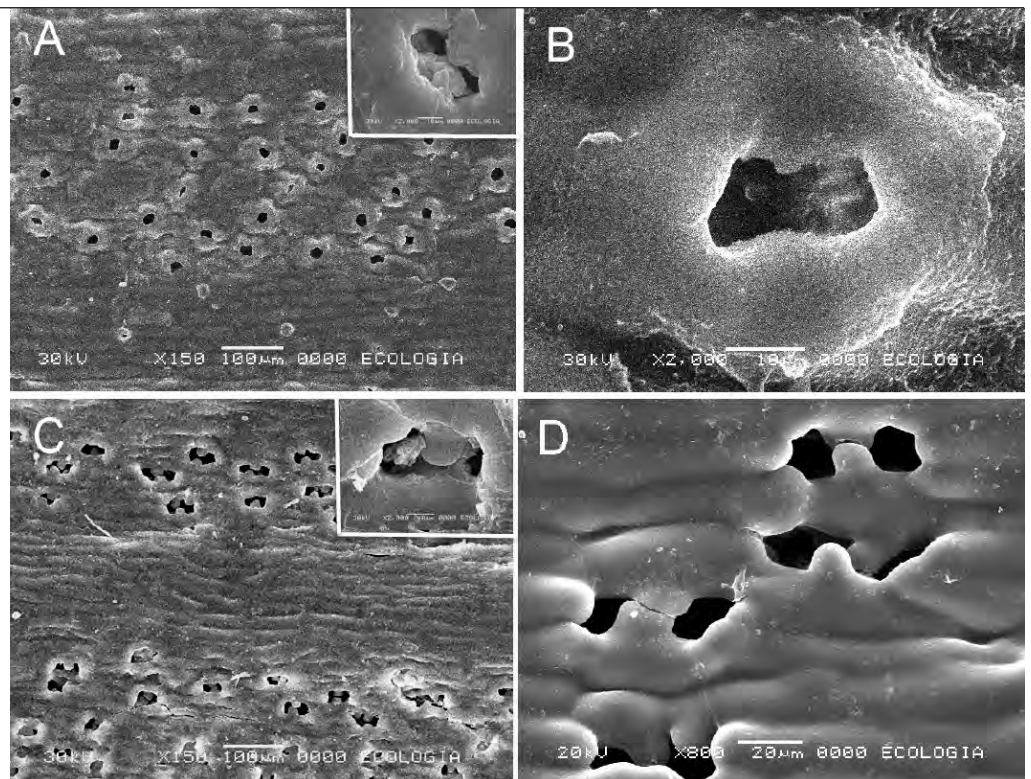


difference is that stomatal plugs, as reported for Podocarpaceae by Stockey *et al.* (1992), were rarely seen in *Dioon* but present occasionally.

The strongly overarching papillate subsidiary cells reported for *Cycas revoluta* by Pant and Mehra (1964) are unlike the structures in *Dioon* though their ontogeny may be similar; figure 1G in Pant and Mehra (1964) shows an overarching papilla of a subsidiary cell in a young stoma, and another subsidiary cell dividing to give rise to one encircling cell, and their figure 3F an adult stoma overarched by thickened papillae of subsidiary cells. In *Dioon* it is the outermost of the three layers of encircling cells with thickened periclinal wall and cuticle that forms the Florin ring-like ring structure.

SEM observations of the cuticle surface of the stomatal band of mature leaflets for all species show a variably undulating surface marking the outline of the epidermal cells. The epistomatal apertures appear raised by the encircling cells forming variably marked structures with regular,

Figure 3. SEM of outer surface of abaxial cuticles of *Dioon*. **A)** *Dioon edule* type Florin rings, insert showing wax plug. **B)** Detail of *D. edule* ring with wax plug. **C)** *D. purpusii* type rings, insert shows aperture with wax plug. **D)** *D. purpusii* detail of pores showing protruding cuticular papillae partially occluding pores. Bar: 100 μm in A and C, 10 μm in B, 20 μm in D.



irregular or slight relief and outline according to species. We observe two types; (1) the *Dioon edule* Lindl. type (Figure 3A) that is a continuous raised ring that slightly overarches the stomatal pore resulting in a volcanic shape that corresponds to the Florin ring type C of Yoshie and Sakai (1985), wax plugs sometimes referred to as Florin plugs have also been observed but are not common (Figures 3A insert and 3B); (2) the *D. purpusii* Rose type (Figure 3C) some presenting wax plugs (Figure 3C insert) that also corresponds to the type C ring but contrasts with the *D. edule* type as irregularly undulating with protruding papillae that vary in number and position, at times partially obscuring the stomatal pore (Figure 3D). The stomatal papillae vary in number, position and depth in the stomatal chamber within and between species that present the *D. purpusii* type structure.

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